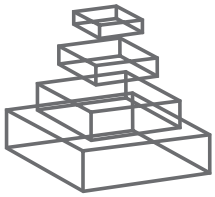
frontiers

RESEARCH TOPICS

SYNTHETIC BIOLOGY APPLICATIONS IN INDUSTRIAL MICROBIOLOGY

Topic Editors
Weiwen Zhang and David R. Nielsen

frontiers in MICROBIOLOGY



frontiers

Cover image provided by Ibbl sarl, Lausanne CH

ISSN 1664-8714
ISBN 978-2-88919-342-4
DOI 10.3389/978-2-88919-342-4

ABOUT FRONTIERS

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

FRONTIERS JOURNAL SERIES

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing.
All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

DEDICATION TO QUALITY

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

WHAT ARE FRONTIERS RESEARCH TOPICS?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area!
Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: researchtopics@frontiersin.org

SYNTHETIC BIOLOGY APPLICATIONS IN INDUSTRIAL MICROBIOLOGY

Topic Editors:
Weiwen Zhang, Tianjin University, China
David R. Nielsen, Arizona State University, USA

Exponentially increasing information on biological organisms coupled with increasing computational power in the past decade have broadened the perspective of fundamental biological research, bringing about considerable promise and unprecedented potential for practical applications in biotechnology. As one emergent discipline, synthetic biology aims to design and engineer novel biologically-based parts, devices, and systems, in addition to redesigning existing, natural biological systems. Although previously relegated to demonstration studies, more recent research in synthetic biology has focused on the rational engineering of industrial microorganisms with the potential to address many of society's critical challenges. Within the realm of industrial microbiology, progress in the field of synthetic biology has enabled the development of, for example, new biosynthetic pathways for the production of renewable fuels and chemicals, programmable logic controls to regulate and optimize cell function, and robust microbes for the destruction of harmful environmental contaminants. Some of the exciting examples included producing anti-malarial drug, anti-cancer taxol precursor and various biofuel molecules in *E. coli* and yeast. In addition, these researches have also greatly enhanced our understanding of the cellular machinery and its regulation in some of the industry important microbes, laying an important foundation for further design and engineering of biological function for even greater application. For these reasons, we present here a collection of articles from the leading edge of the field of synthetic biology, with a specific focus on the development in industrial microorganisms. It is the intent of this collection to reach a wide audience whose interests and expertise spans from development of novel synthetic biology methodologies and theories (both experimental and computational) to practical applications seeking to address issues facing the world today.

Table of Contents

frontiers in
MICROBIOLOGY

EDITORIAL
published: 26 August 2014
doi: 10.3389/fmicb.2014.00451

Synthetic biology applications in industrial microbiology

Weiwen Zhang[1]* and David R. Nielsen[2]*

[1] *Laboratory of Synthetic Microbiology, School of Chemical Engineering and Technology, Tianjin University, Tianjin, China*
[2] *Department of Chemical Engineering, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ, USA*
**Correspondence: wwzhang8@tju.edu.cn; david.r.nielsen@asu.edu*

Edited by:
Peter Neubauer, Technische Universität Berlin, Germany

Reviewed by:
Dong-Yup Lee, National University of Singapore, Singapore
Stephen Fong, Virginia Commonwealth University, USA
Pablo Carbonell, University of Evry, France
Pattanathu K. S. M. Rahman, Teesside University, UK

Keywords: chemicals, microbial systems, synthetic biology, industrial microbiology, biotechnology

With the ability to perform a multitude of unique and complex chemical transformations, microorganisms have long been the "workhorses" of many industrial processes. However, in addition to exploiting the utility of naturally evolved phenotypes, the principles, strategies, and tools of synthetic biology are now being applied to facilitate the engineering of tailor-made microbes capable of tackling some of society's most important and toughest challenges. Fueled in part by exponentially increasing reservoirs of bioinformatic data and coupled with more robust and powerful tools for its processing, research in the past decade has brought about new and broadened perspectives of fundamental biological phenomena. The application of said insight is now beginning to unlock the unprecedented potential of synthetic biology in biotechnology, as well as its considerable promise for addressing previously unsolved global challenges. For example, within the realm of industrial microbiology, progress in the field of synthetic biology has enabled the development of new biosynthetic pathways for the production of renewable fuels and chemicals, programmable logic controls to regulate and optimize complex cellular functions, and robust microbes for the destruction of harmful environmental contaminants. In this Research Topic, a collection of articles—including original research, reviews, and mini-reviews—from leading investigators in the synthetic biology community are presented to capture the current state, recent progress, and over-arching challenges associated with integrating synthetic biology with industrial microbiology and biotechnology.

With improved tools and practices for engineering novel enzymes and pathways, synthetic biology has importantly expanded the diversity of chemical products that can now be synthesized biologically. These previously unattainable molecules serve important industrial roles as monomers for producing bioplastics (Adkins et al., 2012), bulk and fine chemicals, as well as long-chain alcohols (Lamsen and Atsumi, 2012) and other biofuels with improved "drop in" compatibility (Lee et al., 2013). Among those, as comprehensively reviewed by Prof. Tae Seok Moon and co-workers at Washington University in St. Louis, isoprenoids represent a diverse platform of molecules whose microbial production has been greatly enabled by improved understandings of complex regulatory and metabolic networks and the engineering of biological devices from well characterized and standardized genetic parts (Immethun et al., 2013). In fact, in an original study, the research group of Prof. Ganesh Sriram at the University of Maryland at College Park specifically demonstrate how the synthesis of one specific isoprenoid compound, namely dihydroartemisinic acid, by yeast can be improved by identifying non-trivial metabolic engineering targets through the tandem application of both *in silico* and *in vivo* metabolic pathway analyses (Misra et al., 2013).

Meanwhile, of equal importance to the product of interest is the microbial chassis used for its biosynthesis. In an original study, the research group of Prof. Ying-Jin Yuan of Tianjin University reports a strategy combining chassis selection and direct fine-tuning of the balance of key genes to increase ethanol yields in engineered xylose-utilizing *Saccharomyces cerevisiae* (Zha et al., 2012). In a continual push beyond the traditional platform microbes such as *Escherichia coli* and yeast, even cyanobacteria and algae[6] are emerging as amenable platforms for rational engineering, as comprehensively reviewed by Prof. Deirdre Meldrum and co-workers at Arizona State University (Wang et al., 2012). By promoting the use of alternative sources of carbon and energy, including CO_2 and sunlight in the latter case, these efforts are improving the sustainability of renewable product biosynthesis. In natural environments microorganisms commonly exist as communities of multiple species that are capable of performing more varied and complex tasks than in clonal populations. Prof. Travis Bayer and co-worker at Imperial College London review recent efforts to engineer synthetic microbial interactions of multiple microbial species (Brune and Bayer, 2012), emphasizing industrial applications in the field of biomining and bioremediation of acid mine drainage. In addition, Prof. Pier Luisi and co-workers at the University of Roma Tre present a comprehensive review on chemical synthetic biology, a new branch of synthetic biology oriented toward the synthesis of chemical structures alternative to those present in nature (Chiarabelli et al., 2013).

Two articles in this Research Topic focus more on the development and application of new enabling technologies in synthetic

biology (Harrison and Dunlop, 2012; Reyes et al., 2012). The toxicity of biofuels to the producing microorganism is considered as one of the major challenges in achieving high-level fuel production. To address the issue, in an original research article, Prof. Mary Dunlop and co-worker at the University of Vermont develop a mathematical model for cell growth and biofuel production that implements a synthetic feedback loop using a biosensor to control efflux pump expression (Harrison and Dunlop, 2012). The results show that, in comparison to systems that express efflux pumps at a constant level, the feedback sensor increases overall biofuel production by delaying pump expression until it is needed. *In vitro* adaptive evolution has been used extensively for engineering various microbial systems, and the ability to identify the underlying adaptive landscape for a particular phenotype of interest will greatly enhance our abilities to engineer more robust microbial strains. Prof. Katy Kao and co-workers at Texas A&M University College Station present a comprehensive review on visualizing evolution in real-time (VERT), a recently developed methodology based on *in vitro* adaptive evolution that facilitates the identification of mutants with improved fitness throughout the course of evolution (Reyes et al., 2012).

Finally, Prof. Karmella Haynes and co-workers at Arizona State University discuss the importance of several issues surrounding risk mitigation and biocontainment. An important consideration as synthetic biology prepares to emerge from laboratory settings to enter full-scale biorefineries (Moe-Behrens et al., 2013).

As an emerging scientific field, synthetic biology is one of the most dynamic new fields of biotechnology in the past years. Its application in industrial microbiology, although still at very beginning, has demonstrated that it is a powerful technology that could significantly improve the industrial microbiology research to meet the challenges human society faces today.

REFERENCES

Adkins, J., Pugh, S., McKenna, R., and Nielsen, D. R. (2012). Engineering microbial chemical factories to produce renewable biomonomers. *Front. Microbiol.* 3:313. doi: 10.3389/fmicb.2012.00313

Brune, K. D., and Bayer, T. S. (2012). Engineering microbial consortia to enhance biomining and bioremediation. *Front. Microbiol.* 3:203. doi: 10.3389/fmicb.2012.00203

Chiarabelli, C., Stano, P., and Luisi, P. L. (2013). Chemical synthetic biology: a mini-review. *Front. Microbiol.* 4:285. doi: 10.3389/fmicb.2013.00285

Harrison, M. E., and Dunlop, M. J. (2012). Synthetic feedback loop model for increasing microbial biofuel production using a biosensor. *Front. Microbiol.* 3:360. doi: 10.3389/fmicb.2012.00360

Immethun, C. M., Hoynes-O'Connor, A. G., Balassy, A., and Moon, T. S. (2013). Microbial production of isoprenoids enabled by synthetic biology. *Front. Microbiol.* 4:75. doi: 10.3389/fmicb.2013.00075

Lamsen, E. N., and Atsumi, S. (2012). Recent progress in synthetic biology for microbial production of C3-C10 alcohols. *Front. Microbiol.* 3:196. doi: 10.3389/fmicb.2012.00196

Lee, S. J., Lee, S. J., and Lee, D. W. (2013). Design and development of synthetic microbial platform cells for bioenergy. *Front. Microbiol.* 4:92. doi: 10.3389/fmicb.2013.00092

Misra, A., Conway, M. F., Johnnie, J., Qureshi, T. M., Lige, B., Derrick, A. M., et al. (2013). Metabolic analyses elucidate non-trivial gene targets for amplifying dihydroartemisinic acid production in yeast. *Front. Microbiol.* 4:200. doi: 10.3389/fmicb.2013.00200

Moe-Behrens, G. H., Davis, R., and Haynes, K. A. (2013). Preparing synthetic biology for the world. *Front. Microbiol.* 4:5. doi: 10.3389/fmicb.2013.00005

Reyes, L. H., Winkler, J., and Kao, K. C. (2012). Visualizing evolution in real-time method for strain engineering. *Front. Microbiol.* 3:198. doi: 10.3389/fmicb.2012.00198

Wang, B., Wang, J., Zhang, W., and Meldrum, D. R. (2012). Application of synthetic biology in cyanobacteria and algae. *Front. Microbiol.* 3:344. doi: 10.3389/fmicb.2012.00344

Zha, J., Hu, M. L., Shen, M. H., Li, B. Z., Wang, J. Y., and Yuan, Y. J. (2012). Balance of XYL1 and XYL2 expression in different yeast chassis for improved xylose fermentation. *Front. Microbiol.* 3:355. doi: 10.3389/fmicb.2012.00355

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 14 May 2014; accepted: 11 August 2014; published online: 26 August 2014.
Citation: Zhang W and Nielsen DR (2014) Synthetic biology applications in industrial microbiology. Front. Microbiol. ***5****:451. doi: 10.3389/fmicb.2014.00451*
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 08 June 2012
doi: 10.3389/fmicb.2012.00196

Recent progress in synthetic biology for microbial production of C3–C10 alcohols

Edna N. Lamsen and Shota Atsumi*

Department of Chemistry, University of California, Davis, CA, USA

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Ziyu Dai, Pacific Northwest National Laboratory, USA
Shawn Chen, Ohio University, USA

****Correspondence:***
Shota Atsumi, Department of Chemistry, University of California, One Shields Avenue, Davis, CA, USA.
e-mail: atsumi@chem.ucdavis.edu

The growing need to address current energy and environmental problems has sparked an interest in developing improved biological methods to produce liquid fuels from renewable sources. While microbial ethanol production is well established, higher-chain alcohols possess chemical properties that are more similar to gasoline. Unfortunately, these alcohols (except 1-butanol) are not produced efficiently in natural microorganisms, and thus economical production in industrial volumes remains a challenge. Synthetic biology, however, offers additional tools to engineer synthetic pathways in user-friendly hosts to help increase titers and productivity of these advanced biofuels. This review concentrates on recent developments in synthetic biology to produce higher-chain alcohols as viable renewable replacements for traditional fuel.

Keywords: biofuel, butanol, higher-chain alcohol, isobutanol, metabolic engineering, synthetic biology

INTRODUCTION

Depletion of finite liquid fuels is on the horizon, and the growing concern with current energy and environmental problems has ignited an interest in the production of liquid biofuels (Herrera, 2006; Zinoviev et al., 2010; Fairley, 2011). In 2010, petroleum consumption in the United States was about 36 quadrillion BTU, in which 70% consumed was imported (Fichman, 2011). To help increase independence from petroleum-derived fuels as well as its foreign importation, endeavors to develop and improve biological methods to produce renewable liquid fuels are in play (Stephanopoulos, 2007; Fortman et al., 2008; Lee et al., 2008; Sheehan, 2009). Ethanol ranks as the most prominent biofuel to date, reaching a production high of 13.2 billion gallons in 2010 (more than eight times produced a decade ago; Fichman, 2011). Despite its well-developed production and wide use, notably as a gasoline additive and as a high-percentage E85 blend, it falls short of being the ideal "drop-in" fuel to replace gasoline. Ethanol is hygroscopic, making it incompatible with current gasoline infrastructure. Moreover, ethanol is only 65% as energy dense as gasoline (20.8 versus 32.0 MJ/L, respectively; Li et al., 2010b). Higher-chain alcohols, on the other hand, are not hygroscopic, and provide energy densities that are more comparable to gasoline, and thus help to mitigate the problems that ethanol arises.

Unlike ethanol, higher-chain alcohols (except 1-butanol produced naturally by the *Clostridium* species; Jones and Woods, 1986) are neither produced efficiently in nature nor in efficient quantities, and so increased efforts are being made to use microorganisms to cost-effectively produce them from biomass as well as in industrial volumes. Much energy has been put into using metabolic engineering to improve natural producers of these alcohols in the past, but without success in reaching desired industrial-relevant titers and productivity (Lutke-Eversloh and Bahl, 2011). Part of this difficulty stems from a lack of tools and techniques for genetic modification, slow growth, and complex physiology of the natural producer (Ezeji et al., 2007). User-friendly hosts such as *Escherichia coli* and *Saccharomyces cerevisiae*, however, come with a wide range of tools and techniques, are faster growing, and possess a more simple physiology. With these helping hands, many efforts have been put into developing higher-chain alcohol production in non-native hosts.

Outside the advantageous inherent properties of using a well-characterized host and the ease in which metabolic engineering can help optimize pathways and maintain cell fitness, synthetic biology is a relatively nascent practice being used to aid in the development of higher-chain alcohol producers (Agapakis and Silver, 2009; Khalil and Collins, 2010). Synthetic biology provides the ability to piece together biological components from several different origins in order to redesign a natural or construct a novel pathway that the host uses to synthesize a valuable chemical (Keasling, 2008; Connor and Atsumi, 2010). In conjunction with metabolic engineering, development of a more efficient production platform in a non-native but user-friendly host for a desired chemical is made possible (Alper and Stephanopoulos, 2009). This review will concentrate on recent progress in using synthetic biology to engineer user-friendly microbes that produce C3–C10 alcohols.

CoA-DEPENDENT PATHWAYS

Natural production of 1-butanol occurs in the acetone–butanol–ethanol (ABE) pathway of the *Clostridium* species (Gheshlaghi et al., 2009), where starch and simple sugars are fermented into a mixture of solvents, including acetone, lactate, and acetoin. In this pathway, two acetyl-CoA molecules are converted to butyryl-CoA in four enzymatic steps using acetyl-CoA acetyltransferase, 3-hydroxybutyryl-CoA dehydrogenase, crotonase, and butyryl-CoA dehydrogenase (Bennett and Rudolph, 1995). From here, 1-butanol can be produced using a dual-function butyraldehyde/butanol dehydrogenase (Atsumi et al., 2008a).

Since natural alcohol producers like the *Clostridium* species have been difficult to engineer for 1-butanol production, great strides have been made to transfer and improve this traditional fermentation pathway in non-natural producers like *E. coli* and *S. cerevisiae*. For example, *E. coli*, a Gram-negative bacterium, has a long history of being studied and exploited as a model host, due to its rapid growth rate, relatively simple physiology and nutritional needs, as well as ease of cultivation and manipulation. Furthermore, elucidation and characterization of its genetics, physiology, and biochemistry has led to the development of a wide selection of genetic tools for manipulating cell behavior and function.

In general, overexpression of either native or heterologous genes involves construction of plasmids that fuse multiple genes transcriptionally. Overexpression of genes may also be integrated into the genomic DNA (gDNA) of a microorganism using homologous recombination via a plasmid containing the gene of interest and a selectable marker. This approach is used to engineer cyanobacteria such as *Synechococcus elongatus* (Golden et al., 1987). Furthermore, in order to gain control of expression levels, there are various promoters, both native and engineered (Gronenborn, 1976; Brosius et al., 1985; Studier and Moffat, 1986; Elvin et al., 1990; Mumberg et al., 1994; Lutz, 1997; Haldimann et al., 1998; Pátek et al., 2003), available to choose from. While heterologous gene expression – due to various factors like protein misfolding and aggregation (Thomas et al., 1997; Vabulas et al., 2010) – is not always successful, progress has been made to mechanistically understand why these processes can occur (Georgiou and Valax, 1996; Baneyx and Mujacic, 2004; Ami et al., 2009). As a result, several approaches have been developed to circumvent these types of pitfalls and increase the likelihood that heterologous expression is successful (Baneyx and Mujacic, 2004).

To minimize costs for industrial scale production, it is ideal that production can be done in minimum growth media rather than rich media. More often than not, however, rich media are used in order to maximize cell density and optimize cell condition for production in laboratory scale production (Tartoff and Hobbs, 1987; Amberg et al., 2005). Measurement of alcohols and other metabolites can be monitored using various chromatographic methods, typically gas chromatography–mass spectrometry (GC–MS), gas chromatography–flame ionization detector (GC–FID), and high-performance liquid chromatography (HPLC).

The following describes research to increase the titers of 1-butanol, isopropanol, and 1-hexanol in user-friendly hosts, as well as the utilization and engineering of the CoA-dependent pathway (**Figure 1**) to shunt production toward non-natural higher-chain alcohols.

1-BUTANOL PRODUCTION

In order to demonstrate the feasibility of this strategy, Atsumi et al. (2008a) transferred the 1-butanol pathway of *Clostridium acetobutylicum* into *E. coli*. Use of 1-butanol as an additive or replacement for gasoline has been growing in popularity due to its hygroscopicity and comparable energy density (29.2 MJ/L) to gasoline. For production through the *Clostridium* pathway, one 1-butanol molecule is produced per molecule of glucose and four NADH. The strain containing essential genes for 1-butanol production (*thl*, *hbd*, *crt*, *bcd*, *etfAB*, and *adhE2*) were cloned and expressed using a two-plasmid system, where *thl* and *adhE2* were overexpressed under control of the IPTG-inducible $P_L lacO_1$ promoter (Lutz, 1997) in a colE (Kahn et al., 1979) origin plasmid, while *crt*, *bcd*, *etfAB*, and *hbd* were overexpressed under control of the $P_L lacO_1$ promoter in a p15A (Chang and Cohen, 1978) replication origin plasmid. This two-plasmid expression strain produced 13.9 mg/L 1-butanol in 40 h under anaerobic conditions. Elevating oxygen levels increased production in this strain, although these enzymes are from a strict anaerobe. To further improve production, genes responsible for production of metabolic byproducts in the *E. coli* genome were deleted. Namely, Δ*ldhA*, Δ*frdBC*, and Δ*adhE* knockouts were introduced in order to reduce lactate, ethanol, and succinate. Furthermore, *pta* (encoding phosphotransacetylase) was deleted to decrease amount of acetate produced. The *fnr* gene, encoding the regulator Fnr that deactivates the pyruvate dehydrogenase complex under anaerobic conditions, was deleted as well. The resulting strain boosted 1-butanol production to 373 mg/L. Finally, this strain, when grown in Terrific Broth (TB)-enriched, glycerol-supplemented media (Tartoff and Hobbs, 1987), increased 1-butanol titer to 552 mg/L.

A promising strategy to tweak alcohol production further in non-native hosts is to consider the various driving forces that are modified as a consequence to manipulating a host, and making efforts to alleviate negative affects in a resulting strain. When altering a native pathway, many factors such as growth and gene expression have the potential to be compromised, which can diminish cell fitness and create bottlenecks in the engineered system that prevent high titers of a desired product (Fischer et al., 2008). A recurring problem that must be tackled is the ubiquitous occurrence of reversible reactions in metabolic pathways that slow the optimal production of important intermediates essential for propelling a series of steps.

Bond-Watts et al. (2011) explored the chemical nature of enzymes to drive equilibria forward in the design of a 1-butanol platform in *E. coli*. First, in an *E. coli* strain containing *phaA*, *phaB*, and *crt* in a p15A origin (Chang and Cohen, 1978) plasmid, and *ccr* and *adhE2* in a colE origin (Kahn et al., 1979) plasmid (both under control of the IPTG-inducible P_{trc} promoter; Brosius et al., 1985), only 95 mg/L 1-butanol was produced after 6 days in TB supplemented with glucose. To push the equilibrium between crotonyl-CoA and butyryl-CoA forward, *ter*, encoding an NADH-dependent crotonyl-CoA reductase from *Treponema denticola*, replaced *ccr*. This gene replacement led to a 3.5-fold increase in titer (340 mg/L) after 3 days. Upon further investigation for additional bottlenecks, enzymes that generate stereo-specific products were found and tested. After replacing *phaB* with *hbd*, encoding NADH-dependent (*S*)-3-hydroxybutyryl-CoA dehydrogenase from *C. acetobutylicum*, and *crt* with *phaJ*, encoding an R-specific enoyl-CoA hydratase from *Aeromonas caviae*, production rose to 2.95 g/L. Finally, balance of reducing equivalents was taken into consideration. While four NADH molecules needed to make one molecule 1-butanol, glycolysis only produces two NADH per glucose molecule. To increase availability and direct consumption of acetyl-CoA and NADH toward alcohol production and away from lactate and acetate formation, *aceEF-lpd*, encoding the pyruvate dehydrogenase complex, was overexpressed.

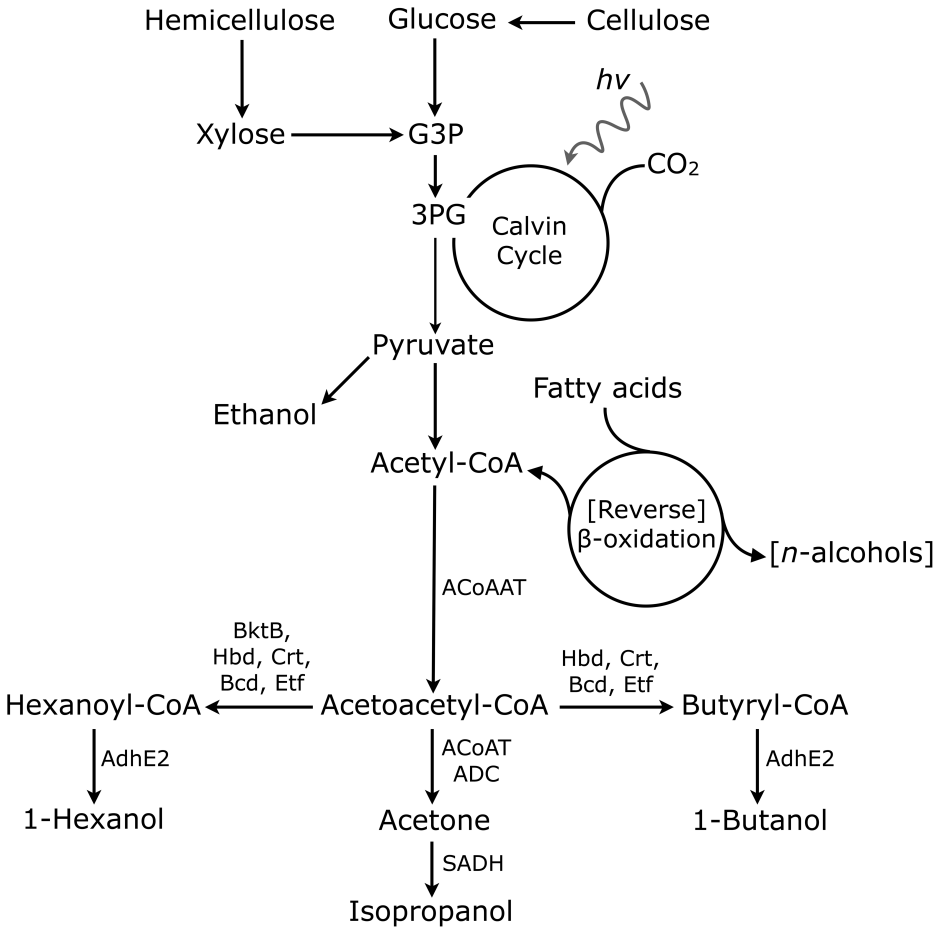


FIGURE 1 | Metabolic representation of CoA-dependent pathways for biofuel production. G3P, glyceraldehyde-3-phosphate; 3PG, 3-phosphoglycerate; ACoAAT, acetyl-CoA acetyltransferase; BktB, 2-ketothiolase; Hbd, 3-hydroxybutyryl-CoA; Crt, crotonase; Bcd, butyryl-CoA dehydrogenase; Etf, electron transfer flavoprotein; ACoAT, acetoacetyl-CoA transferase; ADC, acetoacetate decarboxylase; AdhE2, aldehyde/alcohol dehydrogenase; SADH, primary–secondary alcohol dehydrogenase. Reversal of β-oxidation to make *n*-alcohols (in the presence of a non-fatty acid carbon source) is indicated in brackets.

This enhancement provided the additional two NADH molecules needed to synthesize 1-butanol, and improved production levels to a final titer of 4.65 g/L at 28% the theoretical max from glucose.

Using a similar premise to improve the CoA-dependent pathway in *E. coli*, Shen et al. (2011) utilized a number of driving forces to generate 1-butanol in industrial-relevant quantities. In this study, *atoB* (*E. coli*), *adhE2* (*C. acetobutylicum*), *crt* (*C. acetobutylicum*), *hbd* (*C. acetobutylicum*), and *ter* (*T. denticola*; used to replace the oxygen-sensitive, NADPH-dependent Bcd–EtfAB complex) genes were overexpressed in an *E. coli* Δ*adhE* Δ*ldhA* Δ*frd* strain (JC166), which cannot grow without an additional NADH-consuming pathway. In this two-plasmid system, *atoB*, *adhE2*, *crt*, and *hbd* were overexpressed in a plasmid under control of the $P_L lacO_1$ promoter (Lutz, 1997) with a colE (Kahn et al., 1979) origin, while *ter* was overexpressed in a plasmid under $P_L lacO_1$ control with a colA origin (Zverev and Khmel, 1985). This strain anaerobically produced 1.8 g/L after 24 h in TB supplemented with glucose. Catalysis by Ter to convert crotonyl-CoA to butyryl-CoA was found irreversible in the presence of excess butyryl-CoA and NAD^+ by *in vitro* enzyme assays. Furthermore, Bcd–EtfAB replacement by NADH-dependent Ter successfully restored anaerobic growth of JC166. Next, *fdh*, encoding a formate dehydrogenase from *Candida boidinii*, was overexpressed to reduce excess pyruvate. Without overexpression of *fdh* in the 1-butanol pathway, pyruvate serves as a sink for excess carbon flux and piles up in the cell. On the contrary, when *fdh* is overexpressed, formate is oxidized into CO_2 and NADH to promote accumulation of the additional two NADH equivalents needed for 1-butanol production. Moreover, *pta*, which would have encoded a phosphate acetyltransferase, was deleted in order to build up acetyl-CoA and decrease acetate formation. As a result, 1-butanol production rose to 15 g/L after 3 days at 88% theoretical maximum yield. Lastly, anaerobic, pH-controlled fermentation was performed, in conjunction with continuous gas stripping, using a 1 L stirred-tank bioreactor. Final measurements indicated that 1-butanol levels shot up to 30 g/L after 7 days (∼70% theoretical maximum).

Escherichia coli is not the only user-friendly host available to for 1-butanol production. The robust yeast, *S. cerevisiae*, has been a prominent host for industrial ethanol production as well as the non-native production of a variety of valuable chemicals. It is well-characterized, has a wide range of genetic tools for manipulation, its fermentation is relatively cheap, and its mass production and downstream processing is well-developed. Especially, *S. cerevisiae* has a vast history of being used for the production of ethanol for beer and wine from sugars. Like for *E. coli*, synthetic biology has contributed to the improvement of engineered processes in *S. cerevisiae* (Krivoruchko et al., 2011).

Steen et al. (2008) chose *S. cerevisiae* as the host for 1-butanol production, in which clostridial enzymes were substituted with isozymes from various other species. From an initial group of strains to elucidate a thiolase with the best activity [*phaA* (*Ralstonia eutropha*), *atoB* (*E. coli*), or *ERG10* (*S cerevisiae*)], the highest producer (BY4747) containing *phaA* and *phaB* (*R. eutropha*), *ccr* (*Streptomyces collinus*), and *crt* and *adhE2* (*C. beijerinckii*), under control of either the GAL1 or GAL10 galactose-dependent promoters in 2 μ origin (Huberman et al., 1987) plasmids, generated 1 mg/L 1-butanol. To improve upon this initial strain, an NADH-dependent hydroxybutyryl-CoA dehydrogenase, encoded by Hbd, replaced an NADPH-dependent enzyme, encoded by PhaB. The strain including *ERG10* (encoding a native thiolase) and *hbd* resulted in 2.5 mg/L production in synthetic defined (SD) media (Amberg et al., 2005) supplemented with galactose.

In an interest to compare production in various hosts, Nielsen et al. (2009) reconstructed and engineered the 1-butanol pathway of *C. acetobutylicum* into Gram-negative *Pseudomonas putida* and Gram-positive *Bacillus subtilis*. *P. putida* and *B. subtilis* were chosen as hosts for this biosynthetic pathway due to higher 1-butanol tolerance relative to *E coli* (de Bont, 1998). *P. putida* has been exploited for bioremediation of soil, and possesses a versatile metabolism, an ability to thrive in extreme environmental conditions, simple nutritional requirements, and rapid growth rate (Dos Santos et al., 2004). The *P. putida* strain was cultured in TB under control of the IPTG-inducible T7lac promoter (Studier and Moffat, 1986) and produced 50 mg/L with glucose, and 122 mg/L with glycerol. Results also indicated that the Bcd–EtfAB complex of *C. acetobutylicum* was active in the obligate aerobe. *B. subtilis*, on the other hand, has been extensively studied as a model Gram-positive bacterium, and can live in several different environments, including soil, animal gastrointestinal tracts, and plant roots. Moreover, it possesses the distinct ability to become competent for transformation naturally (Earl et al., 2008). *B. subtilis* genes *amyE* (encoding an α-amylase), *thrC* (encoding a threonine synthase), and *pyrD* (encoding a dihydroorotate dehydrogenase) were deleted from the genome using double-crossover homologous recombination. The *B. subtilis* Δ*amyE* Δ*thrC* Δ*pyrD* strain was cultured in TB under control of the IPTG-inducible promoter (Studier and Moffat, 1986), and produced 23 mg/L with glucose, and 24 mg/L in glycerol under anaerobic conditions.

Production of higher-chain alcohols using bacteria that directly utilize CO_2 and sunlight is another approach being explored. In addition, if an efficient production platform is successfully designed, then production of higher-chain alcohols directly from sunlight would give a higher-value advantage. One photosynthetic organism with a growing genetic toolbox, elucidated genome sequences, and relatively fast growth rate is known as cyanobacteria (Ruffing, 2011), and its capabilities have already been tapped to produce a range of different fuels and chemicals (Machado and Atsumi, 2012). In cyanobacterial systems, sunlight is utilized to fix carbon in the Calvin–Benson–Bassham cycle and produce intermediates that enter and are consumed in glycolysis. Thus, the need to grow and convert biomass into sugars is bypassed.

Lan and Liao (2011) introduced a 1-butanol pathway into *S. elongatus* PCC 7924 by integrating *atoB* (*E. coli*) and *adhE2* (*C. acetobutylicum*), under P_{trc} control (Brosius et al., 1985), into neutral site I (NSI; Bustos and Golden, 1992), and then integrating *hbd*, *crt* (*C. acetobutylicum*), and *ter* (*T. denticola*), under $P_L lacO_1$ control (Lutz, 1997), into neutral site II (NSII; Andersson et al., 2000) on the genome (Lan and Liao, 2011). In this strain, 3.04 mg/L 1-butanol accumulated after 7 days. In an improved strain containing gene rearrangement where a His-tagged *ter* was integrated, under P_{trc} control, into NSI, and *atoB*, *adhE2*, *hbd*, and *crt* genes were integrated, under $P_L lacO_1$ control, into NSII, 13.16 mg/L 1-butanol was produced. Cultures in screw-capped flasks and air-bubbling cultures, however, did not produce 1-butanol. To determine whether light or oxygen halted 1-butanol production, various culturing conditions (i.e., oxic/anoxic, light/dark) in BG-11 media supplemented with $NaHCO_3$ was tested. Delving further into oxygen effects, photosystem II was inhibited with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; Komenda et al., 2000), in order to prevent generation of oxygen under light. Between 2.5 and 5.5 mg/L 1-butanol was produced under continuous light and addition of varying concentrations of DCMU, indicating that elimination of oxygen is key for 1-butanol production in *S. elongatus*. Furthermore, experiments to test AdhE2 activity in oxic and anoxic conditions showed similar activities in both conditions, suggesting that low AdhE2 activity may not be related to the anoxic requirement for 1-butanol production. The best strain produced 14.5 mg/L 1-butanol in 7 days while decreasing in cell density (OD_{730} from 3.9 to 2.4) when incubated in dark anoxic conditions.

ISOPROPANOL PRODUCTION

Isopropanol production using the CoA-dependent pathway has also been explored. Even though isopropanol has an energy density of only 23.9 MJ/L, it has a lower hygroscopicity relative to ethanol. With these properties, isopropanol may be used as a gasoline or diesel additive.

Hanai et al. (2007) constructed the isopropanol pathway in *E. coli*, modeling it after the one in *C. beijerinckii* that converts acetyl-CoA via acetone into isopropanol. First, an acetyl-CoA acetyltransferase condenses two acetyl-CoA molecules into acetoacetyl-CoA. Next, an acetoacetyl-CoA transferase is used to form acetoacetate. Then, an acetoacetate decarboxylase is used to produce acetone and CO_2. Finally, a secondary alcohol dehydrogenase (ADH) reduces acetone into isopropanol. Five combinations of the following genes were overexpressed for production under control of the $P_L lacO_1$ promoter (Lutz, 1997): *atoB* (*E. coli*) or

thl (*C. acetobultylicum*), *ctfAB* (*C. acetobutylicum*) or *atoAD* (*E. coli*), *adc* (*C. acetobutylicum*), and *adh* (*C. beijerinckii* or *Thermoanaerobacter brockii*). All strains [except one where overexpression of an *adh* (*T. brockii*), encoding a primary–secondary alcohol (SADH), produced 1.1 g/L acetone] produced comparable isopropanol titers, ranging between 2.1 and 2.7 g/L, with minute quantities of acetone and ethanol. The strain that produced 2.7 g/L isopropanol overexpressed *adh* in a p15A origin (Chang and Cohen, 1978) plasmid, while *thl*, *atoAD*, and *adc* were overexpressed in a colE origin (Kahn et al., 1979) plasmid. Crude extract assays measured an SADH (*C. beijerinckii*) activity nearly 13 times higher than that of *T. brockii*, thus explaining the stark difference in isopropanol to acetone ratio. Further experiments in SD-8 media (Luli and Strohl, 1990), with varied starting amounts of glucose concentration resulted in isopropanol production peaking at 4.9 g/L after 30.5 h. Furthermore, maximum productivity was reported to be 0.41 g/L/h between 3 and 9.5 h at 43.5% theoretical yield.

Inokuma et al. (2010) further improved isopropanol production in *E. coli* by optimizing culture and media conditions, as well as using gas stripping for product recovery. First, pH-maintained, intermittent-fed fermentation in SD-8 media (Luli and Strohl, 1990) supplemented with glucose resulted in 40.1 g/L isopropanol after 60 h at 73.2% theoretical yield, and a production rate of 0.74 g/L/h between 6 and 60 h. To further increase titer, isopropanol was recovered using a gas-stripping system, where an optimized amount of air was bubbled through the culture in order to remove, transfer, and collect isopropanol and other volatile compounds into two collection bottles. By fusing fed-batch fermentation to the gas-stripping setup, isopropanol concentration increased to 79.6 g/L after 144 h, with a concentration of 19.8 g/L in the culture flask. However, cell density and production rate decreased over time. To alleviate this decrease, concentrated media was intermittently added in order to keep a fresh stock of nutrients available for consumption. Using this approach, titers reached 143 g/L after 240 h at 67.4% maximum theoretical yield, and demonstrated the potential for industrial fermentative production of isopropanol.

1-HEXANOL PRODUCTION

Dekishima et al. (2011) expanded the 1-butanol pathway in *E. coli* in order to produce 1-hexanol. The pathway constructed by Shen et al. (2011) was used as the starting point, where *atoB* (*E. coli*), *adhE2* (*C. acetobutylicum*), *hbd* (*C. acetobutylicum*), and *crt* (*C. acetobutylicum*) were overexpressed in a plasmid under control of the $P_L lacO_1$ promoter (Lutz, 1997) with a colE origin (Kahn et al., 1979) of replication, and *ter* [Eg-*ter* (*Euglena gracilis*) or Td-*ter* (*T. denticola*)] was overexpressed in a plasmid under control of the $P_L lacO_1$ promoter and colA origin (Zverev and Khmel, 1985) to convert two molecules of acetyl-CoA into butyryl-CoA. Finally, butyryl-CoA is subsequently reduced by AdhE2 (*C. acetobutylicum*) into 1-butanol. In order to extend the potential alcohol by two carbons, *bktB* (*R. eutropha*; encodes a 2-ketothiolase) was overexpressed in a plasmid under control of the $P_L lacO_1$ promoter and colA origin to add an acetyl group to butyryl-CoA to generate 3-keto-hexanoyl-CoA. Similar to synthesis of 1-butanol, 3-keto-hexanoyl-CoA can then be converted to 1-hexanol using the same enzymes used to convert acetoacetyl-CoA. First, Hbd converts 3-keto-hexanoyl-CoA into 3-hydroxyhexanoyl-CoA, allowing Crt to catalyze the generation of hexenoyl-CoA. Next, Ter is used to make hexanoyl-CoA. Finally, alcohol/aldehyde dehydrogenase AdhE2 cleaves the CoA group from hexanoyl-CoA and reduces it into 1-hexanol in two steps. An *E. coli* Δ*adhE* Δ*ldhA* Δ*frdBC* strain was used to increase the NADH available to drive the pathway forward. In this strain where only one of the trans-enoyl-CoA reductases, Td-ter, was overexpressed, there was no 1-hexanol detected, but when both Td-ter and Eg-ter were co-expressed, 23 mg/L 1-hexanol after 68 h was produced in TB supplemented with glucose. To further attempt to increase titer, a strain containing Δ*adhE* Δ*ldhA* Δ*frdBC* Δ*pta* knockouts was first constructed. 1-Hexanol titer using this strain was 27 mg/L after 68 h. Then, *fdh* was overexpressed in a plasmid under control of the $P_L lacO_1$ promoter and pSC101* origin (Stoker et al., 1982) in this knockout strain in order to increase the amount of NADH. In a pH-maintained, anaerobic production where the culture was replenished with fresh TB media every 24 h, production of 1-hexanol at 48 h increased to 47 mg/L. Although 1-hexanol concentrations did not reach those of 1-butanol in this system, further improvements to increase enzymatic activity toward C6 relative to C4 intermediates can be done.

C5–C10 ALCOHOL PRODUCTION VIA β-OXIDATION PATHWAY

In contrast to constructing a 1-butanol pathway by overexpressing genes from *C. acetobutylicum*, Gulevich et al. (2012) proposed the use of the native aerobic fatty acid β-oxidation cycle commonly found in bacteria and yeast. Normally, these species use this cycle to break down (C_{n+2})-acyl-CoA molecules into acetyl-CoA and (C_n)-acetyl CoA molecules using four genes (Fujita et al., 2007). Since metabolic pathways are essentially reversible, however, 1-butanol could be made after one turn of this cycle (with chain elongation occurring by addition of acetyl-CoA to another thioester). That is, an acetyl-CoA C-acetyltransferase would first condense two acetyl-CoA molecules into acetoacetyl-CoA. Next, a 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase would reduce the β-keto acid into 3-hydroxybutyryl-CoA, and subsequently to crotonyl-CoA. Then, crotonyl-CoA would be hydrogenated to a butyryl-CoA, where a native CoA-dependent alcohol/aldehyde dehydrogenase would then convert it into 1-butyraldehyde then 1-butanol in two steps. In an *E. coli* strain containing one chromosomal copy of each gene needed for 1-butanol synthesis (*atoB*, *fadA*, *fadB*, *fadE*, and *adhE*), an artificial genetic element, $P_{trc\text{-}ideal\text{-}4}$-SDφ10 (containing a strong LacI-dependent promoter as well as a strong ribosome binding site from phage T7), replaced the native regulatory regions of each of the genes in the proposed pathway. When production was tested in semi-aerobic conditions in M9 media (Miller, 1992) containing glucose and glycerol or LB-containing glucose, 334 and 897 mg/L of 1-butanol was produced, respectively. In addition, 1-butanol in LB was higher, but amount of ethanol produced was the same, and the amount of acetic acid synthesized was lower. On the other hand, in comparison to semi-aerobic conditions, titer was lower in LB (674 mg/L), but higher in M9 media (615 mg/L) under anaerobic conditions.

Dellomonaco et al. (2011) also demonstrated reversal of the β-oxidation cycle to generate higher-chain alcohols and other chemicals. In the presence of a non-fatty acid carbon source, constitutive expression of the fad–ato regulon reversed the beta oxidation cycle (Dellomonaco et al., 2010). To increase efficiency, repressive regulation of the beta oxidation cycle in the presence of glucose was avoided by using a cAMP-independent mutant (*crp**) to replace the native *crp* gene, and by deleting the *arcA* gene in order to eliminate any β-oxidation-encoding operon repression by ArcA (aerobic respiratory control A). In a strain containing these initial genomic modifications in pSC101* origin (Stoker et al., 1982) plasmids under the control of the $P_L tetO_1$ promoter, production of 1-butanol was achieved by overexpression of native *fucO*, encoding a L-1,2-propanediol oxidoreductase, and *yqeF*, encoding an acyl transferase, as well as deletion of the following genes involved in competing pathways: *adhE* (lactate, ethanol, succinate), *pta* (acetate), and *frdA* (succinate), *eutE* and *yqhD* (ethanol). Production in M9 media supplemented with glucose synthesized 2.2 g/L 1-butanol after 24 h at a 28% theoretical yield. When production moved to a bioreactor, however, titer rose to 14 g/L after 36 h at 33% theoretical maximum and a rate of 2 g 1-butanol/g cell dry weight/h. In this pathway, many CoA-thioester intermediates produced could also be converted to the corresponding alcohols and carboxylic acids. By simultaneously overexpressing *tesA* or *tesB* encoding a thioesterase and *yqeF* encoding an acyl-transferase, and deleting of both *fadB* encoding an acetyl-CoA C-acetyltransferase and *ydiO* encoding an acyl-CoA dehydrogenase, 150–500 mg/L amounts of various carboxylic acids were produced. Production of longer even-chain *n*-alcohols (C6, C8, and C10) was achieved by utilizing native ADHs (EutG, YiaY, and BetA). Lastly, generation of longer odd-chain *n*-alcohols (C5, C7, and C9) was achieved by overexpressing *yiaY*, encoding a Fe-containing ADH, and supplementing the media with propionate.

Bokinsky et al. (2011) used consolidated bioprocessing (CBP) (Lynd et al., 2002) to produce three different biofuels, including 1-butanol, using *E. coli* able to grow on and degrade ionic liquid-pretreated switchgrass (Li et al., 2010a), without the addition of exogenous glycoside hydrolases (Bokinsky et al., 2011). To degrade cellulose, they found a cellulase, when fused with OsmY, capable of being transported out of *E. coli*. Out of the two OsmY-cellulase fusions, the one that had the highest endocellulase activity was from *Bacillus* sp. D04. To degrade hemicellulose, a previously found endoxylanase from *C. stercorarium*, Xyn10B (Steen et al., 2010), was also fused to OsmY. Genes encoding this fusion were under control of a native P_{cspD} promoter in a pSC101* origin (Stoker et al., 1982) plasmid. To further break down the cello- and xylodextrins into glucose, a β-glucosidase from *Cellvibrio japonicus*, encoded by *cel3A*, was overexpressed under the control of the native P_{wrbA} promoter to degrade cellobiose, while a xylobiosidase from *C. japonicus*, encoded by *gly43F*, was overexpressed under the control of the native P_{cstA} promoter in a p15A origin (Chang and Cohen, 1978) plasmid to degrade xylodextrins. Resulting plasmids expressing cellulose and hemicellulose breakdown were named pCellulose and pXylan, respectively. By overexpressing a CoA-dependent 1-butanol pathway in an *E. coli* DH1 Δ*adhE* strain, 28 mg/L 1-butanol was produced in EZ-Rich media (Teknova) and 3.3% w/v ionic liquid-pretreated switchgrass as the only carbon source.

2-KETO ACID PATHWAYS FOR C3–C8 ALCOHOLS

Amino acid pathways are universal, and have also been harnessed for the ease in which amino acid precursors can be rerouted for production C3–C6 alcohols (**Figure 2**). The following describes research that emanates from utilization of the last two steps of the Ehrlich pathway (Sentheshanmuganathan and Elsden, 1958) to divert 2-keto acid intermediates to higher-chain alcohols in various hosts.

Atsumi et al. (2008b) reported production of several higher-chain alcohols, including isobutanol, 1-butanol, 2-methyl-1-butanol (2MB), 3-methyl-1-butanol (3MB), and 2-phenylethanol, from glucose by engineering the amino acid biosynthetic pathway in *E. coli*. In the pathway, 2-keto acids are converted to the corresponding aldehyde with a 2-keto acid decarboxylase (KDC) and then to the alcohol using an ADH. First, *ADH2* (*S. cerevisiae*) was overexpressed with genes encoding five different KDCs [Pdc6, Aro10, and Thi3 (*S. cerevisiae*), Kivd (*Lactococcus lactis*), and Pdc (*C. acetobutylicum*)] in colE origin plasmids under control of the $P_L lacO_1$ promoter to determine which combination gave the highest longer-alcohol titer. Results indicated that Kivd was the most active and had the largest substrate range, and produced all of the expected alcohols. About 22 g/L isobutanol (86% theoretical max) was produced under microaerobic conditions in 112 h by overexpressing *alsS* (*B. subtilis*), *ilvC*, *ilvD*, *kivd*, and *ADH2*, by introducing Δ*adhE*, Δ*ldhA*, Δ*frdAB*, Δ*fnr*, Δ*pta*, and Δ*pflB* knockouts in order minimize production from competing pathways. Isobutanol has an energy density of 29.0 MJ/L. In addition, about 541 mg/L 1-propanol and 667 mg/L 1-butanol was produced in M9 media (supplemented with glucose) by the non-native alcohol producing pathway. In this pathway, 2-ketobutyrate (2KB), a precursor to isoleucine, is formed via the threonine pathway, and can be converted to 1-propanol using Kivd and Adh2. On the other hand, 2KB can be extended by one carbon using LeuABCD operon to produce the unnatural amino acid norvaline precursor 2-ketovalerate, which can be converted to 1-butanol using the same KDC and ADH.

ISOBUTANOL PRODUCTION VIA THE 2-KETO ACID PATHWAY

To improve upon the amino acid-based production of isobutanol, Baez et al. (2011) compared aerobic isobutanol production in a 1 L bioreactor connected to a gas-stripping system. Using the engineered isobutanol strain (Atsumi et al., 2008b), 50 g/L isobutanol was produced in the bioreactor after 72 h (68% maximum theoretical yield) under aerobic conditions and 30°C, which is over twice as high as the 22 g/L produced in a shake flask. By-product production (except acetate) was not detectable. Efforts to knockout several acetate-producing pathways (Δ*ack* Δ*pta* Δ*poxB*), however, did not prevent accumulation of acetate.

Microaerobic conditions have been used to produce isobutanol. For a large-scale production of isobutanol, however, anaerobic production is preferred. A major barrier that must be overcome is NADPH dependency. Since two of the enzymes in the pathway are

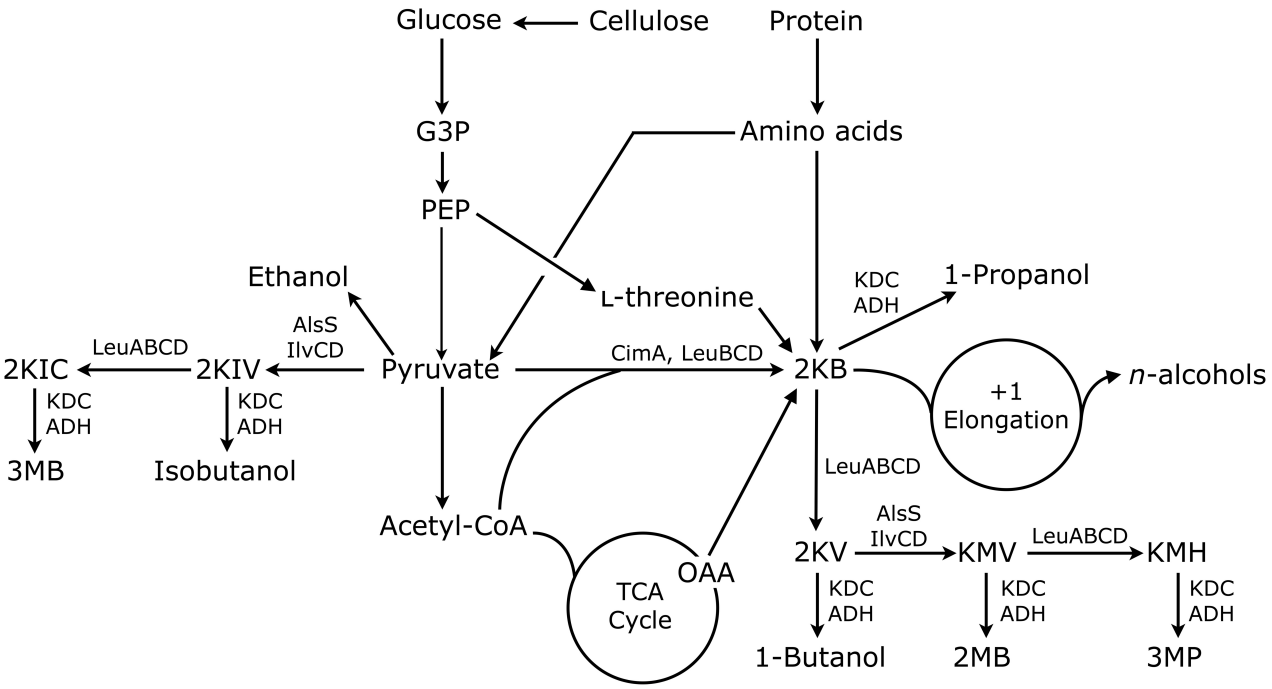


FIGURE 2 | Metabolic representation of 2-keto acid pathways for biofuel production. G3P, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate; AlsS, acetolactate synthase; IlvC, acetohydroxy acid isomeroreductase; IlvD, dihydroxy-acid dehydratase; LeuA, isopropylmalate synthase; LeuB, isopropylmalate dehydrogenase; LeuCD, isopropylmalate dehydratase; CimA, citramalate synthase; 2KIC, 2-ketoisocaproate; 3MB, 3-methyl-1-butanol; 2KIV, 2-ketoisovalerate; 2KB, 2-ketobutyrate; 2KV, 2-ketovalerate; KMV, 2-keto-3-methylvalerate; 2MB, 2-methyl-1-butanol; KMH, 2-keto-4-methylhexanoate; 3MP, 3-methyl-1-pentanol; OAA, oxaloacetate.

NADPH-dependent, the pentose phosphate pathway and tricarboxylic acid cycles can regenerate this cofactor only in the presence of oxygen. Under anaerobic conditions, however, NADH is produced as a result of glycolysis. To overcome this cofactor imbalance issue, Bastian et al. (2011) constructed an NADH-dependent pathway by engineering a ketol-acid reductoisomerase IlvC (*E. coli*) and ADH AdhA (*L. lactis*). Directed evolution approaches (Romero and Arnold, 2009) were applied to IlvC and AdhA to switch cofactor dependence from NADPH to NADH. In an anaerobic, fully NADH-dependent strain containing $AdhA^{RE1}$ and $IlvC^{6E6\text{-}his6}$, 13.4 g/L isobutanol at 100% theoretical maximum yield was produced in M9 media supplemented with glucose, yeast extract, and trace metals (hereafter modified M9). Overexpression of *pntAB*, which enabled reversible transfer of a hydride ion between NADH and NADPH, with *ilvC* and *yqhD* overcame the cofactor imbalance, but produced at a titer and theoretical yield slightly lower than the strain overexpressing NADH-utilizing engineered $IlvC^{6E6\text{-}his6}$ and $AdhA^{RE1}$.

Another host used for isobutanol production is the cyanobacterium *S. elongatus* PCC7942, a photosynthetic organism that Atsumi et al. (2009) engineered to convert CO_2 and light energy directly into isobutyraldehyde, a precursor to isobutanol. To install the isobutyraldehyde production pathway *in S. elongatus*, *kivd* (*L. lactis*), under P_{trc} control, was integrated into NSI (Bustos and Golden, 1992), and *alsS* (*B. subtilis*), *ilvC*, and *ilvD* (*E. coli*), under $P_L lacO_1$ control, was integrated into NSII (Andersson et al., 2000) of the genome by homologous recombination (Golden et al., 1987). Production in a Roux culture bottle at 30°C and subsequent gas-stripping of isobutyraldehyde resulted in 723 mg/L after 12 days and a production rate of 2,500 μg/L·h. No isobutanol was detected, most likely because the endogenous ADH had no detectable activity toward isobutyraldehyde. One known bottleneck to the pathway is a low activity of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) in fixing CO_2 within the Calvin–Benson–Bassham cycle. To improve isobutyraldehyde production, the *rbcLS* genes from a related *S. elongatus*, PCC6301, was inserted downstream from the endogenous *rbcLS* genes. With an increased Rubisco activity, the resulting strain produced 1.1 g/L isobutyraldehyde after 8 days and a production rate of 6,230 μg/L·h. Isobutanol production was also measured by integrating NADPH-dependent ADH, encoded by *yqhD*, in addition to the isobutyraldehyde pathway. This strain produced 450 mg/L isobutanol after 9 days.

Isobutanol production in Gram-positive *Corynebacterium glutamicum*, an industrial amino acid producer (Kinoshita, 1972; Sahm et al., 1996; Wendisch, 2007) has also been achieved. Smith et al. (2010) explored isobutanol production using *C. glutamicum* because *C. glutamicum* was chosen for its rapid growth rate and vast range of tools for genetic manipulation. Furthermore, *C. glutamicum* has been modified to produce L-valine in industrial quantities, and the isobutanol pathway utilizes its precursors. In this system, *alsS* (*B. subtilis*), *ilvC*, *ilvD*, *adhA* (*C. glutamicum*), and *kivd* (*L. lactis*), in a P_{eftu}-controlled, NG2 origin (Messerotti et al., 1990) plasmid, were overexpressed in *C. glutamicum* and

resulted in 2.6 g/L isobutanol, 50 mg/L isobutyraldehyde, 0.4 g/L 3MB, as well as other various higher-chain alcohols, after 48 h in CGIII media supplemented with glucose (Menkel et al., 1989). To prevent direct formation of oxaloacetate from pyruvate, the *pyc* gene (encoding pyruvate carboxylase) was inactivated. In order to inactivate a desired gene in Gram-positive organisms like *C. glutamicum*, a pK-derived mobilizable cloning vector containing a fragment of the gene is first constructed (Schafer et al., 1994). Then, RP4-mediated conjugation occurs (Haase et al., 1995), where the pK-derived plasmid is first incorporated into the genome, then subsequently disrupted. Essentially, a double cross-over event is carried out. As a result of the Δ*pyc* knockout, isobutanol titer increased to 4.3 g/L. Despite this increase, 2.6 g/L of lactate formed. To inhibit formation of lactate from pyruvate and NADH, an additional knockout, Δ*ldh*, which would have encoded lactate dehydrogenase, was introduced. In the highest-producing *C. glutamicum* Δ*pyc* Δ*ldh* knockout strain (containing NG2 origin P_{eftu}::*alsS-ilvCD-adhA-kivd*), 4.9 g/L isobutanol was produced after 120 h (23% theoretical maximum) using a fermenter.

Li et al. (2011) engineered *B. subtilis* for production of isobutanol. *B. subtilis* was a host of interest because it was found to have a higher isobutanol tolerance (2%) than both *C. glutamicum* and *E. coli* (1.9- and 3.8-fold, respectively). In a strain containing Δ*amyE* and a two-plasmid system (P_{43}::*ilvD-ilvC-alsS* and P_{43}::*kivd-adh2*), 2.02 g/L isobutanol was produced after 40 h under 37°C and microaerobic conditions. In addition, lower titers of ethanol, 1-phenylethanol, 2MB, and 3MB were produced. Acetate and lactate were also detected in the production culture. To improve titer, batch and fed-batch fermentations in 1 L shake flasks were performed under microaerobic conditions. After 48 h, the fed-batch fermentation in LB media supplemented with glucose, phosphate buffer, and trace metals produced 2.62 g/L isobutanol at a 0.086 g/L/h production rate.

Higashide et al. (2011) used the cellulolytic mesophile *C. cellulolyticum* to convert crystalline cellulose into isobutanol. *C. cellulolyticum* uses an extracellular, multi-enzymatic cellulosome to breakdown and consume cellulose and other complex polysaccharides efficiently (Desvaux, 2005). In a strain overexpressing *kivd*, *yqhD*, *alsS*, *ilvC*, and *ilvD* under the control of a constitutive ferredoxin *C. pasteurianum* promoter, 364 mg/L isobutanol was produced in 90 h from cellobiose, while 660 mg/L isobutanol in 9 days was produced from cellulose. Two major setbacks to the successful production of isobutanol in *C. cellulolyticum* using a CBP platform were (1) lack of an available inducible expression system and the resulting toxicity of gene products, and (2) lack of detectable activity of IlvC and IlvD (due to differences in GC content and codon usage frequencies between *E. coli* and *C. cellulolyticum*).

Chen et al. (2011) engineered *S. cerevisiae* to produce isobutanol. To construct the pathway, an acetolactate synthase, encoded by *ILV2* (catalytic subunit) and *ILV6* (regulatory subunit), converts two pyruvate molecules into 2-acetolactate, which is subsequently reduced to 2,3-dihydroxyisovalerate by an acetohydroxy acid reductoisomerase, encoded by *ILV5*. Then, 2-ketoisovalerate (2KIV) is produced using a dihydroxyacid dehydratase, encoded by *ILV3*. Next, two branched-chain amino acid aminotransferases, encoded by mitochondrial *BAT1* and cytosolic *BAT2*, reversibly converts 2KIV into L-valine. Afterward, isobutanol is formed using pyruvate decarboxylases (Pdc6, 5, 1) and ADHs. In a strain overexpressing *ILV2*, *ILV3*, and *ILV5* (in plasmids under control of the PGK1 promoter) led to 4.12 mg isobutanol/g glucose when cultivated under aerobic conditions in pH-maintained minimal media supplemented with yeast extract, peptone, dextrose, and 40 g/L glucose. Under anaerobic conditions and minimal media supplemented with 40 g/L glucose, isobutanol yield was 2.13 mg/g glucose in a strain that overexpressed *BAT2* in addition to *ILV2*, *ILV3*, and *ILV5*.

To improve upon the isobutanol titer in *S. cerevisiae*, Kondo et al. (2012) searched for and overexpressed *KDC* and *ADH* genes from different sources. The following three *KDC* enzymes were tested: endogenous phenylpyruvate decarboxylase (Aro10), endogenous 2-ketoisocaproate decarboxylase (Thi3), and 2KIV decarboxylase (Kivd) from *L. lactis*. In addition, six ADH genes from *S. cerevisiae*, encoding for Adh1, Adh2, Adh5, Adh6, Adh7, and Sfa1, were overexpressed in plasmids under control of the PGK1 promoter. Different KDC–ADH combinations were used to measure isobutanol levels under additive additions of 8 g/L 2KIV, the precursor to isobutanol. A *kivd-ADH6* combination gave the highest isobutanol level at 488 mg/L, and was used for isobutanol production. To reduce ethanol production, *ILV2* encoding an acetolactate synthase was overexpressed (catalyzes the first committed step in valine biosynthesis from pyruvate). Furthermore, to prevent acetaldehyde production, *pdc1*, which encodes an isozyme of the pyruvate dehydrogenase complex, was deleted. This strain was cultured in synthetic dextrose media supplemented with yeast nitrogen base and glucose, and produced 75 mg/L after 72 h. Under microaerobic conditions, titer rose to 143 mg/L after 120 h at a yield of 6.6 mg/g glucose.

1-PROPANOL AND 1-BUTANOL PRODUCTION VIA THE 2-KETO ACID PATHWAY

Shen and Liao (2008) improved a keto acid-based pathway in *E. coli* to produce 1-propanol (contains an energy density of 27 MJ/L) and 1-butanol from glucose (Atsumi et al., 2008b; Shen and Liao, 2008). To deregulate threonine synthesis and increase 2-keto acid intermediates for alcohol production, a feedback-resistant $ThrA^{fbr}$ was used. In addition, several competing pathways were deleted to increase flux toward 2-keto acid production. The final strain containing Δ*metA* Δ*tdh* Δ*ilvB* Δ*ilvI* Δ*adhE* and overexpression of *thrA*^{fbr}*BC*, *ilvA*, *leuABCD* (*E. coli*), *kivd* (*L. lactis*), and *ADH2* (*S. cerevisiae*), in p15A (Chang and Cohen, 1978) or colE (Kahn et al., 1979) origin plasmids and all under the control of the $P_L lacO_1$ promoter, produced 2 g/L 1:1 coproduction of 1-butanol and 1-propanol after 72 h in modified M9 media under anaerobic conditions.

Atsumi and Liao (2008) improved production of 1-propanol and 1-butanol by evolving a citramalate synthase (CimA) from the extreme thermophile *Methanococcus jannaschii*. CimA is a homolog of LeuA that implements a more direct route than the threonine pathway toward 2KB. In addition, bypassing the threonine pathway to generate 2KB (Δ*ilvA* and Δ*tdcB*) eliminates the liberation of NH_3 due to deamination of threonine. CimA catalyzes the addition of an acetyl group from acetyl-CoA onto

pyruvate to synthesize citramalate, which then undergoes transformations catalyzed by LeuBCD to produce 2KB. 1-Propanol and 1-butanol can then be produced using the same enzymes expressed by *kivd* (*L. lactis*) and *ADH2* (*S. cerevisiae*; Atsumi et al., 2008b). To improve activity of CimA toward pyruvate, several rounds of random mutagenesis and DNA shuffling were used to mutate the *cimA* gene, and improved variants were screened for using a growth-based selection. That is, in a strain containing Δ*ilvA* and Δ*tdcB*, isoleucine cannot be produced, and thus the strain cannot grow, unless CimA is active in the cell. The strain containing the evolved *cimA*, overexpressed in a $P_L lacO_1$-controlled, p15A origin (Chang and Cohen, 1978) plasmid, anaerobically produced 2.8 g/L 1-propanol and 393 mg/L 1-butanol after 40 h at 30°C in modified M9 media.

3-METHYL-1-BUTANOL PRODUCTION VIA THE 2-KETO ACID PATHWAY

Connor and Liao (2008) took advantage of native amino acid biosynthetic pathways to produce 3MB, another higher-chain alcohol with an energy density of 30.5 MJ/L, in *E. coli* from glucose. To produce 3MB, the *ilvIHCD* genes were overexpressed to convert pyruvate into 2KIV, a precursor to the amino acid valine, which can be converted to 2-ketoisocaproate, a precursor to the amino acid leucine, by LeuABCD enzymes. Finally, 2-ketoisocaproate can then be redirected by Kivd and Adh2 to produce 3MB. The initial strain produced 56 mg/L 3MB after 18 h in modified M9 media under anaerobic conditions at 30°C. To increase 3MB titer, several modifications were introduced. First, *alsS* (*B. subtilis*), previously shown to increase isobutanol production via valine synthesis, was overexpressed in place of *ilvIH* in a pSC101 origin (Stoker et al., 1982) plasmid under $P_L lacO_1$ control (Lutz, 1997). To increase production of 2KIV, the *leuABCD* genes were overexpressed in a colE origin (Kahn et al., 1979), $P_L lacO_1$-controlled plasmid. Furthermore, genes expressing enzymes involved in competing pathways were deleted (Δ*adhE* Δ*frdBC* Δ*ldhA* Δ*pta* Δ*fnr* Δ*pflB* Δ*ilvE* Δ*tyrB*) from the genome. Lastly, a feedback-insensitive $LeuA^{fbr}$ (Gusyatiner et al., 2002) was used to deregulate leucine synthesis and increase flux toward 2-ketoisocaproate. The final strain produced 1.28 g/L 3MB (0.11 g/g yield) and 0.2 g/L isobutanol after 28 h under anaerobic conditions.

Expanding upon the 3MB pathway (Connor and Liao, 2008), Connor et al. (2010) performed several rounds of random whole-cell mutagenesis with *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine and selection with amino acid analog 4-aza-D,L-leucine (AZL) to produce a 3MB hyper-producer. If the cell can outcompete production of this L-leucine analog by producing much more natural L-leucine – in turn increasing production of 3MB precursor 2-ketoisocaproate – then the cell will circumvent the occurrence of fatal incorporation of AZL into polypeptides. Therefore, selecting for mutants able to adapt to the addition of toxic levels of AZL by elevating production of natural amino acids should increase 3MB titer. Using a mutant able to outcompete production of AZL, 4.4 g/L 3MB was produced with a theoretical yield of 30% after 36 h in under anaerobic conditions. In addition to mutagenesis and selection for higher 3MB producers, a two-phase fermentation strategy with oleyl alcohol was implemented in order to separate 3MB from the production phase and minimize toxic concentrations of 3MB, thus extending production lifetime of the engineered 3MB hyper-producer. Using this approach with a mutated 3MB strain resulted in a titer of 9.6 g/L at 33% theoretical maximum after 60 h. Further analysis of the two-phase culture revealed that 90% of 3MB and 80% of isobutanol produced was present in the oleyl alcohol phase.

2-METHYL-1-BUTANOL PRODUCTION VIA THE 2-KETO ACID PATHWAY

Cann and Liao (2008) demonstrated the production of 2MB, a branched-chain alcohol with an energy density of 30.5 MJ/L, via the precursor for isoleucine, 2-keto-3-methylvalerate (KMV), in *E. coli* from glucose. In this study, different isozymes of key enzymes along the constructed 2MB pathway were tested and selected based on 2MB versus 1-propanol titer. Both 2MB and 1-propanol are derivatives of 2-keto acids, and involve the use of a broad-substrate range KDC to convert the corresponding 2-keto acid into an aldehyde, then reduce it into the alcohol using an ADH. Further, an acetohydroxy acid synthase (AHAS) catalyzes the first step in isoleucine synthesis, where 2KB and pyruvate are condensed to make 2-aceto-2-hydroxybutyrate, a precursor to 2MB. Therefore, an ideal AHAS for 2MB production was chosen based on how well it outcompeted KDC for 2KB. If the AHAS outcompetes KDC, then 2MB production would be favored over 1-propanol production. First, several AHAS isozymes were tested for rate of conversion of 2KB to 2MB versus 1-propanol. It was found that AHAS II (encoded by *ilvGM*) from *Salmonella typhimurium* produced the highest amount of 2MB and minimal 1-propanol in modified M9 media supplemented with 8 g/L 2KB. Next, various threonine deaminase isozymes were overexpressed in p15A origin (Chang and Cohen, 1978) plasmids under $P_L lacO_1$ control to analyze how well supplied L-threonine is consumed and converted into 2MB and 1-propanol. The strain containing the best threonine deaminase coded by *ilvA* (*C. glutamicum*) converted about 88% of supplied L-threonine into 2MB and 1-propanol. Then, the threonine pathway was overexpressed (*thrABC*). Lastly, several combinations of Δ*metA* Δ*tdh* Δ*ilvB* Δ*ilvI* Δ*leuA* and Δ*ilvE* deletions were tested for 2MB production. The best combination was determined to be Δ*metA* and Δ*tdh*, which eliminated carbon flux toward methionine and consumption of threonine, respectively. The resulting *E. coli* strain produced, from glucose, 1.25 g/L 2MB (44% theoretical maximum) in 24 h at 30°C under anaerobic conditions.

C6–C8 ALCOHOL PRODUCTION VIA THE 2-KETO ACID PATHWAY

Zhang et al. (2008) expanded the 2-keto acid-based pathway for the production of higher-chain alcohols (C > 5) by using structure-based protein engineering to expand the substrate range of 2-isopropylmalate synthase (LeuA) and 2KIV decarboxylase (Kivd) from *L. lactis*, which are both already known to be promiscuous enzymes. First, Kivd was engineered to be more selective toward 2-keto-4-methylhexanoate, a non-natural precursor of 3-methyl-1-pentanol. To do this, a sequence alignment was done on indolepyruvate decarboxylase (IPDC) of *Enterobacter cloacae* and pyruvate decarboxylase (PDC) from *Zymomonas*

mobilis, each having a 40 and 31% homology to Kivd, respectively. Investigation of residues within the keto acid-binding pocket of each enzyme led to an F381L/V461A Kivd variant. To engineer LeuA, sequence alignment of LeuA from *S. typhimurium* and *Mycobacterium tuberculosis* showed 92 and 21% homology to that of *E. coli*, respectively. In addition to the G462D point mutation to deregulate valine synthesis, S139G mutation was inserted to accommodate the extra methyl group (S)-2-keto-3-methylvalerate contains. In an *E. coli* strain containing both Kivd and LeuA mutants, 793.5 mg/L 3-methyl-1-pentanol was produced in modified M9 media. Additional point mutations into smaller amino acids in Kivd were included to accommodate larger keto acid intermediates. Specifically, the addition of an N167A point mutation to the double mutant allowed for the production of 51.9 mg/L 4-methyl-1-hexanol. Moreover, incorporation of H97A to the triple mutant resulted in production of 57.3 mg/L 4-methyl-1-hexanol, as well as 22.0 mg/L 5-methyl-1-heptanol.

Marcheschi et al. (2012) modified LeuA further with structure-based protein engineering to increase its promiscuity toward larger 2-keto acids. These larger 2-keto acids, in turn, can be directed toward higher-chain alcohol production (C > 5). Furthermore, quantum mechanical modeling and protein–substrate complex modeling were used to simulate and analyze the different transition states of the carbon–carbon bond forming steps that are catalyzed by candidate LeuA variants (LeuA*) in order to determine the best conformers. Using this approach, mutants that cater to a 2-keto acid of interest can be predicted, whether it contains an aromatic, linear-chained, or branched-chain group. In a threonine hyperproducer strain containing a Δ*rhtA* (encoding threonine transporter gene), and three plasmid [with pSC101 (Stoker et al., 1982), p15A (Chang and Cohen, 1978), or colE (Kahn et al., 1979) replication origin], $P_L lacO_1$-controlled (Lutz, 1997) overexpression of *thrABC* (threonine pathway genes), *ilvA* (*B. subtilis*) *leuA*BCD*, *kivd* (F381L/V461A, *L. lactis*), and *ADH6* (*S. cerevisiae*), production of C4–C8 alcohols in modified M9 media was measured. There were four notable LeuA* variants. LeuA* containing S139G/G462D produced 993 mg/L and 2.22 g/L 1-butanol and 1-pentanol, respectively. LeuA* H97A/S139G/G462D produced 302 mg/L 1-hexanol. Lastly, LeuA* H97A/S139G/P169A/G462D produced 80 and 2 mg/L 1-heptanol and 1-octanol, respectively.

PROTEIN CONVERSION TO C4–C5 ALCOHOLS

Apart from the use of glucose as a feedstock for biofuel production, Huo et al. (2011) explored the use of protein biomass as a potential feedstock. *E. coli* was engineered to generate protein hydrolysates in order to breakdown proteins in three exogenous transamination and deamination cycles from bacteria, yeast, and microalgae into amino acids, which can then be converted to the corresponding 2-keto acid intermediates, and finally into C2–C6 alcohols by mimicking the Ehrlich pathway. Any reduced nitrogen, i.e., NH_3, produced is excreted irreversibly from the cell, and can be collected and recycled as fertilizer. In addition, leftover amino acids can be used as raw material for various chemicals and pharmaceutical intermediates, or as a supplement for animal feedstocks. The final strain [YH19Δ*glnA*Δ*gdhA*Δ*lsrA* overexpressing *alsS*, *ilvC*, *ilvD*, and *avtA* in a p15A origin (Chang and Cohen, 1978), $P_L lacO_1$-controlled (Lutz, 1997) plasmid, *kivD*, *yqhD*, *leuDH*, and *ilvE*, in a colE origin (Kahn et al., 1979), $P_L lacO_1$-controlled plasmid, and *sdaB* and *ilvA* in a pSC101 origin (Stoker et al., 1982), $P_L lacO_1$-controlled plasmid] produced 4 g/L C4 and C5 alcohols at 56% theoretical yield from amino acids.

ISOPENTENOL PRODUCTION VIA THE MEVALONATE ISOPRENOID PATHWAY

Isoprenoids have a vast range of uses, including for nutraceuticals, therapeutics, polymers, and fragrances. They are hydrocarbons that stem from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) precursors, which are produced from acetyl-CoA or pyruvate and glyceraldehyde-3-phosphate using the mevalonate (Goldstein and Brown, 1990) or methylerythritol (Lichtenthaler, 2000) pathways, respectively. The mevalonate isoprenoid pathway has been engineered and exploited for production of isopentenol from acetyl-CoA.

To produce isopentenol from isoprenoid precursors IPP and DMAP (**Figure 3**), Withers et al. (2007) first used a growth-based phenotype to screen a library of isoprene synthases from *B. subtilis* 6051 based on ability to minimize toxic levels of prenyl diphosphates by converting them into prenyl alcohols. One of two genes

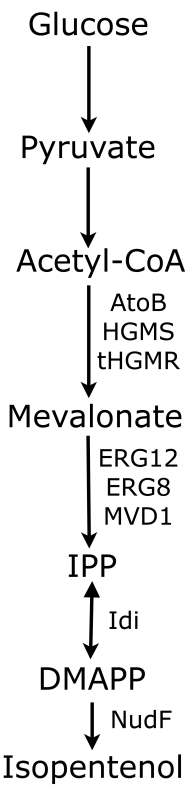


FIGURE 3 | Metabolic representation of mevalonate isoprenoid pathway for biofuel production. AtoB, acetyl-CoA thiolase; HGMS, HMG-CoA synthase; tHGMR, truncated HMG-CoA reductase; ERG12, mevalonate kinase; ERG8, phosphomevalonate kinase; IPP, isopentenyl diphosphate; Idi, IPP isomerase; DMAP, dimethylallyl diphosphate; NudF, ADP-ribose pyrophosphatase.

isolated from a pool of 19,000 clones, *nudF*, was overexpressed in a colE origin (Kahn et al., 1979) plasmid under control of a native *Bacillus* promoter in conjunction with the mevalonate pathway plasmids pMevT (p15 origin) and pMBI (Bbr origin) in the previously optimized *E. coli* DH1 strain (Martin et al., 2003), and produced 110 mg/L isopentenol after 43 h at 30°C under aerobic conditions in 250 mL shake flasks containing M9 media supplemented with 3-(*N*-morpholino)propanesulfonic acid, glucose, and yeast extract.

CONCLUSION

The evolving field of synthetic biology has helped widen the range of modular possibilities in which cell system and behavior can be modified, and will continue to be an influence in the development and improvement of biological methods for biofuel production. In recent years, there has been a growing number of biotech companies that are applying these methods in order to produce higher-chain alcohols commercially. Namely, both Gevo and Butamax have industrialized isobutanol production platforms. But as titers in an engineered host continue to elevate (**Table 1**), and experimental yields start to match up with theoretical maximums, the resulting increase in productivity comes with a price.

One prominent barrier is the toxic accumulation of the target molecule that stunts cell fitness during production, which, in turn, can hinder a newly engineered strain from producing at industrial-relevant titers. Thus, increasing the product tolerance of, as well as simultaneous product removal from, a host is desirable. Fortunately, there are several approaches that have been developed to help mitigate toxicity levels during the production phase (Zheng et al., 2009; Atsumi et al., 2010; Ezeji et al., 2010; Dunlop, 2011). There is, however, much to be elucidated about the convoluted physiological effects that occur in a host when exposed to elevated levels of a final product.

Development of a process to breakdown biomass effectively and efficiently is also vital to decreasing production time and costs. Until then, CBP is yet another approach worth considering in increasing titer, yield, and productivity. A unified production platform in which biomass can be broken down efficiently and subsequently converted into fuel can be practical, especially if paired with product removal methods. Meanwhile, development of a production platform that uses sunlight and CO_2 directly, such as cyanobacteria and microalgae is well worth the effort, and is yet another approach that should be considered in biofuel research. Especially, this approach circumvents the need for a biomass source, and can be cultivated in non-arable areas, i.e., oceans and deserts.

The race to reach a renewable fuel production level of 36 billion gallons by 2022 continues as the production of advanced biofuels gains momentum. For 2012, the Renewable Fuel Standard (RFS2) has established a goal to produce 15.2 billion gallons of biofuels, which already approaches half the mandate for 2022. Although the process to become independent from petroleum-derived fuels is still in its initial stages, efforts to increase synergy between all aspects of strain design, development, optimization, and execution for renewable higher-chain alcohols and other liquid fuel production in non-native hosts will be a great aid in leading the way toward a permanent turn of tables.

Table 1 | Highest reported higher-chain alcohol titers in *E. coli*.

Higher-chain alcohol	Titer (g/L)	Reference
1-Propanol	2.8	Atsumi and Liao (2008)
Isopropanol	143	Inokuma et al. (2010)
1-Butanol	30	Shen et al. (2011)
Isobutanol	50	Baez et al. (2011)
1-Pentanol	2.22	Marcheschi et al. (2012)
Isopentenol	0.11	Martin et al. (2003)
2-Methyl-1-butanol	1.25	Cann and Liao (2008)
3-Methyl-1-butanol	9.6	Connor and Liao (2008)
1-Hexanol	0.047	Dekishima et al. (2011)
3-Methyl-1-pentanol	0.794	Zhang et al. (2008)
1-Heptanol	0.08	Marcheschi et al. (2012)
4-Methyl-1-hexanol	0.057	Zhang et al. (2008)
5-Methyl-1-hexanol	0.022	Zhang et al. (2008)

ACKNOWLEDGMENT

This work was supported partly by NSF grant 1066182.

REFERENCES

Agapakis, C. M., and Silver, P. A. (2009). Synthetic biology: exploring and exploiting genetic modularity through the design of novel biological networks. *Mol. Biosyst.* 5, 704–713.

Alper, H., and Stephanopoulos, G. (2009). Engineering for biofuels: exploiting innate microbial capacity or importing biosynthetic potential? *Nat. Rev. Microbiol.* 7, 715–723.

Amberg, D. C., Burke, D. J., and Strathern, J. N. (2005). *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual.* New York: Cold Spring Harbor Laboratory Press.

Ami, D., Natalello, A., Schultz, T., Gatti-Lafranconi, P., Lotti, M., Doglia, S. M., and De Marco, A. (2009). Effects of recombinant protein misfolding and aggregation on bacterial membranes. *Biochim. Biophys. Acta* 1794, 263–269.

Andersson, C. R., Tsinoremas, N. F., Shelton, J., Lebedeva, N. V., Yarrow, J., Min, H., and Golden, S. S. (2000). Application of bioluminescence to the study of circadian rhythms in cyanobacteria. *Methods Enzymol.* 305, 527–542.

Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J., Hanai, T., and Liao, J. C. (2008a). Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.* 10, 305–311.

Atsumi, S., Hanai, T., and Liao, J. C. (2008b). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89.

Atsumi, S., Higashide, W., and Liao, J. C. (2009). Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* 27, 1177–1180.

Atsumi, S., and Liao, J. C. (2008). Directed evolution of *Methanococcus jannaschii* citramalate synthase for biosynthesis of 1-propanol and 1-butanol by *Escherichia coli*. *Appl. Environ. Microbiol.* 74, 7802–7808.

Atsumi, S., Wu, T. Y., Machado, I. M., Huang, W. C., Chen, P. Y., Pellegrini, M., and Liao, J. C. (2010). Evolution, genomic analysis, and reconstruction of isobutanol tolerance in *Escherichia coli*. *Mol. Syst. Biol.* 6, 449.

Baez, A., Cho, K. M., and Liao, J. C. (2011). High-flux isobutanol production using engineered *Escherichia coli*: a bioreactor study with in situ product removal. *Appl. Microbiol. Biotechnol.* 90, 1681–1690.

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and

misfolding in *Escherichia coli*. *Nat. Biotechnol.* 22, 1399–1408.

Bastian, S., Liu, X., Meyerowitz, J. T., Snow, C. D., Chen, M. M., and Arnold, F. H. (2011). Engineered ketol-acid reductoisomerase and alcohol dehydrogenase enable anaerobic 2-methylpropan-1-ol production at theoretical yield in *Escherichia coli*. *Metab. Eng.* 13, 345–352.

Bennett, G. N., and Rudolph, F. B. (1995). The central metabolic pathway from acetyl-CoA to butyryl-coA in *Clostridium acetobutylicum*. *FEMS Microbiol. Rev.* 17, 241–249.

Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., Soon Lee, T., Tullman-Ercek, D., Voigt, C. A., Simmons, B. A., and Keasling, J. D. (2011). Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19949–19954.

Bond-Watts, B. B., Bellerose, R. J., and Chang, M. C. (2011). Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. *Nat. Chem. Biol.* 7, 222–227.

Brosius, J., Erfle, M., and Storella, J. (1985). Spacing of the -10 and -35 regions in the *tac* promoter. Effect on its in vivo activity. *J. Biol. Chem.* 260, 3539–3541.

Bustos, S. A., and Golden, S. S. (1992). Light-regulated expression of the psbD gene family in *Synechococcus* sp. strain PCC 7942: evidence for the role of duplicated psbD genes in cyanobacteria. *Mol. Gen. Genet.* 232, 221–230.

Cann, A. F., and Liao, J. C. (2008). Production of 2-methyl-1-butanol in engineered *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 81, 89–98.

Chang, A. C., and Cohen, S. N. (1978). Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J. Bacteriol.* 134, 1141–1156.

Chen, X., Nielsen, K. F., Borodina, I., Kielland-Brandt, M. C., and Karhumaa, K. (2011). Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism. *Biotechnol. Biofuels* 4, 21.

Connor, M. R., and Atsumi, S. (2010). Synthetic biology guides biofuel production. *J. Biomed. Biotechnol.* 2010, 541698.

Connor, M. R., Cann, A. F., and Liao, J. C. (2010). 3-Methyl-1-butanol production in *Escherichia coli*: random mutagenesis and two-phase fermentation. *Appl. Microbiol. Biotechnol.* 86, 1155–1164.

Connor, M. R., and Liao, J. C. (2008). Engineering of an *Escherichia coli* strain for the production of 3-methyl-1-butanol. *Appl. Environ. Microbiol.* 74, 5769–5775.

de Bont, J. (1998). Solvent-tolerant bacteria in biocatalysis. *Trends Biotechnol.* 16, 493–499.

Dekishima, Y., Lan, E. I., Shen, C. R., Cho, K. M., and Liao, J. C. (2011). Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered *Escherichia coli*. *J. Am. Chem. Soc.* 133, 11399–11401.

Dellomonaco, C., Clomburg, J. M., Miller, E. N., and Gonzalez, R. (2011). Engineered reversal of the beta-oxidation cycle for the synthesis of fuels and chemicals. *Nature* 476, 355–359.

Dellomonaco, C., Rivera, C., Campbell, P., and Gonzalez, R. (2010). Engineered respiro-fermentative metabolism for the production of biofuels and biochemicals from fatty acid-rich feedstocks. *Appl. Environ. Microbiol.* 76, 5067–5078.

Desvaux, M. (2005). *Clostridium cellulolyticum*: model organism of mesophilic cellulolytic clostridia. *FEMS Microbiol. Rev.* 29, 741–764.

Dos Santos, V. A., Heim, S., Moore, E. R., Stratz, M., and Timmis, K. N. (2004). Insights into the genomic basis of niche specificity of *Pseudomonas putida* KT2440. *Environ. Microbiol.* 6, 1264–1286.

Dunlop, M. J. (2011). Engineering microbes for tolerance to next-generation biofuels. *Biotechnol. Biofuels* 4, 32.

Earl, A. M., Losick, R., and Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. *Trends Microbiol.* 16, 269–275.

Elvin, C. M., Thompson, P. R., Argall, M. E., Hendry, P., Stamford, N. P., Lilley, P. E., and Dixon, N. E. (1990). Modified bacteriophage lambda promoter vectors for overproduction of proteins in *Escherichia coli*. *Gene* 87, 123–126.

Ezeji, T., Milne, C., Price, N. D., and Blaschek, H. P. (2010). Achievements and perspectives to overcome the poor solvent resistance in acetone and butanol-producing microorganisms. *Appl. Microbiol. Biotechnol.* 85, 1697–1712.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. (2007). Bioproduction of butanol from biomass: from genes to bioreactors. *Curr. Opin. Biotechnol.* 18, 220–227.

Fairley, P. (2011). Introduction: next generation biofuels. *Nature* 474, S2–S5.

Fichman, B. T. (2011). Annual energy review 2010. *E. I. Administration*.

Fischer, C. R., Klein-Marcuschamer, D., and Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metab. Eng.* 10, 295–304.

Fortman, J. L., Chhabra, S., Mukhopadhyay, A., Chou, H., Lee, T. S., Steen, E., and Keasling, J. D. (2008). Biofuel alternatives to ethanol: pumping the microbial well. *Trends Biotechnol.* 26, 375–381.

Fujita, Y., Matsuoka, H., and Hirooka, K. (2007). Regulation of fatty acid metabolism in bacteria. *Mol. Microbiol.* 66, 829–839.

Georgiou, G., and Valax, P. (1996). Expression of correctly folded proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 7, 190–197.

Gheshlaghi, R., Scharer, J. M., Moo-Young, M., and Chou, C. P. (2009). Metabolic pathways of clostridia for producing butanol. *Biotechnol. Adv.* 27, 764–781.

Golden, S. S., Brusslan, J., and Haselkorn, R. (1987). Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol.* 153, 215–231.

Goldstein, J. L., and Brown, M. S. (1990). Regulation of the mevalonate pathway. *Nature* 343, 425–430.

Gronenborn, B. (1976). Overproduction of phage lambda repressor under control of the *lac* promotor of *Escherichia coli*. *Mol. Gen. Genet.* 148, 243–250.

Gulevich, A. Y., Skorokhodova, A. Y., Sukhozhenko, A. V., Shakulov, R. S., and Debabov, V. G. (2012). Metabolic engineering of *Escherichia coli* for 1-butanol biosynthesis through the inverted aerobic fatty acid beta-oxidation pathway. *Biotechnol. Lett.* 34, 463–469.

Gusyatiner, M. M., Lunts, M. G., Kozlov, Y. I., Ivanovskaya, L. V., and Voroshilova, E. B. (2002). *DNA coding for mutant isopropylmalate synthase L-leucine producing microorganism and method for producing L-leucine. U.S. Patent 6,403,342.*

Haase, J., Lurz, R., Grahn, A. M., Bamford, D. H., and Lanka, E. (1995). Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. *J. Bacteriol.* 177, 4779–4791.

Haldimann, A., Daniels, L. L., and Wanner, B. L. (1998). Use of new methods for construction of tightly regulated arabinose and rhamnose promoter fusions in studies of the *Escherichia coli* phosphate regulon. *J. Bacteriol.* 180, 1277–1286.

Hanai, T., Atsumi, S., and Liao, J. C. (2007). Engineered synthetic pathway for isopropanol production in *Escherichia coli*. *Appl. Environ. Microbiol.* 73, 7814–7818.

Herrera, S. (2006). Bonkers about biofuels. *Nat. Biotechnol.* 24, 755–760.

Higashide, W., Li, Y., Yang, Y., and Liao, J. C. (2011). Metabolic engineering of *Clostridium cellulolyticum* for production of isobutanol from cellulose. *Appl. Environ. Microbiol.* 77, 2727–2733.

Huberman, J. A., Spotila, L. D., Nawotka, K. A., El-Assouli, S. M., and Davis, L. R. (1987). The in vivo replication origin of the yeast 2 microns plasmid. *Cell* 51, 473–481.

Huo, Y. X., Cho, K. M., Rivera, J. G., Monte, E., Shen, C. R., Yan, Y., and Liao, J. C. (2011). Conversion of proteins into biofuels by engineering nitrogen flux. *Nat. Biotechnol.* 29, 346–351.

Inokuma, K., Liao, J. C., Okamoto, M., and Hanai, T. (2010). Improvement of isopropanol production by metabolically engineered *Escherichia coli* using gas stripping. *J. Biosci. Bioeng.* 110, 696–701.

Jones, D. T., and Woods, D. R. (1986). Acetone-butanol fermentation revisited. *Microbiol. Rev.* 50, 484–524.

Kahn, M., Kolter, R., Thomas, C., Figurski, D., Meyer, R., Remaut, E., and Helinski, D. R. (1979). Plasmid cloning vehicles derived from plasmids ColE1, F, R6K, and RK2. *Methods Enzymol.* 68, 268–280.

Keasling, J. D. (2008). Synthetic biology for synthetic chemistry. *ACS Chem. Biol.* 3, 64–76.

Khalil, A. S., and Collins, J. J. (2010). Synthetic biology: applications come of age. *Nat. Rev. Genet.* 11, 367–379.

Kinoshita, S. (1972). Amino-acid production by the fermentation process. *Nature* 240, 211.

Komenda, J., Koblizek, M., and Prasil, O. (2000). Characterization of processes responsible for the distinct effect of herbicides DCMU and BNT on photosystem II photoinactivation in cells of the cyanobacterium *Synechococcus* sp. PCC 7942. *Photosynth. Res.* 63, 135–144.

Kondo, T., Tezuka, H., Ishii, J., Matsuda, F., Ogino, C., and Kondo, A. (2012). Genetic engineering to enhance the Ehrlich pathway and alter carbon flux for increased isobutanol production from glucose by *Saccharomyces cerevisiae*. *J. Biotechnol.* 159, 32–37.

Krivoruchko, A., Siewers, V., and Nielsen, J. (2011). Opportunities for yeast metabolic engineering: lessons from synthetic biology. *Biotechnol. J.* 6, 262–276.

Lan, E. I., and Liao, J. C. (2011). Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng.* 13, 353–363.

Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., and Keasling, J. D. (2008). Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Curr. Opin. Biotechnol.* 19, 556–563.

Li, C., Knierim, B., Manisseri, C., Arora, R., Scheller, H. V., Auer, M., Vogel, K. P., Simmons, B. A., and Singh, S. (2010a). Comparison of dilute acid and ionic liquid pretreatment of switchgrass: biomass recalcitrance, delignification and enzymatic saccharification. *Bioresour. Technol.* 101, 4900–4906.

Li, H., Cann, A. F., and Liao, J. C. (2010b). Biofuels: biomolecular engineering fundamentals and advances. *Annu. Rev. Chem. Biomol. Eng.* 1, 19–36.

Li, S., Wen, J., and Jia, X. (2011). Engineering *Bacillus subtilis* for isobutanol production by heterologous Ehrlich pathway construction and the biosynthetic 2-ketoisovalerate precursor pathway overexpression. *Appl. Microbiol. Biotechnol.* 91, 577–589.

Lichtenthaler, H. K. (2000). Non-mevalonate isoprenoid biosynthesis: enzymes, genes and inhibitors. *Biochem. Soc. Trans.* 28, 785–789.

Luli, G. W., and Strohl, W. R. (1990). Comparison of growth, acetate production, and acetate inhibition of *Escherichia coli* strains in batch and fed-batch fermentations. *Appl. Environ. Microbiol.* 56, 1004–1011.

Lutke-Eversloh, T., and Bahl, H. (2011). Metabolic engineering of *Clostridium acetobutylicum*: recent advances to improve butanol production. *Curr. Opin. Biotechnol.* 22, 634–647.

Lutz, R. (1997). Independent and tight regulation of transcriptional units in *Escherichia coli* via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res.* 25, 1203–1210.

Lynd, L. R., Weimer, P. J., Van Zyl, W. H., and Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506–577.

Machado, I. M. P., and Atsumi, S. (2012). Cyanobacterial biofuel production. *J. Biotechnol.* doi: 10.1016/j.jbiotec.2012.03.005 [Epub ahead of print].

Marcheschi, R. J., Li, H., Zhang, K., Noey, E. L., Kim, S., Chaubey, A., Houk, K. N., and Liao, J. C. (2012). A synthetic recursive "+1" pathway for carbon chain elongation. *ACS Chem. Biol.* 7, 689–697.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21, 796–802.

Menkel, E., Thierbach, G., Eggeling, L., and Sahm, H. (1989). Influence of increased aspartate availability on lysine formation by a recombinant strain of *Corynebacterium glutamicum* and utilization of fumarate. *Appl. Environ. Microbiol.* 55, 684–688.

Messerotti, L. J., Radford, A. J., and Hodgson, A. L. (1990). Nucleotide sequence of the replication region from the *Mycobacterium–Escherichia coli* shuttle vector pEP2. *Gene* 96, 147–148.

Miller, J. H. (1992). *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Mumberg, D., Muller, R., and Funk, M. (1994). Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. *Nucleic Acids Res.* 22, 5767–5768.

Nielsen, D. R., Leonard, E., Yoon, S. H., Tseng, H. C., Yuan, C., and Prather, K. L. (2009). Engineering alternative butanol production platforms in heterologous bacteria. *Metab. Eng.* 11, 262–273.

Pátek, M., Nešvera, J., Guyonvarch, A., Reyes, O., and Leblon, G. (2003). Promoters of *Corynebacterium glutamicum. J. Biotechnol.* 104, 311–323.

Romero, P. A., and Arnold, F. H. (2009). Exploring protein fitness landscapes by directed evolution. *Nat. Rev. Mol. Cell Biol.* 10, 866–876.

Ruffing, A. M. (2011). Engineered cyanobacteria: teaching an old bug new tricks. *Bioeng. Bugs* 2, 136–149.

Sahm, H., Eggeling, L., Eikmanns, B., and Krämer, R. (1996). Construction of L-lysine-, L-threonine-, or L-isoleucine-overproducing strains of *Corynebacterium glutamicum. Ann. N. Y. Acad. Sci.* 782, 25–39.

Schafer, A., Tauch, A., Jager, W., Kalinowski, J., Thierbach, G., and Puhler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum. Gene* 145, 69–73.

Sentheshanmuganathan, S., and Elsden, S. R. (1958). The mechanism of the formation of tyrosol by *Saccharomyces cerevisiae. Biochem. J.* 69, 210–218.

Sheehan, J. J. (2009). Biofuels and the conundrum of sustainability. *Curr. Opin. Biotechnol.* 20, 318–324.

Shen, C. R., Lan, E. I., Dekishima, Y., Baez, A., Cho, K. M., and Liao, J. C. (2011). Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli. Appl. Environ. Microbiol.* 77, 2905–2915.

Shen, C. R., and Liao, J. C. (2008). Metabolic engineering of *Escherichia coli* for 1-butanol and 1-propanol production via the keto-acid pathways. *Metab. Eng.* 10, 312–320.

Smith, K. M., Cho, K. M., and Liao, J. C. (2010). Engineering *Corynebacterium glutamicum* for isobutanol production. *Appl. Microbiol. Biotechnol.* 87, 1045–1055.

Steen, E. J., Chan, R., Prasad, N., Myers, S., Petzold, C. J., Redding, A., Ouellet, M., and Keasling, J. D. (2008). Metabolic engineering of *Saccharomyces cerevisiae* for the production of n-butanol. *Microb. Cell Fact.* 7, 36.

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., Mcclure, A., Del Cardayre, S. B., and Keasling, J. D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science* 315, 801–804.

Stoker, N. G., Fairweather, N. F., and Spratt, B. G. (1982). Versatile low-copy-number plasmid vectors for cloning in *Escherichia coli. Gene* 18, 335–341.

Studier, F., and Moffat, B. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113–130.

Tartoff, K. D., and Hobbs, C. A. (1987). Improved media for growing plasmid and cosmid clones. *Bethesda Res. Lab. Focus* 9, 12.

Thomas, J. G., Ayling, A., and Baneyx, F. (1997). Molecular chaperones, folding catalysts, and the recovery of active recombinant proteins from *E. coli.* To fold or to refold. *Appl. Biochem. Biotechnol.* 66, 197–238.

Vabulas, R. M., Raychaudhuri, S., Hayer-Hartl, M., and Hartl, F. U. (2010). Protein folding in the cytoplasm and the heat shock response. *Cold Spring Harb. Perspect. Biol.* 2, a004390.

Wendisch, V. F. (2007). *Amino Acid Biosynthesis – Pathways, Regulation, and Metabolic Engineering (Microbiology Monographs).* Berlin: Springer.

Withers, S. T., Gottlieb, S. S., Lieu, B., Newman, J. D., and Keasling, J. D. (2007). Identification of isopentenol biosynthetic genes from *Bacillus subtilis* by a screening method based on isoprenoid precursor toxicity. *Appl. Environ. Microbiol.* 73, 6277–6283.

Zhang, K., Sawaya, M. R., Eisenberg, D. S., and Liao, J. C. (2008). Expanding metabolism for biosynthesis of non-natural alcohols. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20653–20658.

Zheng, Y. N., Li, L. Z., Xian, M., Ma, Y. J., Yang, J. M., Xu, X., and He, D. Z. (2009). Problems with the microbial production of butanol. *J. Ind. Microbiol. Biotechnol.* 36, 1127–1138.

Zinoviev, S., Muller-Langer, F., Das, P., Bertero, N., Fornasiero, P., Kaltschmitt, M., Centi, G., and Miertus, S. (2010). Next-generation biofuels: survey of emerging technologies and sustainability issues. *ChemSusChem* 3, 1106–1133.

Zverev, V. V., and Khmel, I. A. (1985). The nucleotide sequences of the replication origins of plasmids ColA and ColD. *Plasmid* 14, 192–199.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 29 March 2012; accepted: 14 May 2012; published online: 08 June 2012.

Citation: Lamsen EN and Atsumi S (2012) Recent progress in synthetic biology for microbial production of C3–C10 alcohols. Front. Microbio. ***3****:196. doi: 10.3389/fmicb.2012.00196*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 29 May 2012
doi: 10.3389/fmicb.2012.00198

Visualizing evolution in real-time method for strain engineering

Luis H. Reyes, James Winkler and Katy C. Kao*

Department of Chemical Engineering, Texas A&M University, College Station, TX, USA

Edited by:
David Nielsen, Arizona State University, USA

Reviewed by:
Giuseppe Spano, University of Foggia, Italy
Wensheng Lan, Shenzhen Entry-Exit Inspection and Quarantine Bureau, China
Keith Tyo, Northwestern University, USA

Correspondence:
Katy C. Kao, Department of Chemical Engineering, Texas A&M University, 3122 TAMU, College Station, TX 77843-3122, USA.
e-mail: kao.katy@gmail.com

The adaptive landscape for an industrially relevant phenotype is determined by the effects of the genetic determinants on the fitness of the microbial system. Identifying the underlying adaptive landscape for a particular phenotype of interest will greatly enhance our abilities to engineer more robust microbial strains. Visualizing evolution in real-time (VERT) is a recently developed method based on *in vitro* adaptive evolution that facilitates the identification of fitter mutants throughout the course of evolution. Combined with high-throughput genomic tools, VERT can greatly enhance the mapping of adaptive landscapes of industrially relevant phenotypes in microbial systems, thereby expanding our knowledge on the parameters that can be used for strain engineering.

Keywords: adaptive evolution, strain development, population dynamics, evolutionary engineering

INTRODUCTION

The majority of industrially relevant phenotypes in microbial systems involve multiple loci and mechanisms. The identities of these genetic determinants are generally not known, making the rational engineering of strains for these complex phenotypes challenging. Classical strain engineering for these traits generally involve several rounds of random mutagenesis followed by selection. However, with successive rounds of induced mutagenesis, mutations that are deleterious or have negative epistatic effects tend to accumulate by hitchhiking with beneficial alleles. If the desired trait can be coupled with growth, *in vitro* adaptive evolution can be used to improve the desired phenotype. This process is accomplished by applying a selective pressure so that beneficial mutants (mutants with increased fitness) can be obtained through the process of natural selection. The identities of the mutations residing in adaptive mutants obtained through natural selection or mutagenesis and their subsequent effects on cellular processes must be leveraged for further rational engineering. With advances in genomic tools, the genes and mechanisms involved can now be identified using combinations of whole-genome re-sequencing (Comas et al., 2012; Toprak et al., 2012), transcriptomics (Fitzgerald and Musser, 2001; Paulsen et al., 2001), proteomics (Callister et al., 2008; Boulais et al., 2010), and metabolomics (Ding et al., 2010; Goodarzi et al., 2010) studies.

In vitro adaptive evolution has been used extensively for the engineering of microbial system for both tolerances to inhibitors (Minty et al., 2011) and for enhanced product formation (Hu and Wood, 2010). The adaptive landscape, also known as fitness landscape, is used to describe the collection of relative fitness effects of each genotype under a specific condition. Detailed molecular characterization of adaptive mutants isolated from *in vitro* adaptive evolution experiments provides insights into the adaptive landscape for the phenotype of interest. Characterization of the adaptive landscape will significantly enhance our knowledge on the important parameters underlying complex phenotypes needed for the rational engineering of strains.

In adaptive evolution, clones are typically isolated from the evolving population after an arbitrarily elapsed time or at the end of the experiment. However, since the evolving population is heterogeneous, interclonal competition (clonal interference; Shaver et al., 2002; Kao and Sherlock, 2008) may lead to the extinction of beneficial mutants. Depending on the population structure during the course of evolution, the random isolation of adaptive mutants may fail to identify some adaptive mutations that arise during the course of the evolution. This review will (1) discuss factors that influence population structure and the impact of complex population dynamics on evolutionary engineering and (2) describe a novel evolutionary engineering method called visualizing evolution in real-time (VERT), that was recently developed to help address some of these limitations in traditional evolutionary engineering approach.

ADAPTIVE LANDSCAPE

The idea of an adaptive landscape was first introduced as "surfaces of selective value" by Wright in 1931 (Wright, 1931, 1982, 1988). The adaptive landscape is a multi-dimensional surface representation of the biological fitness of an organism in a particular environment. In an adaptive landscape map for a specific condition, each genotype is correlated with a fitness value (see **Figure 1** for an illustration). The resulting landscape can be flat with a single optimum where the evolving population is required to acquire a specific set of mutations, or can be rugged where the accessible optima will depend on the starting point within the landscape. It has been demonstrated that bacteria encounter both types of landscapes in evolution experiments (Orr, 2005; Weinreich et al., 2006;

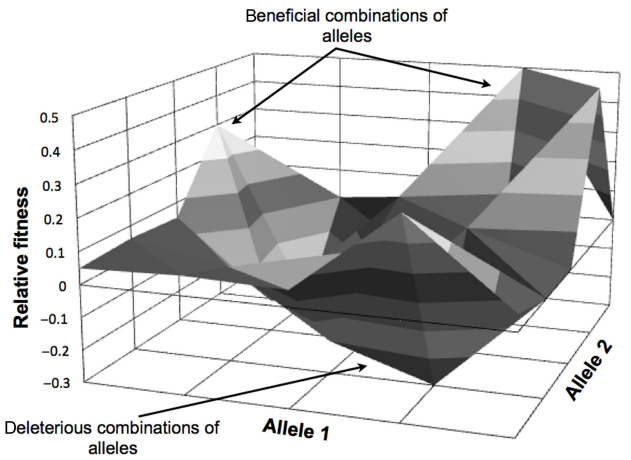


FIGURE 1 | Simplified adaptive landscape for two alleles (for one background genotype in one condition). The figure depicts fitness values for beneficial (positive relative fitness values) and deleterious (negative relative fitness values) combinations of alleles.

Gresham et al., 2008). Natural selection usually drives a population to the closest local optimum, but not necessarily the global optimum. Evolving populations tend to be trapped in suboptimal solutions (Lenski et al., 1998) in asexual systems. Thus to reach the global optimum, processes that allow for large "jumps" in the adaptive landscape, such as recombination and horizontal DNA transfer, are necessary to reach new regions of the adaptive landscape in a semi-rational manner. Recombination allows the combination of beneficial mutations with positive synergy and the removal of deleterious mutations acquired in the evolutionary process while horizontal gene transfer allows the acquisition of new functions.

THEORIES GOVERNING POPULATION STRUCTURE DURING ASEXUAL EVOLUTION

Numerous theories have been proposed for the population structure in *in vitro* adaptive evolution experiments. Several factors, including the selective pressure, size of the population, rate of mutations, frequency of beneficial mutations, and relative fitness of beneficial mutants, are involved in determining the population structure during evolution. In the simplest case, a well-adapted mutant rises in the population, and due to its increased fitness compared to background, the genotype will expand and eventually replace the parental population. This population structure is applicable to situations where the evolution is mutation-limited, the population size is small, and the time between the establishments of successive mutations is much larger than the time it takes for a beneficial mutant to fix in the population (strong positive selection). This theory, called clonal replacement (also called succession-fixation regime or strong-selection weak-mutation regime), implies that only one mutation can become fixed at a time, leading to successive complete selective sweeps (depicted in **Figure 2A**). The resulting population can be assumed to be homogeneous except during the periods when the beneficial mutant is sweeping through (Desai et al., 2007). However,

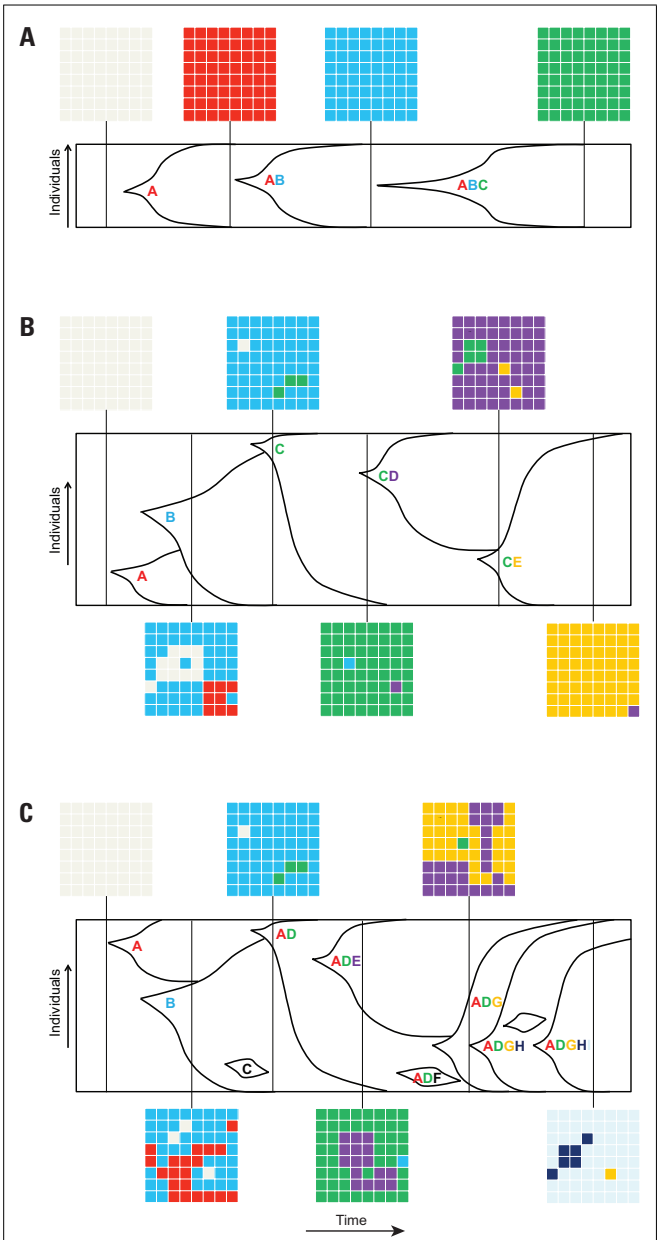


FIGURE 2 | Theories governing population structure during asexual evolution. The graphs represent the population structure as a function of time during asexual evolution. The capital letters represent different beneficial mutations in the population. The gridded boxes represent a snapshot of the frequency of different genotypes in the population at that one point in time. **(A)** Clonal replacement model, where successive sweeps and fixation of different beneficial mutations take place in a small population; snapshots of the genotypes at different elapsed times show that the population is homogeneous except when the beneficial mutant is sweeping through the population. **(B)** Clonal interference model, where different adaptive mutants compete until one with the largest fitness advantage sweeps through and becomes the founding genotype for subsequent evolution (e.g., mutations A, B, and C compete until C completely takes over the population). **(C)** Multiple mutations model, where multiple mutations occur in the same lineage before fixation. In the latter two population structures, some adaptive mutations are lost from the population, and depending on when adaptive mutants are isolated, some mutants (and thus the underlying molecular mechanisms for adaptation) may not be identified.

when the mutations are established at a faster rate than the rate of fixation, multiple mutant lineages can coexist and compete for resources until one with the largest fitness advantage outcompetes all the other genotypes and become the next founding genotype for subsequent evolution. This theory, known as clonal interference (or one-by-one clonal interference), assumes that a single mutation can be fixed at a time, producing heterogeneous populations except immediately after the sweeping of the fittest mutant (depicted in **Figure 2B**); this theory focuses on the competition between different mutations with positive relative fitness (Gerrish and Lenski, 1998; Orr, 2000; Gerrish, 2001; Kim and Stephan, 2003; Campos and de Oliveira, 2004; Wilke, 2004). The two theories described above assume that only one beneficial mutation can be fixed at a time. However, if the population size is large enough or the rate of mutation is high enough, multiple mutations can occur in the same lineage before fixation, leading to the multiple-mutation model (Desai et al., 2007; depicted in **Figure 2C**). The importance of this third theory on population structure has been demonstrated in several theoretical and experimental studies (Yedid and Bell, 2001; Shaver et al., 2002; Bachtrog and Gordo, 2004). In general, the population size in laboratory conditions is large enough that either one-by-one clonal interference or multiple mutations models shape the population structure.

FACTORS INFLUENCING POPULATION DYNAMICS

As mentioned above, factors such as mutation rate, relative fitness advantage, population size, and rate of beneficial mutations are important in shaping the population dynamics during evolution. We will briefly discuss each of these factors and how they impact adaptive evolution in different experimental systems. Since the evolution dynamics is dependent on the mutation rate, one would assume higher mutation rate to be advantageous for speeding up evolution by generating mutational diversity. However, an increase in mutation rates does not necessarily accelerate the pace of adaptation (Arjan et al., 1999). While a low mutation rate would result in a slow discovery of beneficial mutations, prolonged exposure to high mutation rate (such as the use of a mutator strain) increases the occurrence and accumulation of deleterious mutations as well as the hitchhiking of apparent "silent" mutations during the course of evolution, increasing the genetic load (Elena and Lenski, 2003; Gresham et al., 2008; Barrick et al., 2009). This is evidenced by the rarity of mutator strains in Lenski's long-term adaptive evolution experiment with *Escherichia coli*; where mutators were found only after thousands of generations of evolution (Elena and Lenski, 1997; Sniegowski et al., 1997; Arjan et al., 1999; Vulic et al., 1999; Lenski et al., 2003) and the fitness advantage conferred by the mutator strains is most likely a result of overcoming a mutation-limited bottleneck during the evolution. Mutagens are often used to increase genetic diversity in evolution experiments. However, since it is not convenient to periodically mutagenize the evolving population, a controllable mutator system can be used, where the expression of mutator alleles can be induced only when needed (Selifonova et al., 2001).

The time it takes a beneficial mutation to become the majority in the population is called the fixation time and is an important factor in determining the population dynamics during evolution. This fixation time depends mainly on two factors, genetic drift and the fitness advantage of the beneficial mutation in comparison with the background, and is inversely proportional to the relative fitness advantage of the beneficial mutant (Lenski et al., 1991). A beneficial mutation with a 10% relative fitness advantage will become the majority of the population after approximately 250 generation in serial batch transfer experiments (Elena and Lenski, 2003) and 100 generations in continuous culture experiments (Gresham et al., 2008). Genetic drift is defined as the probability that a beneficial mutation survives extinction (Joanna, 2011). In *in vitro* adaptive evolution experiments, the main source of drift is genetic bottleneck due to random sampling. This phenomenon takes place when a significant amount of the population suddenly vanishes, as occurs when a fresh batch culture is inoculated from an overnight culture. The survival probability of an allele carrying a beneficial mutation that arose in the culture will depend on its proportion in the culture at the time of transfer and the amount of inoculum transferred; therefore there is a chance that it could be completely lost due to the stochasticity of sampling. In evolution experiments using serial batch transfers, genetic bottlenecks between transfers affect heterogeneity by transferring a small fraction of the population. A reduction of the effect of genetic bottleneck could be achieved by using continuous culture systems such as chemostats or turbidostats (Conrad et al., 2011), where a much smaller genetic bottleneck is present.

VISUALIZING EVOLUTION IN REAL-TIME

As stated above, the population sizes in most *in vitro* evolution experiments are large enough to result in heterogeneous populations due to the effects of clonal interference and multiple mutations. Thus, adaptive evolution experiments can significantly benefit from a more systematic isolation of adaptive mutants and ramping-up schedules for selective pressures. The VERT system was developed (Kao and Sherlock, 2008; Huang et al., 2011) to address these limitations in traditional adaptive evolution experiments. The basis for VERT is the use of isogenic, but differentially labeled (typically with fluorescent proteins) strains to seed the initial evolving population. As a beneficial mutant arises and expands in the population, the colored subpopulation that it belongs is expected to increase in proportion. Using fluorescent activated cell sorting (FACS), the relative proportions of each of the colored subpopulations at each point in time can be measured. Each sustained expansion in the proportion of a colored subpopulation is called an "adaptive event." Thus, the tracking of the different colored subpopulations can serve as a tool for determining when a fitter mutant arises in the population.

The relative subpopulation frequency data collected throughout the course of adaptive evolution represent the history of the population. The observed increase in the relative proportion of a colored subpopulation from consecutive data points is assumed to be the result of the expansion of an adaptive mutant. Therefore, adaptive mutants can be isolated from samples based on the observed expansions and contractions, by sorting out the colored subpopulation that is expected to contain the adaptive mutant of interest. Since experimental data can suffer from noise,

the identification of adaptive events may be challenging. Visual inspection of the data to identify adaptive events is a crude, but relatively effective method (Kao and Sherlock, 2008; Huang et al., 2011). However, since small changes in relative frequencies may be difficult to distinguish from noise, computational methods will provide less biased annotation of adaptive events; our group recently developed a supervised learning method for analysis of VERT data (Winkler and Kao, 2012).

The basic feature of the VERT system, the number of labeled subpopulations, is the aspect that can most readily be manipulated directly by the experimentalist, but is somewhat restricted by the available equipment and properties of the labels themselves. The number of fluorescent markers used represents distinct subpopulations that can be visualized during the course of an evolution experiment. VERT labels must have distinguishable emission spectra and preferably have no significant fitness effect in the condition of interest. Widely used fluorescent proteins such as GFP, YFP, and RFP can be detected on most FACS machines and usually have little effect on the physiology of their host strains. At a minimum, two labeled subpopulations are trivially required to observe population dynamics. Three subpopulations, employing RFP, GFP, and YFP labeled strains, have been used successfully (Kao and Sherlock, 2008; Huang et al., 2011) in fungal systems. Additional subpopulations can be included if suitable equipment is available. Simulated evolution may prove a useful tool for unraveling the connection between adaptive event discovery and initial population diversity.

Visualizing evolution in real-time-based *in vitro* adaptive evolution experiments can be used in either serial batch transfer or continuous culture systems. Provided that the different fluorescently marked strains show no significant fitness bias, then equal proportions of each strain maybe used to seed the population for evolution. Samples are then withdrawn and quantified using FACS every few generations to track the population dynamics. It is typically assumed that the adaptive mutant will expand until a fitter mutant arises in another subpopulation and expands sufficiently to impede its' expansion. It is further assumed that the generation at which the expanding subpopulation has reached a maximum proportion will contain the largest fraction of the adaptive mutant responsible for the expansion, simplifying the isolation of the mutant considerably.

In traditional adaptive evolution experiments, selective pressure is generally ramped-up at arbitrarily chosen time intervals. An alternative to this approach, based on using a feedback controller to maintain selective pressure so that the overall population growth rate approaches a user-defined set point, was recently introduced by Toprak et al. (2012). Since the use of VERT allows the users to readily identify when adaptive events occur, it can be used to design a more systematic ramp-up schedule. For example, an increase in selective pressure could be applied when a minimum of 2 adaptive events are observed. The optimal frequency of ramp-up as a function of observed number of adaptive events may differ depending on the adaptive landscape for the phenotype of interest and needs to be investigated.

The isolated adaptive mutants can be further characterized to elucidate the molecular mechanisms of resistance in the selective pressure of interest. Whole-genome re-sequencing, transcriptomics, proteomics, and metabolomics analyses can be used to elucidate the evolutionary trajectories during the process of adaptive evolution. The availability and cost of whole-genome re-sequencing has improved significantly, but in most cases is still more expensive than transcriptome analysis using DNA microarrays. VERT tracks the individual subpopulations, making it easier to distinguish whether genome-wide perturbations observed in the transcriptional regulation found in different isolates arose independently or transitively without whole-genome re-sequencing data (if the isolates come from different colored subpopulations). Since not all the observed perturbations are involved in the complex phenotype of interest, common perturbations observed in independent lineages provide a level of confidence for their involvement. The potential adaptive mechanisms identified can serve as targets for further strain engineering.

The original development of VERT used the yeast *Saccharomyces cerevisiae* evolving under glucose-limited conditions; a three-colored VERT system was used to seed eight parallel populations (Kao and Sherlock, 2008). The VERT data from one of the populations is shown in **Figure 3**; generations and subpopulations from which adaptive mutants were isolated from are indicated. Detailed genotypic and transcriptomics analyses of the isolated adaptive mutants showed convergence in the perturbation of the protein kinase A regulatory network in independent lineages (Kao and Sherlock, 2008). Subsequent development and application of a two-colored VERT system in *E. coli* for *n*-butanol tolerance revealed previously undiscovered resistance mechanisms (Reyes et al., 2012).

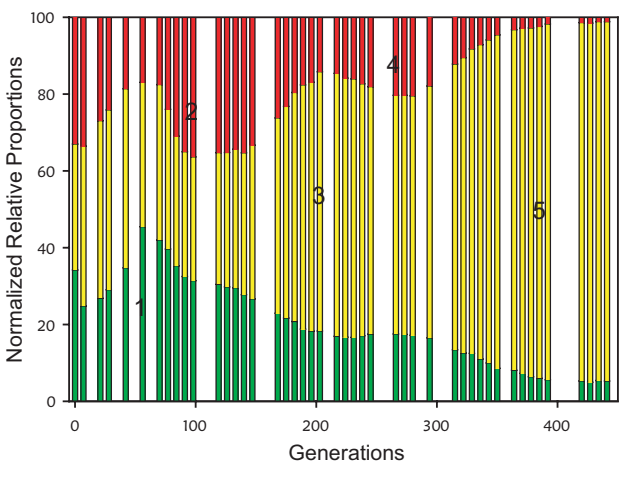


FIGURE 3 | Example population dynamics from a three-colored VERT system (adapted from Kao and Sherlock, 2008). The colored bars represent the relative proportions of each colored subpopulation. An increase in the relative proportion of a colored subpopulation is indicative of the occurrence and expansion of an adaptive mutation in that subpopulation, and is defined as an adaptive event. Under the assumption that the adaptive mutant responsible for the specific adaptive event is at its' highest proportion at the end of the sustained expansion, the adaptive mutants are isolated from the expanding subpopulation from the generation at the end of each expansion. The generation and colored subpopulation from which adaptive mutants were isolated are numbered 1–5.

CONCLUSION

Understanding the adaptive landscape for the phenotypes of interest is important for the rational engineering of strains. The use of evolutionary engineering has been used extensively to improve strains for complex phenotypes where there is limited knowledge on the associated genetic determinants. Advances in molecular biology tools in recent years have significantly improved our ability to obtain insights into the molecular mechanisms involved in the desire phenotypes in isolated adaptive mutants from *in vitro* evolution experiments. VERT was a recently developed tool for evolutionary engineering that can helps to provide a rough population structure for the evolving population, allowing the systematic isolation of adaptive mutants and ramp-up of selective pressure. Combined with advanced genomic tools, use of VERT in evolutionary engineering can help to gain additional insight regarding the adaptive landscape for complex phenotypes.

ACKNOWLEDGMENTS

The authors would like to thank NSF MCB-1054276 (Katy C. Kao), and the Graduate Research Fellowship program (James Winkler) for partial financial support.

REFERENCES

Arjan, J. A., Visser, M., Zeyl, C. W., Gerrish, P. J., Blanchard, J. L., and Lenski, R. E. (1999). Diminishing returns from mutation supply rate in asexual populations. *Science* 283, 404–406.

Bachtrog, D., and Gordo, I. (2004). Adaptive evolution of asexual populations under Muller's ratchet. *Evolution* 58, 1403–1413.

Barrick, J. E., Yu, D. S., Yoon, S. H., Jeong, H., Oh, T. K., Schneider, D., Lenski, R. E., and Kim, J. F. (2009). Genome evolution and adaptation in a long-term experiment with *Escherichia coli*. *Nature* 461, 1243–1274.

Boulais, J., Trost, M., Landry, C. R., Dieckmann, R., Levy, E. D., Soldati, T., Michnick, S. W., Thibault, P., and Desjardins, M. (2010). Molecular characterization of the evolution of phagosomes. *Mol. Syst. Biol.* 6, 423.

Callister, S. J., McCue, L. A., Turse, J. E., Monroe, M. E., Auberry, K. J., Smith, R. D., Adkins, J. N., and Lipton, M. S. (2008). Comparative bacterial proteomics: analysis of the core genome concept. *PLoS ONE* 3, e1542. doi: 10.1371/journal.pone.0001542

Campos, P. R., and de Oliveira, V. M. (2004). Mutational effects on the clonal interference phenomenon. *Evolution* 58, 932–937.

Comas, I., Borrell, S., Roetzer, A., Rose, G., Malla, B., Kato-Maeda, M., Galagan, J., Niemann, S., and Gagneux, S. (2012). Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* 44, 106–110.

Conrad, T. M., Lewis, N. E., and Palsson, B. O. (2011). Microbial laboratory evolution in the era of genome-scale science. *Mol. Syst. Biol.* 7, 509.

Desai, M. M., Fisher, D. S., and Murray, A. W. (2007). The speed of evolution and maintenance of variation in asexual populations. *Curr. Biol.* 17, 385–394.

Ding, M. Z., Zhou, X., and Yuan, Y. J. (2010). Metabolome profiling reveals adaptive evolution of *Saccharomyces cerevisiae* during repeated vacuum fermentations. *Metabolomics* 6, 42–55.

Elena, S. F., and Lenski, R. E. (1997). Long-term experimental evolution in *Escherichia coli*. VII. Mechanisms maintaining genetic variability within populations. *Evolution* 51, 1058–1067.

Elena, S. F., and Lenski, R. E. (2003). Evolution experiments with microorganisms: the dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* 4, 457–469.

Fitzgerald, J. R., and Musser, J. M. (2001). Evolutionary genomics of pathogenic bacteria. *Trends Microbiol.* 9, 547–553.

Gerrish, P. (2001). The rhythm of microbial adaptation. *Nature* 413, 299–302.

Gerrish, P. J., and Lenski, R. E. (1998). The fate of competing beneficial mutations in an asexual population. *Genetica* 102–103, 127–144.

Goodarzi, H., Bennett, B. D., Amini, S., Reaves, M. L., Hottes, A. K., Rabinowitz, J. D., and Tavazoie, S. (2010). Regulatory and metabolic rewiring during laboratory evolution of ethanol tolerance in *E. coli*. *Mol. Syst. Biol.* 6, 378.

Gresham, D., Desai, M. M., Tucker, C. M., Jenq, H. T., Pai, D. A., Ward, A., DeSevo, C. G., Botstein, D., and Dunham, M. J. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* 4, e1000303. doi: 10.1371/journal.pgen.1000303

Hu, H., and Wood, T. K. (2010). An evolved *Escherichia coli* strain for producing hydrogen and ethanol from glycerol. *Biochem. Biophys. Res. Commun.* 391, 1033–1038.

Huang, M., McClellan, M., Berman, J., and Kao, K. C. (2011). Evolutionary dynamics of *Candida albicans* during in vitro evolution. *Eukaryot. Cell* 10, 1413–1421.

Joanna, M. (2011). Genetic drift. *Curr. Biol.* 21, R837–R838.

Kao, K. C., and Sherlock, G. (2008). Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*. *Nat. Genet.* 40, 1499–1504.

Kim, Y., and Stephan, W. (2003). Selective sweeps in the presence of interference among partially linked loci. *Genetics* 164, 389–398.

Kvitek, D. J., and Sherlock, G. (2011). Reciprocal sign epistasis between frequently experimentally evolved adaptive mutations causes a rugged fitness landscape. *PLoS Genet.* 7, e1002056. doi: 10.1371/journal.pgen.1002056

Lenski, R. E., Mongold, J. A., Sniegowski, P. D., Travisano, M., Vasi, F., Gerrish, P. J., and Schmidt, T. M. (1998). Evolution of competitive fitness in experimental populations of *E. coli*: what makes one genotype a better competitor than another? *Antonie van Leeuwenhoek* 73, 35–47.

Lenski, R. E., Rose, M. R., Simpson, S. C., and Tadler, S. C. (1991). Long-term experimental evolution in *Escherichia coli*. 1. Adaptation and divergence during 2,000 generations. *Am. Nat.* 138, 1315–1341.

Lenski, R. E., Winkworth, C. L., and Riley, M. A. (2003). Rates of DNA sequence evolution in experimental populations of *Escherichia coli* during 20,000 generations. *J. Mol. Evol.* 56, 498–508.

Minty, J., Lesnefsky, A., Lin, F., Chen, Y., Zaroff, T., Veloso, A., Xie, B., McConnell, C., Ward, R., Schwartz, D., Rouillard, J.-M., Gao, Y., Gulari, E., and Lin, X. (2011). Evolution combined with genomic study elucidates genetic bases of isobutanol tolerance in *Escherichia coli*. *Microb. Cell Fact.* 10, 18.

Orr, H. A. (2000). The rate of adaptation in asexuals. *Genetics* 155, 961–968.

Orr, H. A. (2005). The genetic theory of adaptation: a brief history. *Nat. Rev. Genet.* 6, 119–127.

Paulsen, I. T., Chen, J., Nelson, K. E., and Saier, M. H. (2001). Comparative genomics of microbial drug efflux systems. *J. Mol. Microbiol. Biotechnol.* 3, 145–150.

Reyes, L. H., Almario, M. P., Winkler, J., Orozco, M. M., and Kao, K. C. (2012). Visualizing evolution in real time to determine the molecular mechanisms of *n*-butanol tolerance in *Escherichia coli*. *Metab. Eng.* (in press).

Selifonova, O., Valle, F., and Schellenberger, V. (2001). Rapid evolution of novel traits in microorganisms. *Appl. Environ. Microbiol.* 67, 3645–3649.

Shaver, A. C., Dombrowski, P. G., Sweeney, J. Y., Treis, T., Zappala, R. M., and Sniegowski, P. D. (2002). Fitness evolution and the rise of mutator alleles in experimental *Escherichia coli* populations. *Genetics* 162, 557–566.

Sniegowski, P. D., Gerrish, P. J., and Lenski, R. E. (1997). Evolution of high mutation rates in experimental populations of *E. coli*. *Nature* 387, 703–705.

Toprak, E., Veres, A., Michel, J.-B., Chait, R., Hartl, D. L., and Kishony, R. (2012). Evolutionary paths to antibiotic resistance under dynamically sustained drug selection. *Nat. Genet.* 44, 101–105.

Vulic, M., Lenski, R. E., and Radman, M. (1999). Mutation, recombination, and incipient speciation of bacteria in the laboratory. *Proc. Natl. Acad. Sci. U.S.A.* 96, 7348–7351.

Weinreich, D. M., Delaney, N. F., DePristo, M. A., and Hartl, D. L. (2006). Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* 312, 111–114.

Wilke, C. O. (2004). The speed of adaptation in large asexual populations. *Genetics* 167, 2045–2053.

Winkler, J., and Kao, K. C. (2012). Computational identification of adaptive mutants using the VERT system. *J. Biol. Eng.* 6, 3.

Wright, S. (1931). Evolution in Mendelian populations. *Genetics* 16, 0097–0159.

Wright, S. (1982). Character change, speciation, and the higher taxa. *Evolution* 36, 427–443.

Wright, S. (1988). Surfaces of selective value revisited. *Am. Nat.* 131, 115–123.

Yedid, G., and Bell, G. (2001). Microevolution in an electronic microcosm. *Am. Nat.* 157, 465–487.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 March 2012; accepted: 14 May 2012; published online: 29 May 2012.

*Citation: Reyes LH, Winkler J and Kao KC (2012) Visualizing evolution in real-time method for strain engineering. Front. Microbio. **3**:198. doi: 10.3389/fmicb.2012.00198*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 05 June 2012
doi: 10.3389/fmicb.2012.00203

Engineering microbial consortia to enhance biomining and bioremediation

Karl D. Brune and Travis S. Bayer*

Centre for Synthetic Biology and Innovation, Division of Molecular Biosciences, Imperial College London, London, UK

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Romy Chakraborty, Lawrence Berkeley National Lab, USA
Lei Chen, Tianjin University, China

****Correspondence:***
Travis S. Bayer, Centre for Synthetic Biology and Innovation, Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, UK. e-mail: t.bayer@imperial.ac.uk

In natural environments microorganisms commonly exist as communities of multiple species that are capable of performing more varied and complicated tasks than clonal populations. Synthetic biologists have engineered clonal populations with characteristics such as differentiation, memory, and pattern formation, which are usually associated with more complex multicellular organisms. The prospect of designing microbial communities has alluring possibilities for environmental, biomedical, and energy applications, and is likely to reveal insight into how natural microbial consortia function. Cell signaling and communication pathways between different species are likely to be key processes for designing novel functions in synthetic and natural consortia. Recent efforts to engineer synthetic microbial interactions will be reviewed here, with particular emphasis given to research with significance for industrial applications in the field of biomining and bioremediation of acid mine drainage.

Keywords: acid mine drainage, bioleaching, biomining, bioremediation, microbial consortia, synthetic biology, synthetic microbial consortia

INTRODUCTION

Natural microbial consortia are known to facilitate a wide range of complex tasks such as inter-species biofilm formation that allows microorganisms to persist in inhospitable environments (Keller and Surette, 2006). Syntrophic degradation of complex molecules also allows two species to complete metabolic reactions from which neither species would gain energy without the cooperation of the other (Zhou et al., 2011). Consortia play a crucial role in the human gut microbiome (Kau et al., 2011) and are known to heavily influence the ecological dynamics of the marine community (Giovannoni and Vergin, 2012). Humans have made use of natural consortia for millennia and selected them for better performance or desired properties in areas such as dairy processing (Reid, 2012), beer, and wine fermentation (Di Maio et al., 2012), and more recently in biogas processing (Lynd et al., 2002) and biomining (Rawlings, 2007).

The field of synthetic biology has developed a wide range of highly engineered clonal populations of bacteria to perform complex tasks such as differentiation (Süel et al., 2006), memory (Ham et al., 2008), counting (Friedland et al., 2009), and pattern formation (Liu et al., 2011a) as well as industrial applications such as production of antimalarial drug precursors (Ro et al., 2006), fuel like long-chain alcohols (Atsumi et al., 2008), and biosensors for arsenic in drinking water (Stocker et al., 2003). The construction and analysis of synthetic gene circuits has not only provided us with new tools for genetic engineering but has given deeper insight into naturally occurring gene circuits, their evolution, architectures, and properties as well (Sprinzak and Elowitz, 2005; Çağatay et al., 2009; Elowitz and Lim, 2010).

Considering that most synthetic circuits have been engineered in clonal populations it has been proposed that engineering synthetic consortia may allow for more complex tasks in industrial applications (Brenner et al., 2008; Sabra et al., 2010; Momeni et al., 2011). Synthetic consortia may empower scientists and engineers to cultivate and make use of some of the 99% percent of microbes that have not been cultured yet and also to elucidate the role of the many genes that have yet unknown function(s), which might well be of particular relevance in microbial consortia (Wintermute and Silver, 2010). So far several proof-of-concept and industrial synthetic consortia have been engineered and reviewed (Brenner et al., 2008; Rollié et al., 2012); highlighted by a recent review focusing on engineered communication and biofuel processing (Shong et al., 2012).

The industrial practice of biomining (Olson et al., 2003) and bioremediation of heavy metal contaminations (Haferburg and Kothe, 2010) could potentially benefit from synthetic consortia as natural consortia have been shown to play crucial roles in these processes. This review focuses on using microbial isolates to construct consortia that would otherwise not be found together in Nature and discusses the potential use of genetically engineered species in bioremediation and biomining processes.

MICROBIAL CONSORTIA IN BIOMINING

BIOLOGICAL PROCESSES IN METAL RECOVERY

Biomining entails the use of acidophilic microbes to facilitate the recovery process of metals from sulfide minerals in the processes of bioleaching and biooxidation. Biooxidation is the enrichment of metals, particularly gold, by mobilization and thus removal of interfering metal sulfides from ores bearing the precious metals (Rohwerder et al., 2003). Bioleaching is the solubilization of metals of interest such as cobalt, copper, and nickel from sulfide minerals. The two processes are industrially well established and are commercially applied worldwide (Rawlings, 2007). The microbes found in these environments are (extreme) acidophiles growing at a pH of 3 or lower and span a wide range of different phyla. The majority belong to the bacterial and archaea domains;

however, unicellular eukaryotes have also been reported (Baker and Banfield, 2003; Bonnefoy and Holmes, 2011).

The impact of microbial consortia in bioleaching, particularly in copper recovery, is widely recognized in literature and industry (Olson et al., 2003; Rohwerder et al., 2003). Microorganisms oxidize both sulfur and iron of sulfide minerals, such as pyrite. It is generally accepted that leaching takes place via an "indirect" mechanism, which can be divided into the "contact" and "non-contact" mode (Baker and Banfield, 2003; Rohwerder et al., 2003). The "indirect" mechanism assumes that chemoautotrophic iron-oxidizing microorganisms like *Acidithiobacillus ferrooxidans* or *Leptospirillum ferrooxidans* generate ferric ions by oxidation of ferrous iron (Rawlings, 2002). During the "non-contact" mode planktonic microbes oxidize aqueous ferrous ions to ferric ions, which in turn attack the mineral surface by chemical oxidation. The "contact" mode assumes a small reaction space between the microbial cell wall and the mineral surface where ferric ions are concentrated in biofilms for a localized attack of the sulfide mineral. Either mode yields different intermediate and final sulfur species depending on the ore leached. The thiosulfate mechanism applies for acid-insoluble metal sulfides such as molybdenite (MoS_2) and pyrite (FeS_2), and eventually yields sulfate (SO_4^{2-}) as the main end product. The so-called polysulfide mechanism applies for acid-soluble metal sulfides such as arsenopyrite (FeAsS) and chalcopyrite ($CuFeS_2$), eventually yielding elemental sulfur as the main end product (Rawlings, 2002; Rohwerder et al., 2003). Accumulating sulfur layers may act as a leaching inhibitor because of sterically impeding the ferric ion attack on the ore and thus affecting the growth of iron-oxidizing microbes. Chalcopyrite leaching is particularly sensitive to inactivation by formation of jarosite layers as a function of redox potential and is thus one of the most recalcitrant ore to leach (Viramontes-Gamboa et al., 2010). These sulfur layers however can be oxidized to soluble sulfate by sulfur-oxidizing bacteria such as *Acidithiobacillus caldus* or *Acidithiobacillus thiooxidans* (Dopson and Lindstrom, 1999; Sand et al., 2001; Mangold et al., 2011). Hence naturally occurring consortia of autotrophic iron-oxidizing microbes and sulfur-oxidizing microbes have been proposed to be symbiotic, potentially mutualistic (Rawlings et al., 1999) or at least synergetic in substrate use (Johnson, 1998; Roger Morin in Donati and Sand, 2007, 136).

COMMUNICATION IN NATURAL BIOMINING CONSORTIA

Research on extracellular polymeric substances (EPS) of *A. ferrooxidans* suggests that biofilm formation, which is crucial for the contact leaching mechanism, leads to an increase in redox potential and thus increase leaching rates as iron ions are trapped in the EPS (Sand and Gehrke, 2006). It is known that communication plays a major role in microbial biofilm formation (McDougald et al., 2012). The model bioleaching organism *A. ferrooxidans* produces and responds to compounds of the acyl homoserine lactones (AHLs) family used in auto-inducer 1 (AI-1) type quorum-sensing (QS) system (Keller and Surette, 2006) as well as to the c-di-GMP pathway (Hengge, 2009), which is also employed in QS. Two loci have been identified encoding for AHL synthases, the classical *LuxI*-type *afeI* acyl synthase (Farah et al., 2005; Rivas et al., 2005) and *act* which is related to the LPA acyltransferase family (Rivas et al., 2007). Both systems are involved in response to iron and sulfur substrates respectively, though *act* may be alternatively (or additionally) involved in cell membrane formation via fatty acid synthesis which is yet to be elucidated (Valdés et al., 2008). Recent studies also suggest a role of AHL-mediated QS in resistance toward high copper concentrations (Wenbin et al., 2011). The existence of a c-di-GMP pathway in *A. ferrooxidans* was discovered by analysis of its genome sequence (Ruiz et al., 2007). The pathway has been shown to respond to changes of the energetic substrate (iron and sulfur) as well as to the lifestyle of the bacteria (planktonic or biofilm-associated growth) by determination of intracellular c-di-GMP levels (Ruiz et al., 2011). Even though AHL type QS systems are absent from *A. caldus* and *A. thiooxidans* as inferred by genome analysis (Valdés et al., 2008), sequence analysis of the psychrotolerant *Acidithiobacillus ferrivorans* SS3 performed in our group (unpublished) suggests presence of an AHL type QS as we were able to identify an *act* homolog with 84% identity giving rise to the possibility for inter-species communication. Potential inter-species communication may also occur via the more common c-di-GMP QS. Related genes and the signaling compound were identified and isolated from *A. caldus* and *A. thiooxidans* (Castro et al., 2009). As biofilm formation is crucial for the leaching process it may be suitable to modulate AHL- and c-di-GMP levels to optimize attachment to ore particles for example, which may enhance bioleaching processes. However, although the existence of the above-mentioned pathways suggests that QS regulated biofilm formation plays a role in mineral solubilization, further experiments are required to prove this assumption.

CONSORTIA OF NATURALLY OCCURRING SPECIES

Whereas most studies have been performed on pure cultures of *A. ferrooxidans*, many early studies characterized mixed cultures of bioleaching organisms, mainly due to the difficulty in full separation of species in natural consortia (Harrison, 1984). Hence naturally occurring consortia have been characterized as well as defined consortia of naturally occurring organisms in order to elucidate mechanisms and synergies that improve the leaching process (Rawlings and Johnson, 2007). The impact of natural consortia was shown to be profound. In one example, a synergetic effect was observed by Qiu et al. (2005) during chalcopyrite leaching with a defined consortia of *A. ferrooxidans* and *A. thiooxidans*. The mixed culture was more efficient at leaching chalcopyrite than the pure cultures. The authors concluded that co-culture reduced the formation of inhibiting jarosite layers by the generation of sulfuric acid due to sulfur oxidation of *A. thiooxidans*.

Employment of heterotrophic acidophiles to remove inhibiting organic compounds that accumulate during growth led to acceleration of the leaching process. This was attributed to the increased growth rate of *A. ferrooxidans* while it was co-cultured with the heterotroph *Acidiphilium acidophilum* (Liu et al., 2011b).

Further examples for advantages of consortia are increased acid production (Okibe and Johnson, 2004), improved attachment to mineral surfaces (Noël et al., 2010), increased growth, and leaching rates (Bacelar-Nicolau and Johnson, 1999; Okibe and Johnson, 2004; Fu et al., 2008; Liu et al., 2011d; Naghavi et al., 2011).

While some of the above-mentioned consortia are most likely to occur in nature, engineering defined natural consortia has opened new possibilities for enhanced bioleaching (Rawlings and Johnson, 2007). Considering that researchers can choose from a wide range of microbes from different geographic locations, there is potential for additional, yet unexplored synergetic effects that may arise as these artificially assembled microbial consortia would not be encountered in nature. The use of consortia assembled from naturally occurring species is furthermore interesting because they would not be considered genetically modified and are hence not susceptible to regulatory procedures.

CONSORTIA OF NATURAL AND ENGINEERED SPECIES

To our best knowledge, hybrid consortia consisting of genetically engineered and naturally occurring bioleaching bacteria have not been reported so far. Even though some bioleaching organisms, in particular those of the *Acidithiobacillus* genus have been successfully transformed, genetic manipulations are difficult as transformation efficiencies are extremely low (Kusano et al., 1992; Peng et al., 1994). In fact only two knockouts (Liu et al., 2000; Wang et al., 2012) and two expression mutants have been reported in the scientific literature. One *rus* overexpressing *A. ferrooxidans* strain and another expressing the *mer* determinant for a mercury resistant *A. caldus* strain (Chen et al., 2011; Liu et al., 2011c). Once more suitable transformation protocols have been developed, it may be feasible to modulate QS signals with engineered microbes by either attenuating or amplifying natural signals or sending artificial signals to promote biofilm formation or mobilization respectively as recently demonstrated with engineered *E. coli* cells (Hong et al., 2012). Interestingly, it has been shown that once an initial consortia has been established, the power of evolution can be used to drive novel species interactions potentially resulting in increased consortia stability and productivity (Hansen et al., 2007).

Ultimately, engineered consortia could be deployed in industrial scale heap and tank leaching operations to improve bioleaching and biooxidation processes. However, as highlighted by Rawlings and Johnson (2007) it is crucial to consider that the tailored consortia have to compete with other microbes and their associated consortia in the non-sterile leaching environment. Usually this is not too much of a problem as the fastest growing species is usually the one leading to increased leaching. Depending on the ore leached, however, in particular chalcopyrite ores, high redox potential, which is associated with dominant iron-oxidizing microbes such as *A. ferrooxidans* or *L. ferrooxidans* is not appreciated as the continuous leaching process will stall after an initial high rate of recovery (Ohata et al., 2010).

Furthermore, hybrid consortia might be used to culture many of the yet uncultured microbes. There are abundant microbes in acid environments which are still to be cultured, characterized and their role in the ecosystem to be elucidated (Baker et al., 2010).

MICROBIAL CONSORTIA IN ACID MINE DRAINAGE BIOREMEDIATION

One major environmental consequence of industries such as mining, galvanic processing, and construction is the possibility of acid rock drainage (ARD) or in the particular case of mining, acid mine drainage (AMD), where wastewater effluents and mine run-off are not properly managed. AMD may occur where access of oxidants to sulfide minerals, in particular pyrite, is facilitated due to mining operations as the surface area of the minerals is increased (Baker and Banfield, 2003). Both inorganic and biological reactions drive the acidification and heavy metal contamination of water due to oxidation of sulfide minerals (Ma and Banfield, 2011). The biological reactions account for the gross of AMD production with estimates as high as 75% (Baker and Banfield, 2003). The same organisms and consortia that are used in biomining operations are the major contributors to AMD generation, though AMD biofilms are mainly dominated by the chemoautotrophic Nitrospirae phylum bacteria *Leptospirillum* spp. (Gadd, 2009; Wilmes et al., 2009). AMD is dealt with usually in two ways, either by migration control or source control in case the axiom "prevention is better than cure" is feasible (Johnson and Hallberg, 2005; Das et al., 2009). In industrial environments AMD is usually managed at source by neutralization of wastewater and run-offs in rather costly abiotic neutralization processes with limestone. Therefore, the above-mentioned microbes and their biofilm environments are an interesting target for AMD management. Abiotic AMD mitigation control options as well as biological remediation processes have been recently reviewed (Johnson and Hallberg, 2005; Gadd, 2009) and are beyond the scope of this review. We will therefore focus on the few examples of biological AMD source control of AMD and discuss potential future applications.

Johnson et al. (2007) reported the use of heterotrophic acidophiles to colonize pyrite prior to exposure to iron-oxidizing bacteria to reduce dissolution of the mineral. The process, named "bioshrouding" was capable of decreasing the dissolution rate between 57 and 75%. This was presumably due to the heterotrophs' biofilm that impeded attachment of the autotrophic iron-oxidizer. It remains to be seen if this approach is viable in industrial scale operations as iron-oxidizing autotrophs may outcompete the heterotrophs in the non-sterile environment though their growth could be impeded by the necessary supply of artificial carbon sources such as yeast extracts to foster the growth of the heterotrophs, which is known to inhibit growth of autotrophic organisms (Harrison, 1984).

Eukaryotic organisms such as algae and fungi have been reported in aquatic AMD environments, and could be engineered to mitigate AMD. Their natural role and potential application in bioremediation of AMD has been recently reviewed (Das et al., 2009). Engineering these eukaryotes for quenching of QS may be a very attractive option as resistance to quenching is unlikely to evolve (Defoirdt et al., 2010) and a broad range of quenching mechanisms are available. One example is the production of AI-1 type interfering halogenated furanones secreted by the algae *Delisea pulchra* (Czajkowski and Jafra, 2009). These small molecules mimic bacterial QS compounds and thus interfere with bacterial signaling and biofilm colonization.

Furthermore, the use of tailored bacteriophages and viruses (Lu and Collins, 2007) could potentially impede biofilm formation of autotrophs as naturally occurring viruses have been reported in AMD environments (Andersson and Banfield, 2008; Denef et al., 2010). Engineers employing this approach would have to

contend with bacterial defense mechanisms. The CRISPR (for Clustered Regularly Interspaced Short Palindromic Repeats) interference mechanisms are one such strategy bacteria and archaea use to evade phage treatment. Exogenous DNA is inactivated and processed to small elements of ∼30 bp due to proteins encoded by the CRISPR-associated (*cas*) genes. These are then incorporated into the CRISPR locus from which they are constitutively expressed, processed and remain with an accompanying flanking region. The resulting CRISPR RNA (crRNA) binds to complementary RNA or DNA molecules and recruits Cas proteins to cleave the targeted nucleic acid depending on the organism (Marraffini and Sontheimer, 2010). This system has been used to rationally engineer crRNA-mediated mRNA cleavage in the extreme thermophilic archaea *Pyrococcus furiosus* using the native Cmr protein (Hale et al., 2012). Desired beneficial strains and consortia could therefore be potentially rendered "immune" by CRISPR engineering.

CONCLUSIONS AND FUTURE PERSPECTIVES

The ability to design and manipulate microbial consortia may allow biologists and engineers to enhance mineral recovery in biomining processes beyond the yields and productivities observed with naturally occurring consortia. Furthermore, it is likely that synthetic consortia will also seep into other industrial sectors, which are currently mainly abiotic processes as microbial consortia are capable of more complex behaviors due to the combined properties of the individual organisms and the additional layers of regulation and adaptation to changing conditions.

The engineering of consortia will also be aided by the further characterization of the diverse species in bioleaching environments, which may have unique metabolic and physiological features. Prime candidates are the abundant archaea in biomining processes, which have not yet been subject to thorough research and are an untapped field of biological resources for industrial applications. The design and construction of synthetic and mixed microbial consortia will not only become a powerful tool in optimizing industrial processes but will also give us an insight into the evolution and emergence of naturally occurring microbial consortia. This will foster our understanding of higher-level system organization that is indispensible for designing complex functions.

ACKNOWLEDGMENTS

The authors would like to thank Axel Nyström for a critical reading of the manuscript. Research on microbial consortia in the Bayer lab is funded by EPSRC, BBSRC, and the Rio Tinto Centre for Advanced Mineral Recovery.

REFERENCES

Andersson, A. F., and Banfield, J. F. (2008). Virus population dynamics and acquired virus resistance in natural microbial communities. *Science* 320, 1047–1050.

Atsumi, S., Hanai, T., and Liao, J. C. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89.

Bacelar-Nicolau, P., and Johnson, D. B. (1999). Leaching of pyrite by acidophilic heterotrophic iron-oxidizing bacteria in pure and mixed cultures. *Appl. Environ. Microbiol.* 65, 585–590.

Baker, B. J., and Banfield, J. F. (2003). Microbial communities in acid mine drainage. *FEMS Microbiol. Ecol.* 44, 139–152.

Baker, B. J., Comolli, L. R., Dick, G. J., Hauser, L. J., Hyatt, D., Dill, B. D., Land, M. L., VerBerkmoes, N. C., Hettich, R. L., and Banfield, J. F. (2010). Enigmatic, ultrasmall, uncultivated Archaea. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8806–8811.

Bonnefoy, V., and Holmes, D. S. (2011). Genomic insights into microbial iron oxidation and iron uptake strategies in extremely acidic environments. *Environ. Microbiol.* doi: 10.1111/j.1462-2920.2011.02626.x [Epub ahead of print].

Brenner, K., You, L., and Arnold, F. H. (2008). Engineering microbial consortia: a new frontier in synthetic biology. *Trends Biotechnol.* 26, 483–489.

Çağatay, T., Turcotte, M., Elowitz, M. B., Garcia-Ojalvo, J., and Süel, G. M. (2009). Architecture-dependent noise discriminates functionally analogous differentiation circuits. *Cell* 139, 512–522.

Castro, M., Ruíz, L. M., Barriga, A., Jerez, C. A., Holmes, D. S., and Guiliani, N. (2009). C-di-GMP pathway in biomining bacteria. *Adv. Mat. Res.* 71–73, 223–226.

Chen, D., Lin, J., Che, Y., Liu, X., and Lin, J. (2011). Construction of recombinant mercury resistant *Acidithiobacillus caldus*. *Microbiol. Res.* 166, 515–520.

Czajkowski, R., and Jafra, S. (2009). Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochim. Pol.* 56, 1–16.

Das, B. K., Roy, A., Koschorreck, M., Mandal, S. M., Wendt-Potthoff, K., and Bhattacharya, J. (2009). Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization. *Water Res.* 43, 883–894.

Defoirdt, T., Boon, N., and Bossier, P. (2010). Can bacteria evolve resistance to quorum sensing disruption? *PLoS Pathog.* 6, e1000989. doi: 10.1371/journal.ppat.1000989

Denef, V. J., Mueller, R. S., and Banfield, J. F. (2010). AMD biofilms: using model communities to study microbial evolution and ecological complexity in nature. *ISME J.* 4, 599–610.

Di Maio, S., Polizzotto, G., Di Gangi, E., Foresta, G., Genna, G., Verzera, A., Scacco, A., Amore, G., and Oliva, D. (2012). Biodiversity of indigenous *Saccharomyces* populations from Old Wineries of South-Eastern Sicily (Italy): preservation and economic potential. *PLoS ONE* 7, e30428. doi: 10.1371/journal.pone.0030428

Donati, E. R., and Sand, W. (2007). *Microbial Processing of Metal Sulfides*. Dordrecht: Springer.

Dopson, M., and Lindstrom, E. B. (1999). Potential role of *Thiobacillus caldus* in arsenopyrite bioleaching. *Appl. Environ. Microbiol.* 65, 36–40.

Elowitz, M., and Lim, W. A. (2010). Build life to understand it. *Nature* 468, 889–890.

Farah, C., Vera, M., Morin, D., Haras, D., Jerez, C. A., and Guiliani, N. (2005). Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* 71, 7033–7040.

Friedland, A. E., Lu, T. K., Wang, X., Shi, D., Church, G., and Collins, J. J. (2009). Synthetic gene networks that count. *Science* 324, 1199–1202.

Fu, B., Zhou, H., Zhang, R., and Qiu, G. (2008). Bioleaching of chalcopyrite by pure and mixed cultures of *Acidithiobacillus* spp. and *Leptospirillum ferriphilum*. *Int. Biodeter. Biodegr.* 62, 109–115.

Gadd, G. M. (2009). Metals, minerals and microbes: geomicrobiology and bioremediation. *Microbiology* 156, 609–643.

Giovannoni, S. J., and Vergin, K. L. (2012). Seasonality in ocean microbial communities. *Science* 335, 671–676.

Haferburg, G., and Kothe, E. (2010). Metallomics: lessons for metalliferous soil remediation. *Appl. Microbiol. Biotechnol.* 87, 1271–1280.

Hale, C. R., Majumdar, S., Elmore, J., Pfister, N., Compton, M., Olson, S., Resch, A. M., Glover, C. V. C., Graveley, B. R., Terns, R. M., and Terns, M. B. (2012). Essential features and rational design of CRISPR RNAs that function with the Cas RAMP module complex to cleave RNAs. *Mol. Cell.* 45, 292–302.

Ham, T. S., Lee, S. K., Keasling, J. D., and Arkin, A. P. (2008). Design and construction of a double inversion recombination switch for heritable sequential genetic memory. *PLoS ONE* 3, e2815. doi: 10.1371/journal.pone.0002815

Hansen, S. K., Rainey, P. B., Haagensen, J. A. J., and Molin, S. (2007). Evolution of species interactions in a biofilm community. *Nature* 445, 533–536.

Harrison, A. P. (1984). The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. *Annu. Rev. Microbiol.* 38, 265–292.

Hengge, R. (2009). Principles of c-di-GMP signalling in bacteria. *Nat. Rev. Microbiol.* 7, 263–273.

Hong, S. H., Hegde, M., Kim, J., Wang, X., Jayaraman, A., and Wood, T. K. (2012). Synthetic quorum-sensing circuit to control consortial biofilm formation and dispersal in a microfluidic device. *Nat. Commun.* 3, 613.

Johnson, D. B. (1998). Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiol. Ecol.* 27, 307–317.

Johnson, D. B., and Hallberg, K. B. (2005). Acid mine drainage remediation options: a review. *Sci. Total. Environ.* 338, 3–14.

Johnson, D. B., Yajie, L., and Okibe, N. (2007). "Bioshrouding" – a novel approach for securing reactive mineral tailings. *Biotechnol. Lett.* 30, 445–449.

Kau, A. L., Ahern, P. P., Griffin, N. W., Goodman, A. L., and Gordon, J. I. (2011). Human nutrition, the gut microbiome and the immune system. *Nature* 474, 327–336.

Keller, L., and Surette, M. G. (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nat. Rev. Microbiol.* 4, 249–258.

Kusano, T., Sugawara, K., Inoue, C., Takeshima, T., Numata, M., and Shiratori, T. (1992). Electrotransformation of *Thiobacillus ferrooxidans* with plasmids containing a mer determinant. *J. Bacteriol.* 174, 6617–6623.

Liu, C., Fu, X., Liu, L., Ren, X., Chau, C. K. L., Li, S., Xiang, L., Zeng, H., Chen, G., Tang, L.-H., Lenz, P., Cui, X., Huang, W., Hwa, T., and Huang, J. D. (2011a). Sequential establishment of stripe patterns in an expanding cell population. *Science* 334, 238–241.

Liu, H., Yin, H., Dai, Y., Dai, Z., Liu, Y., Li, Q., Jiang, H., and Liu, X. (2011b). The co-culture of *Acidithiobacillus ferrooxidans* and *Acidiphilium acidophilum* enhances the growth, iron oxidation, and CO2 fixation. *Arch. Microbiol.* 193, 857–866.

Liu, W., Lin, J., Pang, X., Cui, S., Mi, S., and Lin, J. (2011c). Overexpression of rusticyanin in *Acidithiobacillus ferrooxidans* ATCC19859 increased Fe(II) oxidation activity. *Curr. Microbiol.* 62, 320–324.

Liu, Y., Yin, H., Zeng, W., Liang, Y., Liu, Y., Baba, N., Qiu, G., Shen, L., Fu, X., and Liu, X. (2011d). The effect of the introduction of exogenous strain *Acidithiobacillus thiooxidans* A01 on functional gene expression, structure and function of indigenous consortium during pyrite bioleaching. *Bioresour. Technol.* 102, 8092–8098.

Liu, Z., Guiliani, N., Appia-Ayme, C., Borne, F., Ratouchniak, J., and Bonnefoy, V. (2000). Construction and characterization of a recA mutant of *Thiobacillus ferrooxidans* by marker exchange mutagenesis. *J. Bacteriol.* 182, 2269–2276.

Lu, T. K., and Collins, J. J. (2007). Dispersing biofilms with engineered enzymatic bacteriophage. *Proc. Natl. Acad. Sci. U.S.A.* 104, 11197–11202.

Lynd, L. R., Weimer, P. J., van Zyl, W. H., and Pretorius, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev.* 66, 506–577.

Ma, S., and Banfield, J. F. (2011). Micron-scale Fe^{2+}/Fe^{3+}, intermediate sulfur species and O_2 gradients across the biofilm–solution–sediment interface control biofilm organization. *Geochim. Cosmochim. Acta* 75, 3568–3580.

Mangold, S., Valdés, J., Holmes, D. S., and Dopson, M. (2011). Sulfur metabolism in the extreme acidophile *Acidithiobacillus caldus*. *Front. Microbiol.* 2:17. doi: 10.3389/fmicb.2011.00017

Marraffini, L. A., and Sontheimer, E. J. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.* 11, 181–190.

McDougald, D., Rice, S. A., Barraud, N., Steinberg, P. D., and Kjelleberg, S. (2012). Should we stay or should we go: mechanisms and ecological consequences for biofilm dispersal. *Nat. Rev. Microbiol.* 10, 39–50.

Momeni, B., Chen, C.-C., Hillesland, K. L., Waite, A., and Shou, W. (2011). Using artificial systems to explore the ecology and evolution of symbioses. *Cell. Mol. Life Sci.* 68, 1353–1368.

Naghavi, N. S., Emami, Z. D., and Emtiazi, G. (2011). Synergistic copper extraction activity of *Acidithiobacillus ferrooxidans* isolated from copper coal mining areas. *Asian J. Appl. Sci.* 4, 447–452.

Noël, N., Florian, B., and Sand, W. (2010). AFM & EFM study on attachment of acidophilic leaching organisms. *Hydrometallurgy* 104, 370–375.

Ohata, A., Manabe, M., and ParadaValdecantos, P. A. (2010). United States Patent: 7700343 – Sulfur-oxidizing bacteria and their use in bioleaching processes for sulfured copper minerals. Available at: http://patft.uspto.gov/netacgi/nph-Parser?Sect2=PTO1&Sect2=HITOFF&p=1&u=/netahtml/PTO/search-bool.html&r=1&f=G&l=50&d=PALL&RefSrch=yes&Query=PN/7700343 (Accessed March 28, 2012).

Okibe, N., and Johnson, D. B. (2004). Biooxidation of pyrite by defined mixed cultures of moderately thermophilic acidophiles in pH-controlled bioreactors: significance of microbial interactions. *Biotechnol. Bioeng.* 87, 574–583.

Olson, G. J., Brierley, J. A., and Brierley, C. L. (2003). Bioleaching review part B: progress in bioleaching: applications of microbial processes by the minerals industries. *Appl. Microbiol. Biotechnol.* 63, 249–257.

Peng, J. B., Yan, W. M., and Bao, X. Z. (1994). Plasmid and transposon transfer to *Thiobacillus ferrooxidans*. *J. Bacteriol.* 176, 2892–2897.

Qiu, M., Xiong, S., Zhang, W., and Wang, G. (2005). A comparison of bioleaching of chalcopyrite using pure culture or a mixed culture. *Miner. Eng.* 18, 987–990.

Rawlings, D. E. (2002). Heavy metal mining using microbes. *Annu. Rev. Microbiol.* 56, 65–91.

Rawlings, D. E. (2007). *Biomining*. Berlin: Springer.

Rawlings, D. E., and Johnson, D. B. (2007). The microbiology of biomining: development and optimization of mineral-oxidizing microbial consortia. *Microbiology* 153, 315–324.

Rawlings, D. E., Tributsch, H., and Hansford, G. S. (1999). Reasons why "*Leptospirillum*"-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for the biooxidation of pyrite and related ores. *Microbiology (Reading, Engl.)* 145(Pt 1), 5–13.

Reid, A. J. (2012). Adapting to domesticity. *Nat. Rev. Microbiol.* 10, 163.

Rivas, M., Seeger, M., Holmes, D. S., and Jedlicki, E. (2005). A Lux-like quorum sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Biol. Res.* 38, 283–297.

Rivas, M., Seeger, M., Jedlicki, E., and Holmes, D. S. (2007). Second acyl homoserine lactone production system in the extreme acidophile *Acidithiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* 73, 3225–3231.

Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943.

Rohwerder, T., Gehrke, T., Kinzler, K., and Sand, W. (2003). Bioleaching review part A: progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation. *Appl. Microbiol. Biotechnol.* 63, 239–248.

Rollié, S., Mangold, M., and Sundmacher, K. (2012). Designing biological systems: Systems Engineering meets Synthetic Biology. *Chem. Eng. Sci.* 69, 1–29.

Ruiz, L. M., Castro, M., Barriga, A., Jerez, C. A., and Guiliani, N. (2011). The extremophile *Acidithiobacillus ferrooxidans* possesses a c-di-GMP signalling pathway that could play a significant role during bioleaching of minerals. *Lett. Appl. Microbiol.* 54, 133–139.

Ruiz, L. M., Sand, W., Jerez, C. A., and Guiliani, N. (2007). C-di-GMP pathway in *Acidithiobacillus ferrooxidans*: analysis of putative diguanyl atecyclases (DGCs) and phosphodiesterases (PDEs) bifunctional proteins. *Adv. Mat. Res.* 20–21, 551–555.

Sabra, W., Dietz, D., Tjahjasari, D., and Zeng, A.-P. (2010). Biosystems analysis and engineering of microbial consortia for industrial biotechnology. *Eng. Life Sci.* 10, 407–421.

Sand, W., and Gehrke, T. (2006). Extracellular polymeric substances mediate bioleaching/biocorrosion via interfacial processes involving iron(III) ions and acidophilic bacteria. *Res. Microbiol.* 157, 49–56.

Sand, W., Gehrke, T., Jozsa, P.-G., and Schippers, A. (2001). (Bio)chemistry of bacterial leaching – direct vs. indirect bioleaching. *Hydrometallurgy* 59, 159–175.

Shong, J., Jimenez Diaz, M. R., and Collins, C. H. (2012). Towards synthetic microbial consortia for bioprocessing. *Curr. Opin. Biotechnol.* doi: 10.1016/j.copbio.2012.02.001 [Epub ahead of print].

Sprinzak, D., and Elowitz, M. B. (2005). Reconstruction of genetic circuits. *Nature* 438, 443–448.

Stocker, J., Balluch, D., Gsell, M., Harms, H., Feliciano, J., Daunert, S., Malik, K. A., and van der Meer, J. R. (2003). Development of a set of simple bacterial biosensors for quantitative and rapid measurements of arsenite and arsenate in potable water. *Environ. Sci. Technol.* 37, 4743–4750.

Süel, G. M., Garcia-Ojalvo, J., Liberman, L. M., and Elowitz, M. B. (2006). An excitable gene regulatory circuit induces transient cellular differentiation. *Nature* 440, 545–550.

Valdés, J., Pedroso, I., Quatrini, R., and Holmes, D. S. (2008). Comparative genome analysis of *Acidithiobacillus ferrooxidans*, *A. thiooxidans* and *A. caldus*: insights into their metabolism and ecophysiology. *Hydrometallurgy* 94, 180–184.

Viramontes-Gamboa, G., Peña-Gomar, M. M., and Dixon, D. G. (2010). Electrochemical hysteresis and bistability in chalcopyrite passivation. *Hydrometallurgy* 105, 140–147.

Wang, H., Liu, X., Liu, S., Yu, Y., Lin, J., Lin, J., Pang, X., and Zhao, J. (2012). Development of a markerless gene replacement system for *Acidithiobacillus ferrooxidans* and construction of a pfkB mutant. *Appl. Environ. Microbiol.* 78, 1826–1835.

Wenbin, N., Dejuan, Z., Feifan, L., Lei, Y., Peng, C., Xiaoxuan, Y., and Hongyu, L. (2011). Quorum-sensing system in *Acidithiobacillus ferrooxidans* involved in its resistance to Cu^{2+}. *Lett. Appl. Microbiol.* 53, 84–91.

Wilmes, P., Simmons, S. L., Denef, V. J., and Banfield, J. F. (2009). The dynamic genetic repertoire of microbial communities. *FEMS Microbiol. Rev.* 33, 109–132.

Wintermute, E. H., and Silver, P. A. (2010). Dynamics in the mixed microbial concourse. *Gene. Dev.* 24, 2603–2614.

Zhou, J., He, Q., Hemme, C. L., Mukhopadhyay, A., Hillesland, K., Zhou, A., He, Z., Van Nostrand, J. D., Hazen, T. C., Stahl, D. A., Wall, J. D., and Arkin, A. P. (2011). How sulphate-reducing microorganisms cope with stress: lessons from systems biology. *Nat. Rev. Microbiol.* 9, 452–466.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 10 April 2012; accepted: 17 May 2012; published online: 05 June 2012.

Citation: Brune KD and Bayer TS (2012) Engineering microbial consortia to enhance biomining and bioremediation. Front. Microbio. ***3****:203. doi: 10.3389/fmicb.2012.00203*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 30 August 2012
doi: 10.3389/fmicb.2012.00313

Engineering microbial chemical factories to produce renewable "biomonomers"

Jake Adkins, Shawn Pugh, Rebekah McKenna and David R. Nielsen*

Chemical Engineering, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ, USA

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Kesaven Bhubalan, Universiti Malaysia Terengganu, Malaysia
Martin Siemann-Herzberg, University Stuttgart, Germany

***Correspondence:**
David R. Nielsen, Chemical Engineering, School for Engineering of Matter, Transport, and Energy, Arizona State University, Tempe, AZ 85287-6106, USA.
e-mail: david.r.nielsen@asu.edu

By applying metabolic engineering tools and strategies to engineer synthetic enzyme pathways, the number and diversity of commodity and specialty chemicals that can be derived directly from renewable feedstocks is rapidly and continually expanding. This of course includes a number of monomer building-block chemicals that can be used to produce replacements to many conventional plastic materials. This review aims to highlight numerous recent and important advancements in the microbial production of these so-called "biomonomers." Relative to naturally-occurring renewable bioplastics, biomonomers offer several important advantages, including improved control over the final polymer structure and purity, the ability to synthesize non-natural copolymers, and allowing products to be excreted from cells which ultimately streamlines downstream recovery and purification. To highlight these features, a handful of biomonomers have been selected as illustrative examples of recent works, including polyamide monomers, styrenic vinyls, hydroxyacids, and diols. Where appropriate, examples of their industrial penetration to date and end-product uses are also highlighted. Novel biomonomers such as these are ultimately paving the way toward new classes of renewable bioplastics that possess a broader diversity of properties than ever before possible.

Keywords: bioplastics, biopolymers, monomers, metabolic engineering

INTRODUCTION

At present, nearly all conventional plastics are derived from non-renewable natural gas and petroleum resources. Global annual plastics production exceeded 260 million tons in 2009, accounting for roughly 8% of total worldwide oil consumption (Thompson et al., 2009). By just 2010, the total mark had grown to 265 million tons, with major producers China at 23.5% of total production, the European Union at 21.5%, and the rest of Asia contributing another 20% (Plastics Europe, 2011). North American producers, meanwhile, contributed to nearly 20.5% of that mark, consuming approximately 331 million barrels of liquid petroleum gases (LPG) and natural gas liquids (NGL) and nearly 11 billion ft^3 of natural gas as raw materials in the process (US Energy Information Administration, 2011), in addition to sizable energy demands. All told, in the US, for example, plastics production is responsible for about 4.6% of total consumption of LPG and NGL, 1.5% of total natural gas consumption, and ∼1% of total electricity usage. In view of the declining availability of petrochemical feedstocks, the expectation that global plastics production will surpass 297 million tons by 2015 (Global Industry Analysts, 2012), and rising consumer demand for "greener" products, there is increased interest to develop alternative plastics from renewable and sustainable resources.

In contrast to petroleum and natural gas derived plastics, "bioplastics" are produced from the carbon and energy stored in renewable biomass feedstocks. As such, bioplastics offer the promise to reduce dependence on non-renewable oil and natural gas, and can positively impact the carbon cycle by consuming atmospheric CO_2 (a greenhouse gas) during biomass feedstock cultivation. As the global demand for bioplastics is predicted to triple by 2015 to reach >1 million tons per year to represent a $2.9 billion market (Freedonia Group, 2009), this field is clearly ripe for opportunity and advancements. Whereas technologies exist to convert biomass to plastics through either chemocatalytic processing or by using microbial biocatalysts, the focus here will strictly be on the latter. Together with other recent and complementary reviews (Erickson et al., 2011; Lee et al., 2011a,b, 2012; Curran and Alper, 2012), this article seeks to illustrate how advancements in metabolic and pathway engineering are leading to the development of novel biocatalysts and, in so doing, they are also helping to shape the future of the bioplastics industry.

NATURALLY-OCCURRING BIOPLASTICS

A number of naturally-occurring biopolymers produced by microorganisms from renewable resources have been found to be useful as bioplastics. The most notable are polyhydroxyalkanoates, or PHAs, which have been extensively reviewed over the years (Braunegg et al., 1998; Chen, 2009; Keshavarz and Roy, 2010). PHAs are biodegradable, linear polyesters composed primarily of monomer subunits of (*R*)-3-hydroxybutyrate (3HB) and are produced as carbon and energy storage molecules by numerous different microbes from both simple and complex biomass feedstocks (note that monomers of 3HB and other β-hydroxyacids will be discussed in detail below). Well-studied examples of bacterial PHA producers include species of *Ralstonia* (Reinecke and Steinbuchel, 2009), *Bacillus* (Singh et al., 2009),

and *Pseudomonas* (Rojas-Rosas et al., 2007), as well as phototrophic cyanobacteria (Asada et al., 1999).

Although PHAs are efficiently produced by microbes via naturally evolved biosynthesis routes, expressing the requisite pathway enzymes from various natural producers has also enabled their production at high levels in recombinant *Escherichia coli*, a more tractable host platform (Schubert et al., 1988). All told, bacteria have been engineered to achieve PHA biosynthesis at up to 80% of cell dry weight and at productivities as high as 4 g/L-h from substrates such as glucose (Lee et al., 1999). As crystalline and heat resistant polymers, with glass transition and melting temperatures of around 5°C and 175°C, respectively, PHAs are most well poised as replacements to polyethylene (PE) and polypropylene (PP) (Pei et al., 2011). However, unlike PE and PP, the natural biodegradability PHAs ensures that they will not leave a legacy in landfills at their end of use. Commercial interest in PHAs has been growing, with Metabolix (Cambridge, MA), for example, pursuing their large scale development through their Mirel™ and Mvera™ brands, which collectively are expected to have applications in the production of fibers, films, and coatings, as well as for molded and extruded products.

In spite of numerous advantages, PHA production also suffers from other inherent shortcomings which may ultimately impede their long-term and wide-spread utility. For instance, while their biodegradable nature can help reduce municipal waste, this attribute renders PHAs unsuitable for use in applications requiring long term durability and/or environmental exposure. As all PHAs are thermoplastic polyesters the achievable range of physicochemical and material properties, though diverse, is inherently limited. PHA chain length and purity are difficult to tune and control with high quality assurance in an *in vivo* setting. Lastly, as they are macromolecules, PHAs do not readily diffuse out from cells, but rather accumulate intracellularly as cytosolic inclusion bodies. Thus, since PHAs can only be harvested after the microbial biocatalyst is lysed, prospects for the continuous bioprocessing of PHAs remain unviable.

Another import class of bioplastics that microorganisms naturally produce are those that can be derived from sugars. For example, select strains of *Acetobacter*, most notably *A. xylinum*, are capable of producing high quality cellulose by polymerizing linear chains of β-1,4-glucan (Krystynowicz et al., 2002). Natural cellulose polymers are most attractive due to their inherent biodegradability. Today, most high-grade cellulose is extracted from agricultural sources, for example, fast growing trees or cotton linters. Bacterial cellulose is an attractive alternative, however, because it is free of lignin and hemicelluloses and, with a molecular weight 100-fold lower than plant cellulose, it also possesses greater mechanical strength (Czaja et al., 2006). After synthesizing linear β-1,4-glucan chains and secreting them through pores in their outer membrane, their assembly into microfibril bundles then takes place outside the cell (Ross et al., 1991). Thus, in contrast to PHAs, bacterial cellulose is more amenable to downstream recovery and purification; however, just as PHAs, it still suffers from the same quality assurance and quality control concerns. Nevertheless, bacterial cellulose has found many successful commercial applications. For example, bacterial cellulose is used in Brazil by BioFill Produtos Biotecnologicos in their line of medical films (Biofill®, Bioprocess®, and Gengiflex®) (Hoenich, 2006), whereas Xylos Corp. (Langhorne, PA) also uses it to produce a line of wound care products (XCell®) (Czaja et al., 2006).

NATURALLY-OCCURRING BIOMONOMERS

Limitations associated with naturally-occurring bioplastics have motivated a recent shift in focus to the alternative production of "biomonomers." Biomonomers are small molecules (i.e., monomeric subunits) that can undergo *ex situ* chemocatalytic polymerization to produce plastics. This strategy offers several distinct advantages. First, since most biomonomers are often naturally excreted from microorganisms the need for cell collection and lysis is eliminated, greatly reducing operating expenditures and facilitating downstream product recovery. Polymerization of biomonomers in highly controlled chemocatalytic environments leads to bioplastics with finely tuned and predictable properties and at high purities to satisfy quality control specifications. Lastly, the ability to co-polymerize biomonomers with other desired monomers (biologically or otherwise derived) increases the diversity of plastics that can be produced from renewable resources, widening the range of achievable chemistries and material properties.

In general, there are two classes of biomonomers of industrial interest. The first can be produced from natural metabolites with the aid of a few simple catalytic post-processing steps. For example, the natural fermentation product ethanol can undergo dehydration to ethylene over a solid acid catalyst (Takahara et al., 2005; Hu et al., 2010), before later being polymerized to poly(ethylene) or its co-polymers. Braskem is taking this approach to convert sugarcane-derived ethanol to bioplastics in Brazil (Phillips, 2008). The second class of biomonomers, which will be the central focus of this review, are those metabolites that themselves are directly suitable for polymerization into bioplastics. Wherever possible, the microbial production of "drop in compatible" biomonomers by this approach is advantageous because, as illustrated in **Figure 1**, it ensures a seamless interface between emerging biotechnologies and the existing polymer industry. This strategy enables decades of technological expertise and existing process infrastructure to be efficiently leveraged, and effectively

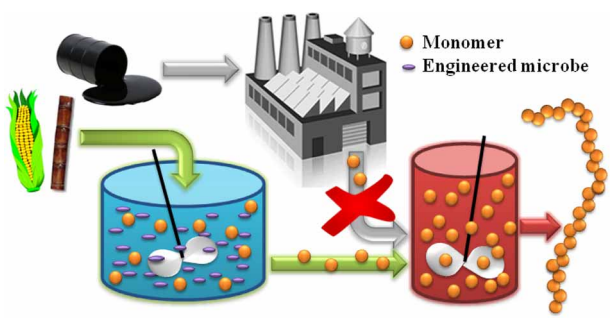


FIGURE 1 | The alternative production of "drop in compatible" monomer compounds from biomass feedstocks enables the development of new classes of bio-derived polymers by coupling emerging biotechnologies with mature polymer processing technologies.

allows conventional chemical and plastics processing to "pick up where biology leaves off."

The most prominent example of a naturally-occurring biomonomer is perhaps *L*(+)-lactic acid, whose polymerization leads to the polyester poly(lactic acid), or PLA. *L*(+)-Lactic acid is a natural fermentation product of numerous microbes, of which prominent examples include lactic acid bacteria (including numerous *Lactobacilli* sp.) and filamentous fungi (Kosakai et al., 1997; Zhou et al., 1999). As microbial lactic acid production has been extensively reviewed in the past (Wee et al., 2006; Reddy et al., 2008), it will not be addressed in detail here. It is worth noting, however, that *E. coli* been engineered to overproduce both *L*(+)- (Zhou et al., 1999) and *D*(−)- (Zhou et al., 2003; Mazumdar et al., 2010) stereroisomers of lactic acid as optically pure products. This is important because lactic acid stereochemistry greatly controls relevant physical properties of PLA including, for example, crystallinity and in turn melting point (Lunt, 1998; Sodergard and Stolt, 2002). In a related and interesting study, Lee and coworkers recently engineered *E. coli* for the direct *in vivo* production of PLA by modifying the substrate specificity of PHA synthase 1 of a *Pseudomonas* sp. and promoting the production of lactyl-CoA, the monomer precursor, by introducing an evolved propionate CoA-transferase from *Clostridium propionicum* (Jung et al., 2010; Yang et al., 2010). However, just as PHA is confined within cells, so too is *in vivo* produced PLA. Thus, it is not yet clear how easily or well this specific approach will translate to the commercial scale. Today, NatureWorks LLC (Minnetonka, MN) already produces > 20 different grades of their Ingeo™ PLA bioplastics with structures that range from amorphous to crystalline and uses spanning from films to foams. Like PHAs, PLA continues to be a desirable target due in large part to its biodegradability.

A second naturally-occurring biomonomer that has been extensively investigated to date is succinic acid. This 4-carbon diacid is a natural fermentation product of numerous bacteria, including *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, and *Mannheimia succiniciproducens,* which have been reviewed elsewhere (Song and Lee, 2006). Moreover, *E. coli's metabolism* has again also been extensively engineered to overproduce this minor fermentation product to near theoretical yields (Thakker et al., 2012). Succinic acid is particularly useful for producing both polyesters and polyamides, and the first commercial bioplastics derived from this biomonomer will likely be polyamide-5,4 (Qian et al., 2009) and/or poly(butylene-*co*-succinate) (PBS) (Bechthold et al., 2008), a biodegradable thermoplastic suitable for extrusion, injection molding, thermoforming, and film blowing (Xu and Guo, 2010). As microbial succinic acid production is now becoming a mature technology, several producers, including both Myriant (Quincy, MA) and BioAmber (Plymouth, MN), are now developing commercial scale fermentation processes. BioAmber, for example, currently operates a 3000 ton capacity plant in France, with plans to commission a 17,000 ton facility in Canada in 2013.

ENGINEERING NOVEL BIOMONOMERS

Despite the potential advantages of using biomonomers to produce renewable plastics, a limited pool of useful naturally-occurring metabolites ultimately constricts the diversity of bioplastics that can be produced by this approach. However, by applying metabolic engineering tools and strategies, *de novo* metabolic pathway engineering provides the potential to produce non-natural biomonomers, and novel bioplastics, that were never before possible.

Through the rational re-engineering of microbial metabolism "microbial chemical factories" can be constructed to convert biomass feedstocks into chemical products of high value and/or utility as greener and more sustainable alternatives to petrochemicals (Carothers et al., 2009; Martin et al., 2009). This approach has already led to significant and recent improvements to the production of renewable fuels (Atsumi et al., 2008; Atsumi and Liao, 2008; Peralta-Yahya and Keasling, 2010; Schirmer et al., 2010; Steen et al., 2010; Mendez-Perez et al., 2011; Rude et al., 2011), chemicals (Wierckx et al., 2005; Qian et al., 2009; Kind et al., 2010a; Schirmer et al., 2010; Whited et al., 2010), and active pharmaceutical ingredients (Ro et al., 2006; Leonard et al., 2009; Tsuruta et al., 2009; Campbell and Vederas, 2010). As illustrated by several examples in **Figure 2**, these strategies now offer the same potential to enhance production of novel biomonomers and, as the following selected examples will illustrate, significant progress is already being made in this regard.

POLYAMIDE BIOMONOMERS

Polyamides (PAs; also known as nylons) are a class of plastics that balance mechanical strength and durability with chemical resistance, and which find extensive use as textiles and mechanical parts. PAs are formed either as homopolymers of amino acids or as co-polymers via condensation of diamines with diacids. The most common commercial PAs are PA-6 (a homopolymer of 6-aminohexanoic acid) and PA-6,6 (a copolymer of adipic acid and 1,6-hexanediamine) which account for more than 85–90% of the global market, which will approach 6.6 million tons by 2015 (Nexant Chem Systems, 2009). As carbon chain length between amide bonds (typically 2–12) strongly influences material properties, a large diversity of PAs can be generated from a limited monomer pool by a combinatorial approach. Here, however, we specifically focus on recent progress and opportunities related to the microbial production of 4- through 6-carbon linear diamines, amino acids, and diacids (**Figure 3**).

Putrescine, the 4-carbon diamine counterpart to succinic acid, occurs as an amino acid biodegradation product, however no natural over-producers have been isolated to date. Putrescine can be produced by *E. coli* by two different pathways that stem from either L-arginine (through agmatine by SpeAB) or L-ornithine (from L-glutamate by SpeC and SpeF) (Tabor and Tabor, 1985; Igarashi and Kashiwagi, 2000). Several researchers have taken to rationally engineering putrescine over-producing microbes including Qian et al. who, for example, developed a putrescine over-producing *E. coli* strain by increasing metabolite flux toward L-ornithine (Qian et al., 2009). This was achieved through both promoter replacements and targeted deletions to overcome allosteric and transcriptional regulation of the L-ornithine biosynthesis pathway. Transcriptional repression by L-arginine upon the native promoters P_{argCBH}, P_{argD}, and P_{argE} was alleviated by their replacement with the strong

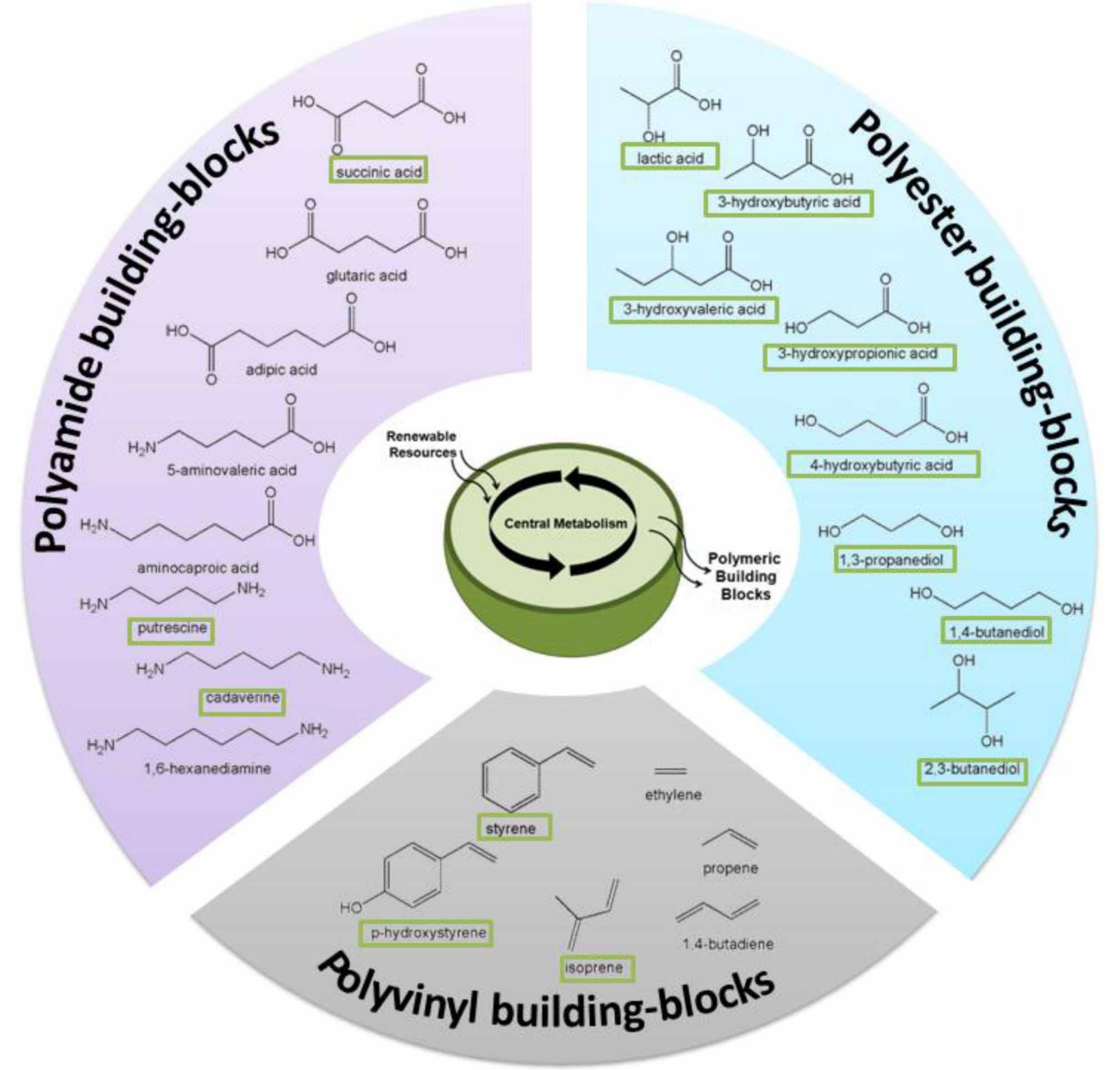


FIGURE 2 | Numerous biomonomers can now be produced as a result of metabolic and pathway engineering; examples discussed in this review are depicted here. "Boxed" names signify molecules that have been produced directly from renewable resources, whereas others have been produced via a hybrid, biocatalytic-chemocatalytic approach.

promoter P_{trc}. Meanwhile, P_{speC} and $P_{sepFpotE}$ were also replaced with P_{trc} to relieve feed-back regulation by putrescine and to increase putrescine export through the *potE* exporter, respectively. Multiple competing pathways were deleted and the resultant *E. coli* strain produced 24.2 g/L putrescine from glucose in fed-batch cultures (Qian et al., 2009). *Corynebacterium glutamicum* has also been used for engineering putrescine over-production by both the L-ornithine and L-arginine pathways, however, the L-arginine pathway was ∼40-fold less effective, most likely due to the inhibitory effects of the urea byproduct (Schneider and Wendisch, 2010). With the L-ornithine pathway, expression of L-ornithine decarboxylase from *E. coli* resulted in putrescine titers of up to 6 g/L (Schneider and Wendisch, 2010). Thus, the relative utility of the L-ornithine-derived pathway is conserved between both host platforms investigated. DSM Engineering Plastics (The Netherlands) currently uses putrescine derived from castor oil to produce PA-4,6 and PA-4,10, marketed as Stanyl™ and EcoPaXX™, respectively. Microbial putrescine production from simple sugars, however, would add economic and sustainability benefits.

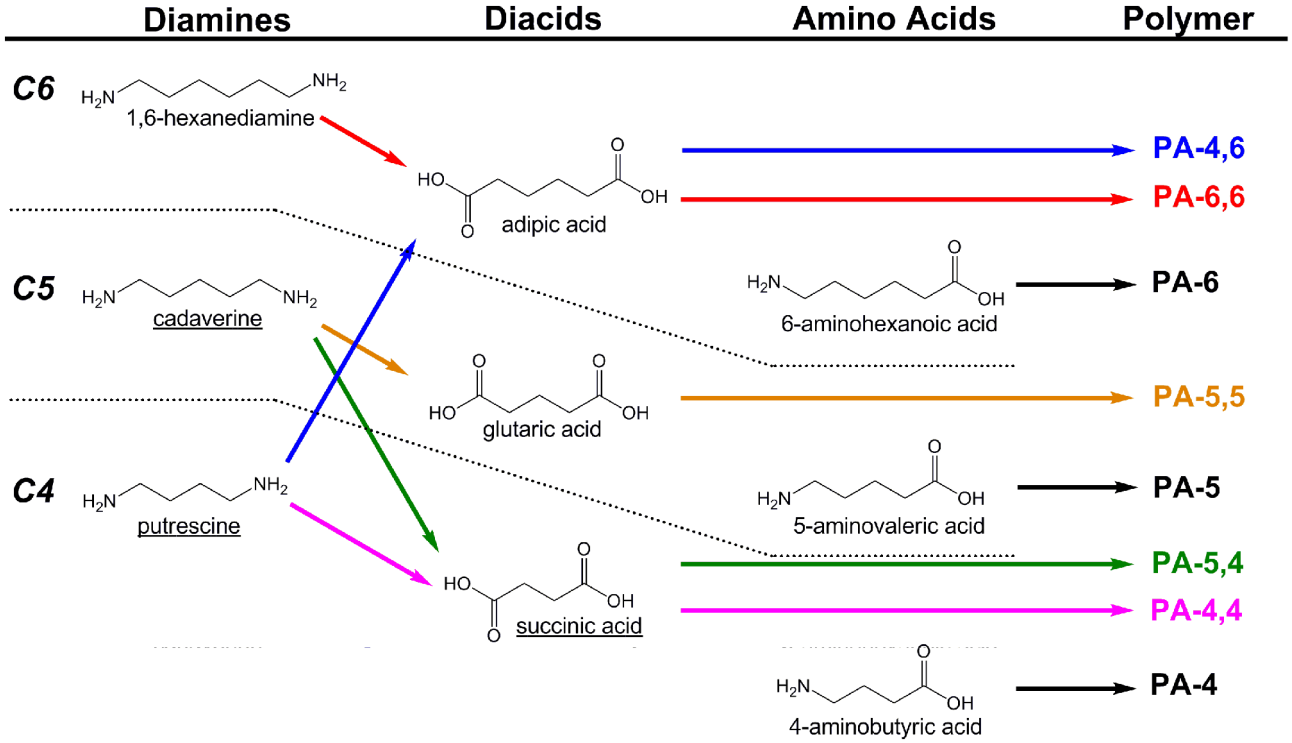


FIGURE 3 | Examples of polyamides (PAs) produced from combinations of C4–C6 diamines, diacids, and amino acids. To date, molecules with names underlined have been produced microbially from renewable resources with the aid of metabolic engineering.

Similar to putrescine, the 5-carbon diamine cadaverine also naturally occurs as an amino acid decarboxylation product, and only at low levels. In this case, it is the decarboxylation of L-lysine that forms cadaverine. PAs derived from cadaverine possess such desirable properties as high melting points and low water absorption (Kind and Wittmann, 2011). In addition, PA-5,4 and PA-5,10 have been proposed as bio-based alternatives to several petroleum-derived PAs (Kind and Wittmann, 2011; Qian et al., 2011). Mimitsuka et al. were first to engineer a microbe for cadaverine over-production from glucose by inserting the acid inducible lysine decarboxylase (encoded by *cadA*) of *E. coli* into the genome of a L-lysine over-producing strain of *C. glutamicum*, as seen in **Figure 4** (Mimitsuka et al., 2007). The same basic strategy has since been implemented in both *E. coli* and *C. glutamicum* which, through additional engineering, have produced cadaverine up to 9.6 g/L in *E. coli* and over 10 g/L in *C. glutamicum* on renewable substrates (Mimitsuka et al., 2007; Tateno et al., 2009; Kind et al., 2010a; Qian et al., 2011). For example, to further enhance production in *E. coli*, Qian et al. subsequently deleted the native enzymes spermacetyl transferase, spermidine synthase, cadaverine oxidase, and putrescine aminotransferase (encoded by *speG*, *speE*, *ygjG*, and *puuA*, respectively) which use cadaverine as substrate (Qian et al., 2011). In *C. glutamicum*, Kind et al. recently showed *ldcC* of *E. coli* functions better than *cadA* as a result of differences in pH optima (Kind et al., 2010a), and codon-optimization further aided its function. To increase the availability of L-lysine, *lysE* was deleted to eliminate losses due to lysine export from *C. glutamicum* (**Figure 4**) (Kind et al., 2010b). Genome-wide transcript analysis identified a putative permease (encoded by *cg2893*) that was up-regulated in the presence of cadaverine. While deletion of *cg2893* reduced cadaverine production by 90%, its over-expression using the strong promoter P_{sod} improved cadaverine yields by 20% (Kind et al., 2011). These results show that if cadaverine can be effectively exported from cells, the negative impacts of allosteric product feed-back inhibition on engineered pathways can be reduced. Most importantly, strategies analogous to those developed here may be of broader utility to the production of other biomonomers and biochemicals.

PA homopolymers are produced from amino acid monomers, a 4-carbon example of which is 4- or γ-aminobutyrate (GABA). GABA is naturally produced by many organisms from L-glutamate via glutamate decarboxylase. Whereas a microbe that produces GABA directly from glucose has yet to be reported, GABA production from exogenously fed L-glutamate has been investigated in strains engineered to over-express heterologous glutamate decarboxylase. For example, up to 5.5 g/L GABA was produced from 10 g/L of glutamate by *E. coli* expressing glutamate decarboxylase (encoded by *gadB*) and the GABA/Glutamate antiporter (encoded by *gadC*) (Le Vo et al., 2012). Additionally, the GABA aminotransferase (encoded by *gabT*) was deleted to prevent endogenous GABA degradation. Future engineering of L-glutamate over-producing hosts will no doubt lead to strains that can achieve high-level GABA production directly from glucose.

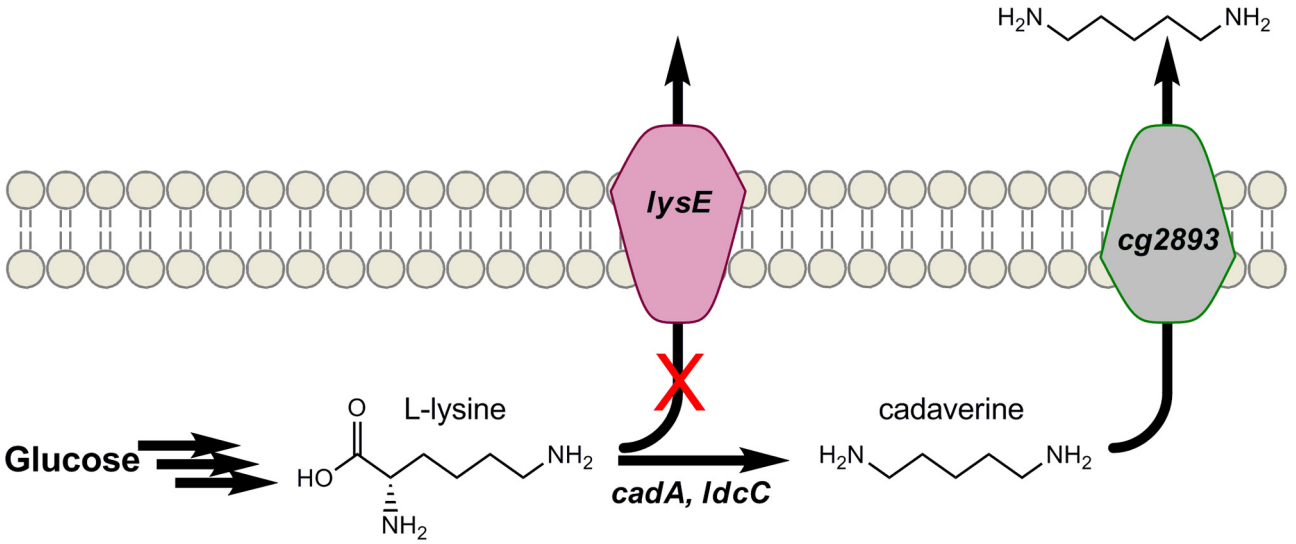


FIGURE 4 | Engineering cadaverine production in Corynebacterium glutamicum by (1) over-expressing L-lysine decarboxylase (*cadA*), (2) disrupting L-lysine the exporter (*lysE*), and (3) over-expressing an identified cadaverine exporter (*cg2893*).

As illustrated in **Figure 3**, the bio-production of several other useful PA monomers has yet to be realized. Like cadaverine, the 5-carbon diacid glutaric acid is known to be a natural L-lysine degradation product. Numerous *Pseudomonas* sp., for example, are known to degrade L-lysine through glutaric acid via 5-aminovaleric acid (AMV) in the AMV pathway (Revelles et al., 2007). Thus, if the AMV pathway can be functionally reconstructed in a L-lysine over-producing host, renewable routes to PA-5 and PA-5,5 would be possible. While PA-5 has not achieved commercial realization, it is known to possess properties close to PA-4,6 and could serve as a suitable substitute (Bermudez et al., 2000). Meanwhile, the microbial production of three of the most important conventional PA monomers, namely 6-aminohexanoic acid (precursor to PA-6), adipic acid, and 1,6-hexanediamine (both precursors to PA-6,6) has yet to be demonstrated. The closest demonstration was of adipic acid production, from Niu et al. who engineered *E. coli* to produce *cis,cis*-muconic acid from glucose at final concentrations of 36 g/L (Niu et al., 2002). *cis,cis*-Muconic acid can be hydrogenated over a palladium catalyst at high pressures to yield adipic acid. Certainly, however, a "one pot" biosynthetic approach would be preferred.

STYRENIC VINYLS

Styrenic vinyls are a family of polymers and co-polymers that use styrene or substituted styrene as a key monomer building-block. Today, nearly 60% of styrene's global annual production (about six million tons in the US manufacturers and 24 million tons globally), for example, supports plastics production (Sri, 2010). Examples include polystyrene (PS), acrylonitrile-butadiene-styrene (ABS), styrene-acrylonitrile (SAN), and styrene butadiene rubber (SBR). Low density PS foams, for instance, are widely used in packaging and as thermal insulators (Thorball, 1967), while the "rubbery" characteristics of SBR make it a natural rubber alternative and the major constituent of tires and many gaskets. Today, all styrenic plastics are produced using petroleum-derived monomers. For example, styrene is produced via ethylbenzene dehydrogenation (Wu et al., 1981). This reaction, however, demands over 3 metric tons of steam per ton of styrene produced, rendering styrene as one of the most energy-intensive commodity petrochemicals (US Department of Energy, 2002). The recent engineering of novel enzymatic routes to both p-hydroxystyrene (pHS) and styrene and from glucose (**Figure 5**), however, may soon enable their renewable production.

The first styrenic biomonomer to be synthesized from renewable resources was pHS (Qi et al., 2007). Its innate optical properties make pHS useful for producing photoresist polymers used in semiconductor manufacturing, for example (Pawlowski et al., 1991). As seen in **Figure 5**, the engineered pHS biosynthesis pathway stemmed from L-tyrosine as its immediate endogenous precursor. Only two enzymatic steps were then required to convert L-tyrosine to pHS. First, the bi-functional phenylalanine/tyrosine ammonia lyase (PAL/TAL) of the yeast *Rhodotorula glutinis* catalyzed p-coumaric acid formation. Then, p-coumaric acid was subsequently decarboxylated by phenylacrylate decarboxylase of either *Bacillus subtilis* (encoded by *pdc*) or *Lactobacillus plantarum* (encoded by *padC*). Initial *E. coli* strains expressing the pHS pathway achieved final titers as high as only 400 mg/L. Accumulation beyond this mark was severely limited as a result of pHS toxicity imposed on the *E. coli* host (Qi et al., 2007). To overcome toxicity limitations, the well-known aromatic-tolerant *Pseudomonas putida* S12 was next investigated as a pHS production platform. Utilizing two strains derived from *P. putida* S12 to overproduce precursor L-tyrosine as hosts, the pHS pathway was introduced. Due to the innate ability of *P. putida* S12 to degrade numerous aromatic solvents, multiple competing pathway genes were also targeted for deletion. These included both *smo*, which encodes styrene monooxygenase, and *fcs*, the first gene in the p-coumaric acid degradation pathway.

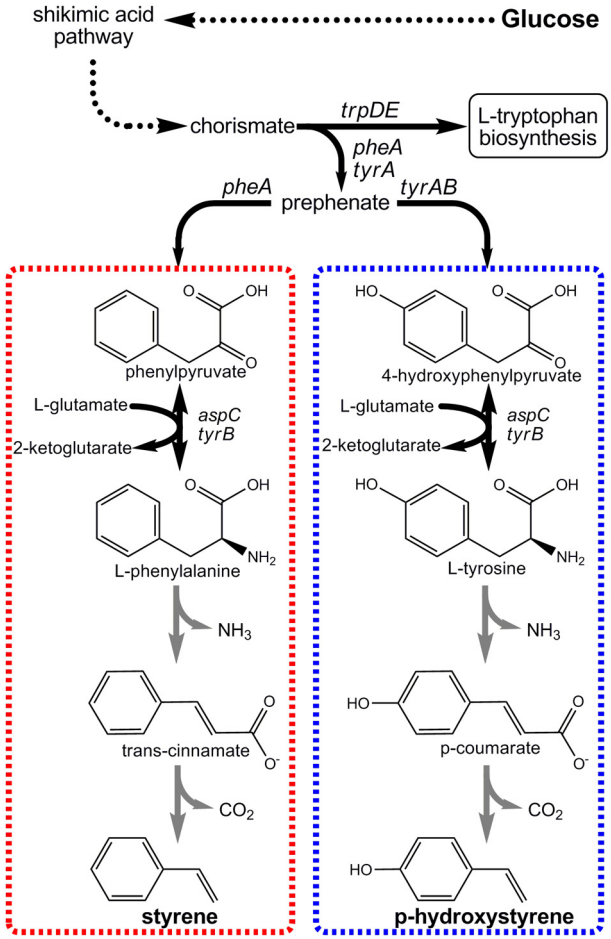


FIGURE 5 | Novel pathways engineered for the production of p-hydroxystyrene and styrene from renewable glucose via aromatic amino acid precursors.

All told, engineered *P. putida* S12 strains were able to reach and tolerate pHS titers as high as 545 mg/L, nearly a 50% improvement.

More recently, our own group engineered a novel pathway to enable *E. coli* to produce styrene directly from glucose (McKenna and Nielsen, 2011). The styrene biosynthetic pathway is analogous to the pHS pathway (**Figure 5**), except in that it stems from L-phenylalanine as its endogenous precursor and as such requires different pathway enzyme "parts." In this case, L-phenylalanine ammonia lyase (PAL) first converts L-phenylalanine to trans-cinnamic acid which is then decarboxylated to styrene. Upon screening numerous enzyme candidates, it was ultimately found that PAL2 from *Arabidopsis thaliana* and FDC1 from *Saccharomyces cerevisiae* were most effective for these steps, respectively. By over-expressing PAL2 and FDC1 in a L-phenylalanine over-producing *E. coli* host, styrene titers as high as 264 mg/L were achieved. As with pHS, styrene titers too approached *E. coli*'s toxicity limit (~300 mg/L) and must similarly be overcome if economical styrene biomonomer production is to be achieved.

HYDROXYACIDS

In recent years, much attention has been given to the bioproduction of the enantiomerically pure hydroxyacids. Not only is this class of molecules useful as precursors for the production of pharmaceuticals, vitamins, antibiotics, and flavor compounds (Tseng et al., 2009), but hydroxyacids can also serve as biomonomers to derive other renewable polyesters. Hydroxyacids of interest in bioplastics include terminal hydroxyacids such as 3-hydroxypropionate (3HP), as well as the β-hydroxyacids 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV). Each of these hydroxyacid monomers have been produced microbially from renewable resources via pathway engineering.

The shortest of the hydroxyacid monomers is 3HP. 3HP was previously named one of the 12 "Top Value Added Chemicals from Biomass" by the US Department of Energy (2004). In addition to serving as polyester monomer, 3HP can also be used to produce several other specialty and commodity chemicals through additional chemocatalytic processing (Rathnasingh et al., 2009), including the conventional monomers acrylamide, propiolactone, malonic acid, and 1,3-propanediol. The most notable of which, however, is acrylic acid produced by the dehydration of 3HP. Acrylic acid and its esters are used in polymeric flocculants, dispersants, coatings, paints, and adhesives (Straathof et al., 2005) and represent a $10 billion global market. Several companies are now pursuing the commercial development of 3HP-derived acrylic acid, including, for example, OPX Biotechnologies (Boulder, CO).

While several distinct enzymatic routes have been proposed for microbial 3HP production (Jiang et al., 2009), two have demonstrated the greatest promise to date. The first was a two-step pathway consisting of an adenosylcobalamin dependent glycerol dehydratase to catalyze the dehydration of glycerol to 3-hydroxypropionaldehyde (3HPA), and an aldehyde dehydrogenase to oxidize 3HPA to 3HP (Suthers and Cameron, 2000). Using this pathway, Rathnasingh and coworkers engineered *E. coli* to produce 38.7 g/L 3HP in a fed-batch bioreactor with glycerol as substrate. In their strain, glycerol dehydratase (DhaB) from *Klebsiella pneumoniae* DSM 2026 and α-ketoglutaric semialdehyde dehydrogenase (KGSADH) from *Azospirillum brasilense* were selected (Rathnasingh et al., 2009). A key shortcoming of this approach, however, was the uneconomical requirement for exogenous coenzyme-B12 supplementation. To address this limitation, *E. coli* was later engineered to produce 3HP from glucose in a coenzyme-B12-independent manner. This second 3HP pathway consisted of over-expressing the acetyl-CoA carboxylase and biotinilase genes of *E. coli* K-12 (*accADBCb*) to convert endogenously produced acetyl-CoA to malonyl-CoA, and expressing the NADPH-dependent malonyl-CoA reductase gene (*mcr*) of *Chloroflexus aurantiacus* DSM 635 to reduce malonyl-CoA to 3HP (Rathnasingh et al., 2012). This second pathway, however, initially suffered from inherent NADPH limitations under aerobic conditions. This was ultimately addressed by co-expressing nicotinamide nucleotide transhydrogenase (*pntAB* from *E. coli*) to convert NADH (the more abundant reducing equivalent) to NADPH, leading to 3HP titers of up to 0.19 g/L (Rathnasingh et al., 2012).

Although both 3HP pathways possess their own unique limitations which hinder their industrial potential, these pathways represent platforms to which subsequent improvements can now be made via continued strain development. For example, the B12-dependent pathway from glycerol may be well-suited for use in natural organisms which are employed for the industrial production of coenzyme-B12, such as *Propionibacterium shermanii* or *Pseudomonas denitrificans*, for example (Martens et al., 2002).

Microbial production of *(R)*-3HB by recombinant *E. coli* was first reported by Gao et al. Their engineered pathway was composed of *phaA* and *phaB* from *Ralstonia eutropha* (a natural PHB producer), encoding β-ketothiolase and an (*R*)-specific 3-hydroxybutyryl-CoA (*R*-3HB-CoA) dehydrogenase, respectively, to convert endogenous acetyl-CoA to 3-HB-CoA through acetoacetyl-CoA (**Figure 6**). The *ptb-buk* operon from *Clostridium acetobutylicum* was lastly co-expressed to convert *R*-3-HB-CoA to *R*-3HB (via *R*-3-hydroxybutyryl phosphate), and shake flask titers exceeding 2 g/L resulted (Gao et al., 2002). Tseng et al. later built upon these works, adding the ability to produce both (*R*)- and (*S*)-3HB isomers with high stereoselectivity and increasing product titers (Tseng et al., 2009). Stereoselective control was achieved by utilizing two different enantioselective 3HB-CoA dehydrogenases: (*R*)-selective PhaB from *R. eutropha* and (*S*)-selective Hbd from *Clostridium acetobutylicum*. It was also demonstrated that the broad-substrate thioesterase II of *E. coli* (encoded by *tesB*) effectively converted both *(R)*- and *(S)*-3HB-CoA to their respective 3HB isomer products, whereas *ptb-buk* was (*R*)-specific (Tseng et al., 2009). In the end, (*R*)- and (*S*)-3HB titers as high as 2.92 and 2.08 g/L, respectively, were attained. (*R*)-3HB biomonomer, can be used to make biodegradable PHB with well-controlled properties. (*S*)-3HB biomonomer, meanwhile, can be used to produce novel PHBs with new features and properties.

Prather and coworkers subsequently expanded upon their efforts to produce, for the first time, enantiomerically pure stereoisomers of the 5-carbon β-hydroxyacid 3HV (Tseng et al., 2010). This was achieved using a single renewable substrate (glucose) by first engineering *E. coli* to over-produce precursor propionyl-CoA via manipulation of the native L-threonine biosynthesis pathway. Propionyl-CoA was condensed with acetyl-CoA via a thiolase displaying broad substrate specificity (BktB of *R. eutropha*). The intermediate product, 3-ketovaleryl-CoA, is the 5-carbon analog to acetoacetyl-CoA in the 3HB pathway (**Figure 6**). Two analogous steps were then used for its stereoselective reduction to (*R*)- or (*S*)-3HV-CoA and conversion to the respective hydroxyacids. The use of BktB was essential in demonstrating the ability of biomonomer chain elongation at the initial condensation step. One disadvantage to their approach, however,

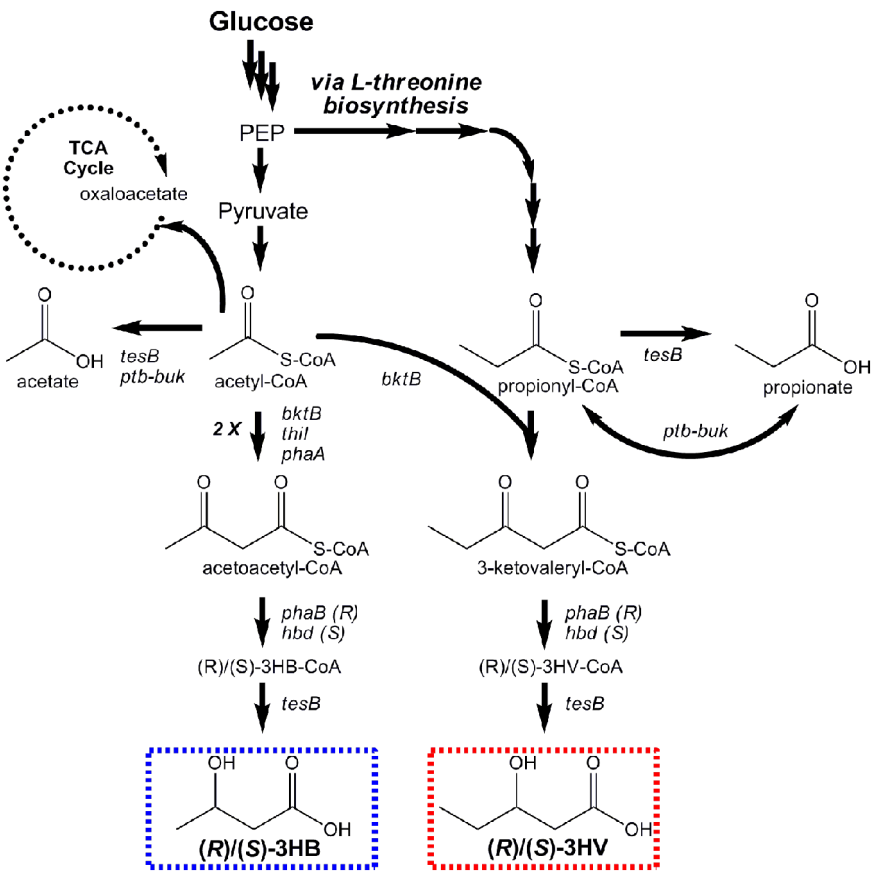


FIGURE 6 | Pathways engineered for stereoselective production of *(R)*- and *(S)*-3-hydroxybutyrate and *(R)*- and *(S)*-3-hydroxyvalerate from glucose.

resulted from the promiscuity of BktB, which led to the simultaneous production of acetoacetyl-CoA, and hence 3HB. This realization implies that control over product purity had diminished. Nevertheless, in glucose fed cultures, titers of 0.50 and 0.31 g/L of *(R)*- and *(S)*-3HV, respectively, were demonstrated. The subsequent discovery that NADP(H) limitations of PhaB reduced conversion to *(R)*-3HV was later overcome using glycerol as substrate, which improved final titers to as high as 0.96 g/L. Whereas 3HV production has yet be commercially explored, as a biomononomer it offers the potential develop bioplastics with broader ranges of properties.

DIOLS

Short-chain diols suitable for bioplastics applications have been produced microbially via both natural and engineered pathways. For example, the four-carbon diol 2,3-butanediol (2,3-BDO) is a natural fermentation product of many microbes, including many species of *Klebsiella*, *Bacillus*, and lactic acid bacteria (Voloch et al., 1985). Microbial production of 2,3-BDO by both natural and engineered strains will not be discussed here, however, as it has been extensively reviewed over the years (Garg and Jain, 1995; Celinska and Grajek, 2009; Ji et al., 2010; Nielsen et al., 2010; Ji et al., 2011). Today, companies including LanzaTech (New Zealand) are developing 2,3-BDO as a biomonomer platform following its chemocatalytic conversion to butadiene. More recent efforts have focused on engineering microbes to produce the three-carbon diol 1,3-propanediol (1,3-PDO) and the four-carbon diol 1,4-butanediol (1,4-BDO). Both 1,3-PDO and 1,4-BDO are non-natural metabolites, produced only with the aid of novel, engineered metabolic pathways. Like 2,3-BDO, 1,4-BDO can be similarly converted to butadiene. More importantly, however, both 1,3-PDO and 1,4-BDO are directly useful as biomonomers for the polyesters poly(propylene terephthalate) (PPT) and poly(butylene terephthalate) (PBT). Both PPT and PBT have the potential to steal market share from what is perhaps the oldest polyester, polyethylene terephthalate (PET) (Haas et al., 2005).

While microbial production of 1,3-PDO has been recently and comprehensively reviewed (Nakamura and Whited, 2003; Sauer et al., 2008; Saxena et al., 2009; Celinska, 2010), the engineering of *E. coli* to produce up to 18 g/L 1,4-BDO from glucose (as well as other substrates) was only recently reported for the first time (Yim et al., 2011). Bioproduction of 1,4-BDO was accomplished through the development of two novel pathways (**Figure 7**), the design of which was facilitated using the SimPheny Biopathway Predictor; a design algorithm that considers chemical structure to predict possible routes from central metabolite precursors to end-product targets (Smolke, 2009). It is also noted that, as seen in **Figure 7**, both 1,4-BDO pathways include the common intermediate 4-hydroxybutyrate (4HB), a hydroxyacid. As described above, hydroxyacids are excellent precursors for polyester production. Thus, by further leveraging this technology an additional, non-natural hydroxyacid building block could also be added to the list of potential polyester biomonomers. As their flagship molecule, Genomatica (San Diego, CA) is now developing 1,4-BDO at the commercial scale. Moreover, NatureWorks and BioAmber recently formed a joint venture (AmberWorks) to explore the development of 100% renewable polyester copolymers of 1,4-BDO and succinic acid (PBS).

CONCLUSIONS

Whereas most monomer compounds and their respective polymers are presently derived from non-renewable petroleum, by application of metabolic engineering to create novel enzyme

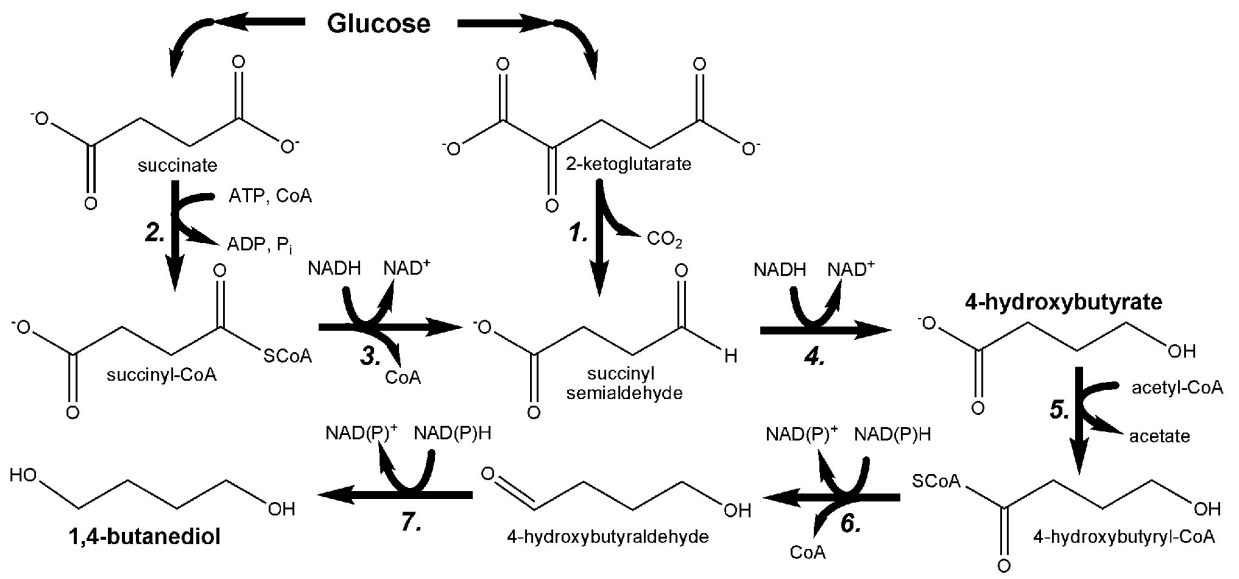


FIGURE 7 | Pathway engineered by Yim et al. for the microbial production of 1,4-butanediol (1,4-BDO) through the hydroxyacid intermediate 4-hydroxybutyrate (4HB). *1.* 2-oxoglutarate decarboxylase; *2.* succinyl-CoA synthetase; *3.* CoA-dependent succinate semialdehyde dehydrogenase; *4.* 4-hydroxybutyrate dehydrogenase; *5.* 4-hydroxybutyryl-CoA transferase; *6.* 4-hydroxybutyryl-CoA reductase; *7.* alcohol dehydrogenase.

pathways this is beginning to change. The rapidly expanding number and diversity of biomonomers will continue to support the development of new markets for "green" and sustainable bioplastics with properties identical to their petroleum-derived counterparts. With the aid of continued research and development, renewable biomonomers will ultimately help to reduce global oil consumption while also promoting greater environmental sustainability.

REFERENCES

Asada, Y., Miyake, M., Miyake, J., Kurane, R., and Tokiwa, Y. (1999). Photosynthetic accumulation of poly-(hydroxybutyrate) by cyanobacteria—the metabolism and potential for CO_2 recycling. *Int. J. Biol. Macromol.* 25, 37–42.

Atsumi, S., and Liao, J. C. (2008). Metabolic engineering for advanced biofuels production from *Escherichia coli*. *Curr. Opin. Biotechnol.* 19, 414–419.

Atsumi, S., Hanai, T., and Liao, J. C. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89.

Bechthold, I., Bretz, K., Kabasci, S., Kopitzky, R., and Springer, A. (2008). Succinic acid: a new platform chemical for biobased polymers from renewable resources. *Chem. Eng. Technol.* 31, 647–654.

Bermudez, M., Leon, S., Aleman, C., and Munoz-Guerra, S. (2000). Comparison of lamellar crystal structure and morphology of nylon 46 and nylon 5. *Polymer* 41, 8961–8973.

Braunegg, G., Lefebvre, G., and Genser, K. F. (1998). Polyhydroxyalkanoates, biopolyesters from renewable resources: physiological and engineering aspects. *J. Biotechnol.* 65, 127–161.

Campbell, C. D., and Vederas, J. C. (2010). Biosynthesis of lovastatin and related metabolites formed by fungal iterative PKS enzymes. *Biopolymers* 93, 755–763.

Carothers, J. M., Goler, J. A., and Keasling, J. D. (2009). Chemical synthesis using synthetic biology. *Curr. Opin. Biotechnol.* 20, 498–503.

Celinska, E. (2010). Debottlenecking the 1, 3-propanediol pathway by metabolic engineering. *Biotechnol. Adv.* 28, 519–530.

Celinska, E., and Grajek, W. (2009). Biotechnological production of 2, 3-butanediol-Current state and prospects. *Biotechnol. Adv.* 27, 715–725.

Chen, G. Q. (2009). A microbial polyhydroxyalkanoates (PHA) based bio- and materials industry. *Chem. Soc. Rev.* 38, 2434–2446.

Curran, K. A., and Alper, H. S. (2012). Expanding the chemical palate of cells by combining systems biology and metabolic engineering. *Metab. Eng.* 14, 289–297.

Czaja, W., Krystynowicz, A., Bielecki, S., and Brown, R. M. (2006). Microbial cellulose—the natural power to heal wounds. *Biomaterials* 27, 145–151.

Erickson, B., Nelson, and Winters, P. (2011). Perspective on opportunities in industrial biotechnology in renewable chemicals. *Biotechnol. J.* 7, 176–185.

Freedonia Group. (2009). *World Bioplastics to 2013*. Cleveland, OH: Freedonia.

Gao, H. J., Wu, Q., and Chen, G. Q. (2002). Enhanced production of D-(-)-3-hydroxybutyric acid by recombinant *Escherichia coli*. *FEMS Microbiol. Lett.* 213, 59–65.

Garg, S. K., and Jain, A. (1995). Fermentative production of 2, 3-butanediol – a review. *Bioresour. Technol.* 51, 103–109.

Global Industry Analysts. (2012). *Plastics: A Global Outlook*. San Jose, CA: GIA.

Haas, T., Jaeger, B., Weber, R., Mitchell, S. F., and King, C. F. (2005). New diol processes: 1, 3-propanediol and 1, 4-butanediol. *Appl. Catal. A Gen.* 280, 83–88.

Hoenich, N. (2006). Cellulose for medical applications: past, present, and future. *Bioresources* 1, 270–280.

Hu, Y. C., Zhan, N. N., Dou, C., Huang, H., Han, Y. W., Yu, D. H., and Hu, Y. (2010). Selective dehydration of bio-ethanol to ethylene catalyzed by lanthanum-phosphorous-modified HZSM-5, influence of the fusel. *Biotechnol. J.* 5, 1186–1191.

Igarashi, K., and Kashiwagi, K. (2000). Polyamines: mysterious modulators of cellular functions. *Biochem. Biophys. Res. Commun.* 271, 559–564.

Jiang, X., Meng, X., and Xian, M. (2009). Biosynthetic pathways for 3-hydroxypropionic acid production. *Appl. Microbiol. Biotechnol.* 82, 995–1003.

Ji, X. J., Huang, H., and Ouyang, P. K. (2011). Microbial 2, 3-butanediol production: a state-of-the-art review. *Biotechnol. Adv.* 29, 351–364.

Ji, X. J., Nie, Z. K., Li, Z. Y., Gao, Z., and Huang, H. (2010). Biotechnological production of 2, 3-butanediol. *Prog. Chem.* 22, 2450–2461.

Jung, Y. K., Kim, T. Y., Park, S. J., and Lee, S. Y. (2010). Metabolic engineering of *Escherichia coli* for the production of polylactic acid and its copolymers. *Biotechnol. Bioeng.* 105, 161–171.

Keshavarz, T., and Roy, I. (2010). Polyhydroxyalkanoates: bioplastics with a green agenda. *Curr. Opin. Microbiol.* 13, 321–326.

Kind, S., Jeong, W. K., Schroder, H., and Wittmann, C. (2010a). Systems-wide metabolic pathway engineering in *Corynebacterium glutamicum* for bio-based production of diaminopentane. *Metab. Eng.* 12, 341–351.

Kind, S., Jeong, W. K., Schroder, H., Zelder, O., and Wittmann, C. (2010b). Identification and elimination of the competing N-acetyldiaminopentane pathway for improved production of diaminopentane by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 76, 5175–5180.

Kind, S., Kreye, S., and Wittmann, C. (2011). Metabolic engineering of cellular transport for overproduction of the platform chemical 1, 5-diaminopentane in *Corynebacterium glutamicum*. *Metab. Eng.* 13, 617–627.

Kind, S., and Wittmann, C. (2011). Bio-based production of the platform chemical 1, 5-diaminopentane. *Appl. Microbiol. Biotechnol.* 91, 1287–1296.

Kosakai, Y., Soo Park, Y., and Okabe, M. (1997). Enhancement of L+-lactic acid production using mycelial flocs of Rhizopus oryzae. *Biotechnol. Bioeng.* 55, 461–470.

Krystynowicz, A., Czaja, W., Wiktorowska-Jezierska, A., Goncalves-Miskiewicz, M., Turkiewicz, M., and Bielecki, S. (2002). Factors affecting the yield and properties of bacterial cellulose. *J. Ind. Microbiol. Biotechnol.* 29, 189–195.

Lee, J. W., Kim, H. U., Choi, S., Yi, J., and Lee, S. Y. (2011a). Microbial production of building block chemicals and polymers. *Curr. Opin. Biotechnol.* 22, 758–767.

Lee, J. W., Kim, T. Y., Jang, Y. S., Choi, S., and Lee, S. Y. (2011b). Systems metabolic engineering for chemicals and materials. *Trends Biotechnol.* 29, 370–378.

Lee, J., Na, D., Park, J., Lee, J., Choi, S., and Lee, S. (2012). Systems metabolic engineering of microorganisms for natural and non-natural chemical. *Nat. Chem. Biol.* 8, 536–546.

Lee, S. Y., Choi, J. I., and Wong, H. H. (1999). Recent advances in polyhydroxyalkanoate production by bacterial fermentation: mini-review. *Int. J. Biol. Macromol.* 25, 31–36.

Le Vo, T., Kim, T., and Hong, S. (2012). Effects of glutamate decarboxylase and gamma-aminobutyric acid (GABA) transporter on the bioconversion of GABA in engineered *Escherichia coli*. *Bioprocess Biosyst. Eng.* 35, 645–650.

Leonard, E., Runguphan, W., O'Connor, S., and Prather, K. J. (2009). Opportunities in metabolic engineering to facilitate scalable alkaloid production. *Nat. Chem. Biol.* 5, 292–300.

Lunt, J. (1998). Large-scale production, properties and commercial applications of polylactic acid polymers. *Polym. Degrad. Stabil.* 59, 145–152.

Martens, J. H., Barg, H., Warren, M. J., and Jahn, D. (2002). Microbial production of vitamin B-12. *Appl. Microbiol. Biotechnol.* 58, 275–285.

Martin, C. H., Nielsen, D. R., Solomon, K. V., and Prather, K. L. J. (2009). Synthetic metabolism: engineering biology at the protein and pathway scales. *Chem. Biol.* 16, 277–286.

Mazumdar, S., Clomburg, J. M., and Gonzalez, R. (2010). *Escherichia coli* strains engineered for homofermentative production of D-lactic acid from glycerol. *Appl. Environ. Microbiol.* 76, 4327–4336.

McKenna, R., and Nielsen, D. R. (2011). Styrene biosynthesis from glucose by engineered *E. coli*. *Metab. Eng.* 13, 544–554.

Mendez-Perez, D., Begemann, M. B., and Pfleger, B. F. (2011). Modular synthase-encoding gene involved in alpha-olefin biosynthesis in *Synechococcus sp.* strain PCC (7002). *Appl. Environ. Microbiol.* 77, 4264–4267.

Mimitsuka, T., Sawai, H., Hatsu, M., and Yamada, K. (2007). Metabolic engineering of *Corynebacterium glutamicum* for cadaverine

fermentation. *Biosci. Biotechnol. Biochem.* 71, 2130–2135.

Nakamura, C. E., and Whited, G. M. (2003). Metabolic engineering for the microbial production of 1, 3-propanediol. *Curr. Opin. Biotechnol.* 14, 454–459.

Nexant Chem Systems. (2009). *New Process Evaluation and Research Program: Nylon 6 and Nylon 6, 6.* San Francisco, CA: Nexant.

Nielsen, D. R., Yoon, S. H., Yuan, C. J., and Prather, K. L. (2010). Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in *E. coli*. *Biotechnol. J.* 5, 274–284.

Niu, W., Draths, K. M., and Frost, J. W. (2002). Benzene-free synthesis of adipic acid. *Biotechnol. Prog.* 18, 201–211.

Pawlowski, G., Dammel, R., Eckes, C., Lindley, C. R., Meier, W., Przybilla, K. J., Roschert, H., and Spiess, W. (1991). Substituted polyhydroxystyrenes as matrix resins for chemically amplified deep uv resist materials. *Microelectron. Eng.* 13, 29–32.

Pei, L., Schmidt, M., and Wei, W. (2011). "Conversion of biomass into bioplastics and their potential environmental impacts," in *Biotechnology of Biopolymers,* ed M. Elnashar (Rijeka, Croatia: InTech), 57–74.

Peralta-Yahya, P. P., and Keasling, J. D. (2010). Advanced biofuel production in microbes. *Biotechnol. J.* 5, 147–162.

Phillips, A. L. (2008). Bioplastics boom. *Am. Sci.* 96, 109.

Plastics Europe. (2011). *Plastics Europe Market Research Group, Plastics—The Facts 2011.* Brussels, Belgium: Plastics Europe.

Qian, Z. G., Xia, X. X., and Lee, S. Y. (2009). Metabolic engineering of *Escherichia coli* for the production of putrescine: a four carbon diamine. *Biotechnol. Bioeng.* 104, 651–662.

Qian, Z. G., Xia, X. X., and Lee, S. Y. (2011). Metabolic engineering of *Escherichia coli* for the production of cadaverine: a five carbon diamine. *Biotechnol. Bioeng.* 108, 93–103.

Qi, W. W., Vannelli, T., Breinig, S., Ben-Bassat, A., Gatenby, A. A., Haynie, S. L., and Sariaslani, F. S. (2007). Functional expression of prokaryotic and eukaryotic genes in *Escherichia coli* for conversion of glucose to p-hydroxystyrene. *Metab. Eng.* 9, 268–276.

Rathnasingh, C., Raj, S. M., Jo, J. E., and Park, S. (2009). Development and evaluation of efficient recombinant *Escherichia coli* strains for the production of 3-hydroxypropionic acid from glycerol. *Biotechnol. Bioeng.* 104, 729–739.

Rathnasingh, C., Raj, S. M., Lee, Y., Catherine, C., Ashok, S., and Park, S. (2012). Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant *Escherichia coli* strains. *J. Biotechnol.* 157, 633–640.

Reddy, G., Altaf, M., Naveena, B. J., Venkateshwar, M., and Kumar, E. V. (2008). Amylolytic bacterial lactic acid fermentation–a review. *Biotechnol. Adv.* 26, 22–34.

Reinecke, F., and Steinbuchel, A. (2009). Ralstonia eutropha strain H16 as model organism for PHA metabolism and for biotechnological production of technically interesting biopolymers. *J. Mol. Microbiol. Biotechnol.* 16, 91–108.

Revelles, O., Wittich, R.-M., and Ramos, J. L. (2007). Identification of the initial steps in d-lysine catabolism in *Pseudomonas putida*. *J. Bacteriol.* 189, 2787–2792.

Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943.

Rojas-Rosas, O., Villafana-Rojas, J., Lopez-Dellamary, F. A., Nungaray-Arellano, J., and Gonzalez-Reynoso, O. (2007). Production and characterization of polyhydroxyalkanoates in *Pseudomonas aeruginosa* ATCC (9027). from glucose, an unrelated carbon source. *Can. J. Microbiol.* 53, 840–851.

Ross, P., Mayer, R., and Benziman, M. (1991). Cellulose biosynthesis and function in bacteria. *Microbiol. Rev.* 55, 35–58.

Rude, M. A., Baron, T. S., Brubaker, S., Alibhai, M., Del Cardayre, S. B., and Schirmer, A. (2011). Terminal olefin (1-alkene) biosynthesis by a novel p450 fatty acid decarboxylase from *Jeotgalicoccus* species. *Appl. Environ. Microbiol.* 77, 1718–1727.

Sauer, M., Marx, H., and Mattanovich, D. (2008). Microbial production of 1, 3-propanediol. *Recent Pat. Biotechnol.* 2, 191–197.

Saxena, R. K., Anand, P., Saran, S., and Isar, J. (2009). Microbial production of 1, 3-propanediol: recent developments and emerging opportunities. *Biotechnol. Adv.* 27, 895–913.

Schirmer, A., Rude, M. A., Li, X., Popova, E., and Del Cardayre, S. B. (2010). Microbial biosynthesis of alkanes. *Science* 329, 559–562.

Schneider, J., and Wendisch, V. (2010). Putrescine production by engineered *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 88, 859–868.

Schubert, P., Steinbuchel, A., and Schlegel, H. G. (1988). Cloning of the *Alcaligenes eutrophus* genes for synthesis of poly-beta-hydroxybutyric acid (PHB) and synthesis of PHB in *Escherichia coli*. *J. Bacteriol.* 170, 5837–5847.

Singh, M., Patel, S. K. S., and Kalia, V. C. (2009). *Bacillus subtilis* as potential producer for polyhydroxyalkanoates. *Microbial. Cell Fact.* 8, 38–48.

Smolke, C. D. (2009). *The Metabolic Pathway Engineering Handbook Fundamentals.* Boca Raton, FL: CRC Press/Taylor and Francis.

Sodergard, A., and Stolt, M. (2002). Properties of lactic acid based polymers and their correlation with composition. *Prog. Polym. Sci.* 27, 1123–1163.

Song, H., and Lee, S. Y. (2006). Production of succinic acid by bacterial fermentation. *Enzyme Microb. Technol.* 39, 352–361.

Sri. (2010). *Styrene.* Access Intelligence LLC Inc.

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., Mcclure, A., Del Cardayre, S. B., and Keasling, J. D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562.

Straathof, A. J. J., Sie, S., Franco, T. T., and Van Der Wielen, L. A. M. (2005). Feasibility of acrylic acid production by fermentation. *Appl. Microbiol. Biotechnol.* 67, 727–734.

Suthers, P. F., and Cameron, D.C. (2000). Production of 3-hydroxypropionic acid in recombinant organisms. US Patent 6852517.

Tabor, C. W., and Tabor, H. (1985). Polyamines in microorganisms. *Microbiol. Rev.* 49, 81–99.

Takahara, I., Saito, M., Inaba, M., and Murata, K. (2005). Dehydration of ethanol into ethylene over solid acid catalysts. *Catal. Lett.* 105, 249–252.

Tateno, T., Okada, Y., Tsuchidate, T., Tanaka, T., Fukuda, H., and Kondo, A. (2009). Direct production of cadaverine from soluble starch using *Corynebacterium glutamicum* coexpressing alpha-amylase and lysine decarboxylase. *Appl. Microbiol. Biotechnol.* 82, 115–121.

Thakker, C., Martinez, I., San, K. Y., and Bennett, G. N. (2012). Succinate production in *Escherichia coli*. *Biotechnol. J.* 7, 213–224.

Thompson, R. C., Swan, S. H., Moore, C. J., and Vom Saal, F. S. (2009). Our plastic age. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 1973–1976.

Thorball, V. (1967). Styrofoam—an extruded polystyrene foam for low temperature insulation. *Mod. Refrig. Air Cond.* 70, 51.

Tseng, H. C., Harwell, C. L., Martin, C. H., and Prather, K. L. (2010). Biosynthesis of chiral 3-hydroxyvalerate from single propionate-unrelated carbon sources in metabolically engineered *E.* coli. *Microb. Cell Fact.* 9, 96.

Tseng, H. C., Martin, C. H., Nielsen, D. R., and Prather, K. L. (2009). Metabolic engineering of *Escherichia coli* for enhanced production of (R)- and (S)-3-hydroxybutyrate. *Appl. Environ. Microbiol.* 75, 3137–3145.

Tsuruta, H., Paddon, C. J., Eng, D., Lenihan, J. R., Horning, T., Anthony, L. C., Regentin, R., Keasling, J. D., Renninger, N. S., and Newman, J. D. (2009). High-level production of amorpha-4, 11-diene, a precursor of the antimalarial agent artemisinin, in *Escherichia coli*. *PLoS ONE* 4:e4489. doi: 10.1371/journal.pone.0004489

US Department of Energy. (2002). Steam System Opportunity Assessment for the Pulp and Paper, Chemical Manufacturing, and Petroleum Refining Industries. Washington, DC.

US Department of Energy. (2004). Top Value Added Chemicals from Biomass. Washington, DC.

US Energy Information Administration. (2011). Available online at www.eia.gov.

Voloch, M., Jjansen, N. B., Ladisch, M., Tsao, G. T., Narayan, R., and Rodwell, V. W. (1985). "2, 3-Butanediol," in *Comprehensive Biotechnology: The Principles, Applications and Regulations of Biotechnology in Industry, Agriculture and Medicine,* 1st Edn, ed M. Moo-Young (New York, NY: Pergamon Press).

Wee, Y. J., Kim, J. N., and Ryu, H. W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technol. Biotechnol.* 44, 163–175.

Whited, G., Feher, F., Benko, D., Cervin, M., Chotani, G., Mcauliffe, J., Laduca, R., Ben-Shoshan, E., and Sanford, K. (2010). Development of a gas-phase bioprocess for isoprene-monomer production using metabolic pathway engineering. *Ind. Biotechnol.* 6, 152–163.

Wierckx, N. J., Ballerstedt, H., De Bont, J. A., and Wery, J. (2005). Engineering of solvent-tolerant *Pseudomonas putida* S12 for bioproduction of phenol from glucose. *Appl. Environ. Microbiol.* 71, 8221–8227.

Wu, C., Koylinski, T., and Bozik, J. (1981). Preparation of styrene from ethylbenzene US Patent 4255599.

Xu, J., and Guo, B. H. (2010). Poly(butylene succinate) and its copolymers: research, development and industrialization. *Biotechnol. J.* 5, 1149–1163.

Yang, T. H., Kim, T. W., Kang, H. O., Lee, S. H., Lee, E. J., Lim, S. C., Oh, S. O., Song, A. J., Park, S. J., and Lee, S. Y. (2010). Biosynthesis of polylactic acid and its copolymers using evolved propionate CoA transferase and PHA synthase. *Biotechnol. Bioeng.* 105, 150–160.

Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick, J. D., Osterhout, R. E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H. B., Andrae, S., Yang, T. H., Lee, S. Y., Burk, M. J., and Van Dien, S. (2011). Metabolic engineering of *Escherichia coli* for direct production of 1, 4-butanediol. *Nat. Chem. Biol.* 7, 445–452.

Zhou, S., Causey, T. B., Hasona, A., Shanmugam, K. T., and Ingram, L. O. (2003). Production of optically pure D-lactic acid in mineral salts medium by metabolically engineered *Escherichia coli* W3110. *Appl. Environ. Microbiol.* 69, 399–407.

Zhou, Y., Dominguez, J. M., Cao, N., Du, J., and Tsao, G. T. (1999). Optimization of L-lactic acid production from glucose by *Rhizopus oryzae* ATCC 52311. *Appl. Biochem. Biotechnol.* 77–79, 401–407.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 04 June 2012; accepted: 08 August 2012; published online: 30 August 2012.

Citation: Adkins J, Pugh S, McKenna R and Nielsen DR (2012) Engineering microbial chemical factories to produce renewable "biomonomers". Front. Microbio. ***3****:313. doi: 10.3389/fmicb.2012.00313*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 19 September 2012
doi: 10.3389/fmicb.2012.00344

Application of synthetic biology in cyanobacteria and algae

Bo Wang[1,2]*, Jiangxin Wang[1], Weiwen Zhang[1†] and Deirdre R. Meldrum[1]*

[1] Center for Biosignatures Discovery Automation, The Biodesign Institute, Arizona State University, Tempe, AZ, USA
[2] Biological Design Graduate Program, Arizona State University, Tempe, AZ, USA

Edited by:
David Nielsen, Arizona State University, USA

Reviewed by:
Shota Atsumi, University of California at Davis, USA
Christie A. M. Peebles, Colorado State University, USA

***Correspondence:**
Bo Wang and Deirdre R. Meldrum, Center for Biosignatures Discovery Automation, The Biodesign Institute, Arizona State University, 1001 South McAllister Avenue, Tempe, AZ 85287-6501, USA.
e-mail: bo.wang.6@asu.edu; deirdre.meldrum@asu.edu

†Current address:
Weiwen Zhang, School of Chemical Engineering and Technology, Tianjin University, Tianjin 300072, People's Republic of China

Cyanobacteria and algae are becoming increasingly attractive cell factories for producing renewable biofuels and chemicals due to their ability to capture solar energy and CO_2 and their relatively simple genetic background for genetic manipulation. Increasing research efforts from the synthetic biology approach have been made in recent years to modify cyanobacteria and algae for various biotechnological applications. In this article, we critically review recent progresses in developing genetic tools for characterizing or manipulating cyanobacteria and algae, the applications of genetically modified strains for synthesizing renewable products such as biofuels and chemicals. In addition, the emergent challenges in the development and application of synthetic biology for cyanobacteria and algae are also discussed.

Keywords: cyanobacteria, algae, synthetic biology, biofuel, green chemistry

INTRODUCTION

Cyanobacteria and algae are endowed with the complex photosynthesis systems (Mulkidjanian et al., 2006) which can absorb solar irradiation with a broad wave length and thereafter channel the absorbed energy to other forms of energy carriers such as chemicals (Chisti, 2007; Takahashi et al., 1998; van de Meene et al., 2006) and electricity (Furukawa et al., 2006; Pisciotta et al., 2010). Solar irradiation is a clean, abundant, and renewable energy resource and, if being properly and efficiently transferred, would be more then enough to power the entire human society (Rittmann, 2008). In addition, growing cyanobacteria and algae do not require arable land, which would eventually alleviate the increasing food prices due to the growing crop-based microbial industries (Rittmann, 2008). In contrast, they can fix carbon dioxide (CO_2), a type of greenhouse gas, during photosynthesis. Furthermore, cyanobacteria and algae grow faster than plants and bear relatively simple genetic background which is relatively easy to manipulate (Koksharova and Wolk, 2002).

As an emerging discipline that tackles biotechnology from a rational-design approach, synthetic biology aims to redesign existing biological systems or create artificial life (Benner, 2003; Endy, 2005; Mukherji and van Oudenaarden, 2009). In recent years, synthetic biology research has been focused on model species such as *Escherichia coli* and yeast, and has greatly boosted not only the in-depth understanding of the biological mechanisms in these cells, but also the capability and efficiency of these systems in biological production of various useful products (Benner, 2003; Lee and Lee, 2003; Martin et al., 2003; Isaacs et al., 2004; Ro et al., 2006; Dwyer et al., 2007; Atsumi et al., 2008b; Inui et al., 2008; Keasling, 2008; Prather and Martin, 2008; Zhang et al., 2008, 2012; Bayer et al., 2009; Ma et al., 2009; Mukherji and van Oudenaarden, 2009; Steen et al., 2010; Yim et al., 2011). However, with over 40 cyanobacterial genome sequences[1] and more than 60 algal genome sequences[2] being completed and published, application of synthetic biology in cyanobacteria and algae has significantly lagged behind those in *E. coli* and yeast. Considering the aforementioned inherent merits of the photosynthetic microbes, we believe it would be of great scientific and application values to further develop synthetic biology tools and apply them in cyanobacteria and algae. We herein review the recent progresses and the challenges in developing and applying synthetic biology for cyanobacteria and algae.

TOOLS FOR SYNTHETIC BIOLOGY IN CYANOBACTERIA AND ALGAE

DEVELOPMENT OF "BIOBRICKS" FOR CYANOBACTERIA AND ALGAE

"BioBricks" stand for standardized DNA parts with common interface and can be assembled in living organisms. They are the basic interchangeable elements for regulating the genetics[3]. Here we focus on the development of the most common BioBricks for cyanobacteria and algae (i.e., promoters, transtriptional terminators, ribosome binding sites, and other regulatory factors).

[1] http://www.genomesonline.org/
[2] http://genome.jgi.doe.gov/
[3] http://biobricks.org/

Promoters

Both native and foreign promoters have been evaluated in cyanobacteria, mostly using *Synechococcus elongatus* PCC 7942 (hereafter *Synechococcus* 7942) and *Synechocystis* sp. PCC 6803 (hereafter *Synechocystis* 6803) as model species (**Table 1**). The native promoters used are usually from genes essential to photosynthesis such as CO_2 fixation (P_{rbcL}, P_{cmp}, P_{sbt}), photosystem I (PSI; P_{psaA},P_{psaD}), PSII (P_{psbA1},P_{psbA2}), and photosynthesis antenna protein phycocyanin (P_{cpc}). A native nickel-inducible promoter, P_{nrsB}, has also been successfully utilized to express phage lysis genes in *Synechocystis* 6803 (Liu and Curtiss, 2009). Besides the native promoters, a limited number of foreign promoters have also been characterized in cyanobacteria. The chimeric P_{tac}/P_{trc} promoter, a strong promoter in *E. coli*, has been used in *Synechococcus* and *Synechocystis* species to initiate high-level expressions of the interest genes (Geerts et al., 1995; Ng et al., 2000; Atsumi et al., 2009; Huang et al., 2010; Niederholtmeyer et al., 2010; Lan and Liao, 2011). It is noteworthy that the composition of the cyanobacterial holopolymerase is quite different from those in most bacteria (including *E. coli*), so commonly used *E. coli* promoters might perform differently when introduced into cyanobacteria (Heidorn et al., 2011). A recent study on gene expression analysis in *Synechocystis* 6803 showed that the strength of P_{trc1O} (a version of the P_{trc}/P_{tac} promoter) was more than fourfold higher than all versions of the promoter of native ribulose bisphosphate carboxylase/oxygenase (RuBisCO) large subunit, P_{rbcL}, whereas the common *E. coli* promoters P_{lac}, P_{tet}, and λ P_R exhibited very low or no detectable activities in the same system (Huang et al., 2010). Since currently very little is known about the performance of various native and foreign promoters in cyanobacteria, a systematic investigation on behaviors of various promoters in cyanobacteria would be important.

In algae, CaMV 35S and SV40 promoters from viruses have been used to express target genes (Benfey et al., 1990; Wang et al., 2010). However, the most effective promoters have been derived from highly expressed algal genes. For example, the widely used promoters for *Chlamydomonas* transformation have been derived from the 5′ untranslated region of the *Chlamydomonas reinhardtii* RuBisCO small subunit gene (*rbcS2* ;Stevens et al., 1996), *Chlamydomonas* heat shock protein 70A gene *hsp70A* (Schroda et al., 2000), marine diatom fucoxanthin-chlorophyll a/c binding protein gene *fcp* (Apt et al., 1996; Miyagawa-Yamaguchi et al., 2011), *Dunaliella* duplicated carbonic anhydrase 1 (DCA1; Li et al., 2010; Lu et al., 2011), *Porphyra yezoensis* actin1 gene (PyAct1; Takahashi et al., 2010), and two *Nannochloropsis* unlinked violaxanthin/chlorophyll a-binding protein (VCP) genes, *VCP1* and *VCP2* (Kilian et al., 2011).

Table 1 | Selected promoters used in cyanobacteria.

Promoters	Sources	Gene(s)	Expression hosts	Reference
P_{rbc}	*Synechococcus* 6301, *Synechocystis* 6803, *Synechococcus* 7942	firefly luciferase, *pdc, adh, far, accBCDA, accD, accA, fatB2*	*Synechococcus* 6301, *Synechocystis* 6803, *Synechococcus* 7942	Takeshima et al. (1994), Deng and Coleman (1999), Liu et al. (2011b), Tan et al. (2011)
P_{petE}	*Synechocystis* 6803	*far, far1, far2, accBCDA*	*Synechocystis* 6803	Tan et al. (2011)
P_{psbA1}	*Synechococcus* 7942	*efe, hydEF, hydG, cvrbcLS*	*Synechococcus* 7942	Sakai et al. (1997), Takahama et al. (2003), Ducat et al. (2011a)
P_{psbA2}	*Synechocystis* 6803	*pdc, adh, cvrbcLS, ispS, tesA, fatB1, fatB2*	*Synechocystis* 6803, *Synechococcus* 7942	Iwaki et al. (2006), Dexter and Fu (2009), Lindberg et al. (2010), Liu et al. (2011b)
P_{psaA}	*Synechocystis* 6803	*luxAB*	*Synechocystis* 6803	Muramatsu and Hihara (2006)
P_{psaD}	*Synechocystis* 6803	*luxAB*	*Synechocystis* 6803	Muramatsu and Hihara (2007)
P_{cpc}	*Synechocystis* 6714	*luxAB, accB, accC*	*Synechococcus* 7942	Imashimizu et al. (2003)
P_{rnpB}	*Synechocystis* 6803	GFP	*Synechocystis* 6803	Huang et al. (2010)
P_{cmp}	*Synechocystis* 6803	*fol, gpl, shl*	*Synechocystis* 6803	Liu et al. (2011a)
P_{sbt}	*Synechocystis* 6803	*gpl*	*Synechocystis* 6803	Liu et al. (2011a)
P_{nrsB}	*Synechocystis* 6803	holin, endolysin, auxiliary lysis enzyme	*Synechocystis* 6803	Liu and Curtiss (2009)
P_{T7}	Coliphage T7	*luxAB*	*Anabaena* sp. 7120	Wolk et al. (1993)
P_{lac}	*E. coli*	*atoB, adhE2, ter, hbd, crt, hydA, alsS, ilvC, ilvD,*	*Synechococcus* 7942	Atsumi et al. (2009), Ducat et al. (2011a), Lan and Liao (2011)
P_{trc}/P_{tac}	*E. coli*	*petE, atoB, adhE2, ter, hbd, crt, kivd, rbcLS, invA, glf, ldhA, lldP, phrA,* GFP, EYFP	*Synechococcus* 7942, *Synechocystis* 6803	Geerts et al. (1995), Ng et al. (2000), Atsumi et al. (2009), Huang et al. (2010), Niederholtmeyer et al. (2010), Lan and Liao (2011)
P_{tet}	*E. coli*	GFP	*Synechocystis* 6803	Huang et al. (2010)

Transcriptional terminators

Placing a transcription terminator downstream of the introduced genes will prevent effects on the expression of genes adjacent to the insertion loci; meanwhile, placing a terminator upstream of the promoter of an introduced gene will also prevent any background transcription effect on the upstream genes (Adhya and Gottesman, 1982). So far only a few native and foreign terminators have been utilized in cyanobacteria, including the cyanobacterial RuBisCO terminator (Takeshima et al., 1994) and strong *E. coli* terminators such as *rrnB* terminator (Geerts et al., 1995; Takahashi et al., 1998; Atsumi et al., 2009), bacteriophage T7 terminator (Lang and Haselkorn, 1991; Argueta et al., 2004), and *rrnBT1-T7TE* double terminator[4] (Huang et al., 2010). Very little work has been conducted to characterize the termination efficiencies in cyanobacteria and algae.

Ribosome binding sites

The ribosome binding sites (RBS) play a crucial role in initiating the translation of downstream target genes. Upon translation initiation, the 3′-terminal sequence of the 16S rRNA interacts with the core Shine–Dalgarno (SD) sequence of RBS by complementary pairing of the nucleic acids. For example, in cyanobacterium *Synechocystis* 6803, the 3′-terminal sequence of the 16S rRNA is AUCACCUCCUUU (Kaneko et al., 1996; Ma et al., 2002) and therefore the optimal complementary SD sequence should be AAAGGAGGUGAU (core SD sequence underlined). Heidorn et al. (2011) studied the efficiencies of different RBS in expressing GFP in *Synechocystis* 6803 and found that the RBS sequence UAGUGGAGGU gave about twofold higher translation efficiency than a RBS sequence AUUAAAGAGGAGAAA and about fourfold higher than those of sequences UCACACAGGAAAG and AAAGAGGAGAAA. However, it is found that the efficiency of the same RBS might vary across species, such as *E. coli* vs. *Synechocystis* (Heidorn et al., 2011). The efficiency of a given RBS also depends on the surrounding nucleotide sequence that may result in secondary structures and the spacing between the SD sequence and the translation start codon AUG (de Smit and van Duin, 1990; Chen et al., 1994; Pfleger et al., 2006). In order to predict the translation efficiency of a given RBS in various genetic contexts, Salis et al. (2009) have established a thermodynamic model that calculates the impact from the SD sequence, the start codon, the spacing between the SD sequence and the start codon, and the mRNA secondary structure; the model can accurately predict protein expression levels within a factor of 2.3 over a range of 100,000-fold in *E. coli*. Similar model should be employed to optimize the RBS for gene expression in cyanobacteria.

Negative regulation of gene expression

The down-regulation of the target gene expression has been studied at transcriptional, translational, and post-translational levels in cyanobacteria. The negative transcriptional factor, LacI, has been utilized as a repressor in regulating the P_{tac}/P_{trc} controlled target gene expression (Geerts et al., 1995; Atsumi et al., 2009; Huang et al., 2010; Niederholtmeyer et al., 2010; Ducat et al., 2011a; Lan and Liao, 2011). However, lacI-P_{tac}/P_{trc} expression system can result in severe leaky expression of target genes (Huang et al., 2010). By placing dual *lac* operators upstream of P_{tac}/P_{trc} promoter the leaky expression of downstream target genes was significantly repressed; however, this also resulted in a limited induction of the promoter with presence of the inducer IPTG (Huang et al., 2010). Degradation tags which can be fused to the target proteins through genetic engineering have also been investigated in cyanobacteria. Three *ssrA* protease degradation tags including ASV, AAV, and LVA were fused to EYFP and expressed in *Synechocystis* 6803. The results indicated that LVA is the strongest degradation tag, AAV is the weaker one and ASV is the weakest (Huang et al., 2010). Recently, it has been discovered that antisense RNAs (asRNAs) play an important role in cyanobacterial gene regulation (Hernández et al., 2006; Georg et al., 2009; Cerutti et al., 2011; Mitschke et al., 2011). asRNAs thus provide another approach for gene silencing in cyanobacteria. For example, Mussgnug et al. (2007) have successfully down-regulated the expression of light-harvesting antenna complexes through RNA interference.

Endogenous enhancers

The transcription of an interest gene can be positively affected by placing in the gene cluster an enhancer, a short DNA fragment which interacts with certain proteins to enhance the transcription. In cyanobacteria, some light-responsive elements exhibit enhancer activities. For example, the 5′-untranslated regions of the *psbAII* and *psbAIII* genes of *Synechococcus* 7942 have been found as enhancers which can increase the expression of downstream genes by 4- to 11-fold when combined with an *E. coli* promoter (*conII*) in the *Synechococcus* 7942 host strain (Li and Golden, 1993). A recent study by Eichler-Stahlberg et al. (2009) showed that inserting three introns from the native alga *C. reinhardtii RBCS2* gene into the recombinant luciferase and erythropoietin resulted in up to fourfold increase of the expression levels. By fusing the recombinant luciferase with the endogenous RuBisCO LSU protein, Muto et al. (2009) has achieved enhanced luciferase expression by 33-fold.

PLASMID VECTORS

Both integrative and replicative plasmids have been developed for cyanobacteria. In cyanobacteria, integrative plasmids are usually utilized as vectors to integrate foreign genes into the cyanobacterial genomes via homologous recombination (Golden et al., 1987; Eaton-Rye, 2004; Heidorn et al., 2011). Integrative plasmids usually cannot replicate themselves and would eventually be eliminated through cell division. Replicative plasmids are those which can replicate in host cyanobacteria and the replication properties can be descended to daughter cells. Replicative cyanobacterial plasmids can be classified into two types: those with replicons of broad-host range plasmids (Mermet-Bouvier et al., 1993; Mermet-Bouvier and Chauvat, 1994; Ng et al., 2000; Huang et al., 2010) and those derived from endogenous cryptic plasmids (Reaston et al., 1982; Wolk et al., 1984; Lang and Haselkorn, 1991; Summers et al., 1995; Deng and Coleman, 1999; Argueta et al., 2004; Iwaki et al., 2006). Representative shuttle vectors for cyanobacteria are listed in **Table 2**. The copy numbers of the broad-host

[4]http://partsregistry.org/Part:BBa_B0015

Table 2 | Representative shuttle vectors for cyanobacteria.

Cyanobacterial replicons	*E. coli* replicons	Representative vectors	Host cyanobacteria	Reference
pDU1	pMB1	pRL1, pJL3	*Anabaena* 7120, *Anabaena* 7118, *Anabaena* M-131, *Nostoc* 7524	Reaston et al. (1982), Wolk et al. (1984), Lang and Haselkorn (1991)
pDC1	pMB1	pSCR119/202, pSUN119/202	*Nostoc* sp. MAC 8009, *Nostoc punctiforme*, *Nostoc* ATCC 29133	Lambert and Carr (1983), Summers et al. (1995), Argueta et al. (2004)
pUH24	P15A	pUC303	*Synechococcus* 7942	Kuhlemeier et al. (1983), Iwaki et al. (2006)
pUH24	pMB1	pCB4, pSG111	*Synechococcus* 7942	Golden and Sherman (1983), Luinenburg and Coleman (1993), Deng and Coleman (1999)
pAQ1	pMB1	pAQE17	*Synechococcus* 7002	Buzby et al. (1985)
PBA1	pMB1	pARUB19	*Synechococcus* 6301	Takeshima et al. (1994)
RSF1010	RSF1010	pFC1, pSL1211, pPMQAK1,	*Synechosystis* 6803, *Synechosystis* 6714, *Synechococcus* 7942, *Synechococcus* 6301, *Anabaena* 7120, *Nostoc* ATCC 29133	Mermet-Bouvier et al. (1993), Mermet-Bouvier and Chauvat (1994), Ng et al. (2000), Huang et al. (2010)

range RSF1010-derived plasmids have been reported as about 10 per chromosome in *E. coli* cells and 10–30 per cell in *Synechocystis* (Ng et al., 2000; Huang et al., 2010) which is slightly higher than the average copy number (approximately 10) of the *Synechocystis* chromosome (Eaton-Rye, 2004). Due to lack of an active partitioning mechanism, RSF1010-derived plasmids tend to be eliminated in cells and thus antibiotic selection pressure is required for the maintenance (Becker and Meyer, 1997; Meyer, 2009).

Plasmid vectors have been developed to transform algae (León-Bañares et al., 2004). Recombinant eukaryotic algal viruses as transformation vectors (Langridge et al., 1986) and *Agrobacterium tumefaciens*-mediated method (Kumar et al., 2004) were also successfully developed for both marine and freshwater algae (Wang et al., 2010).

CODON USAGE

Since different organisms usually bear particular codon usage patterns, when a gene is cloned from one species and expressed in a second organism, some codons might become rare codons in the new host, leading to poor translation efficiency (Kane, 1995). Genes in cyanobacteria show a bias in use of synonymous codons (Campbell and Gowri, 1990; Nakamura et al., 2000; Beck et al., 2012; Yu et al., 2012); it is thus very important to examine the difference in codon usage of a heterologous gene before it is expressed in cyanobacteria. In a recent study, Lindberg et al. (2010) studied the effects of codon usage on heterologous expression of kudzu *IspS* gene (encoding the isoprene synthase) in *Synechocystis* 6803. The results showed that the codon-optimized *IspS* showed remarkable improved expression, 10-fold higher than that of the native *IspS* gene under control of the same promoter. The importance of codon optimization in algal genetic applications is also increasingly acknowledged. For instance, it has been shown that codon bias significantly affects the GFP expression in *C. reinhardtii* (Heitzer et al., 2007). As a result, in recent transgenic research, the codon-optimized luciferase gene was used in a green alga *Gonium pectorale* (Lerche and Hallmann, 2009) and the codon-modified β-glucuronidase gene was transformed in a red seaweed *P. yezoensis* (Takahashi et al., 2010).

TRANSFORMATION OF CYANOBACTERIA AND ALGAE

Methods to introduce DNA into cyanobacteria include conjugation (Thiel and Wolk, 1987; Elhai and Wolk, 1988), electroporation (Zang et al., 2007), and natural transformation (Shestakov and Khyen, 1970; Grigorieva and Shestakov, 1982; Kuhlemeier and van Arkel, 1987). The methods have been well summarized in several recent reviews (Eaton-Rye, 2004; Koksharova and Wolk, 2002; Heidorn et al., 2011) and we suggest readers to refer these excellent reviews for details. Compared to cyanobacteria, transformation methods for algae are less developed and more complicated. Since the chloroplast and nucleus of alga *C. reinhardtii* were stably transformed more than two decades ago (Boynton et al., 1988; Debuchy et al., 1989; Fernández et al., 1989), different methods have been employed in algal transformation which include, but not limited to, particle bombardment, glass bead agitation, microinjection, electroporation and *A. tumefaciens*-mediated transformation (León-Bañares et al., 2004; Coll, 2006; León and Fernández, 2007; Potvin and Zhang, 2010). Specifically, bombardment of target cells with DNA-coated metal particles turns to be an effective and highly reproducible method to transform algae. This method has been so far applied in the transformation of nuclear and chloroplast of many algal species such as *C. reinhardtii*, *Volvox carteri*, *Chlorella sorokiniana*, *Chlorella ellipsoidea*, *Chlorella kessleri*, *Haematococcus pluvialis, Phaeodactylum tricornutum*, and *G. pectorale* (Boynton and Gillham, 1993; Potvin and Zhang, 2010). In addition, agitation of the cell wall-deficient algal cells with glass beads, polyethylene glycol (PEG) and foreign DNA has been used to transform algae such as *C. reinhardtii*, *Dunaliella salina*, and red alga *Porphyra haitanensis* (Kindle, 1990; Feng et al., 2009; Wang et al., 2010). Microinjection of the viral SV40 DNA or the chimeric construction pSV2neo into the marine unicellular green alga *Acetabularia mediterranea* also resulted in a high yield and stable nuclear transformation (Neuhaus et al., 1986);

nevertheless, it is hard to operate and the throughput of transformation is low. *Agrobacterium tumefaciens* has been used to mediate the transformation of *C. reinhardtii* (Kumar et al., 2004) and *H. pluvialis* (Kathiresan et al., 2009). Recently, it was discovered that the industrially relevant oil-producing alga *Nannochloropsis* sp. is haploid and can be transformed with high efficiency using high electric field electroporation. It has also been found that efficient stable transformation of this species via homologous recombination requires using linear DNA fragment rather than circular plasmid DNA (Kilian et al., 2011). However, the mechanism for the high homologous recombination efficiency is to be elucidated.

APPLICATIONS OF MODIFIED CYANOBACTERIA AND ALGAE

We focus here on recent progress in producing biofuels and other useful chemicals using genetically modified cyanobacteria and algae. For other applications, readers can refer to several other excellent reviews published recently (Radakovits et al., 2010; Ruffing, 2011; Qin et al., 2012).

BIOFUELS

United States consumed 13.3 million barrels of petroleum per day for transportation purposes in 2009, accounting for 71% of all petroleum used (Energy Information Administration, 2010). Many alternatives to current liquid fuels have been proposed, including ethanol, 1-butanol, isobutanol, short-chain alcohols, short-chain alkanes, biodiesel (FAME, fatty acid methyl esters), fatty alcohols, alkanes, linear and cyclic isoprenoids (Lee et al., 2008; Connor and Atsumi, 2010). Current routes for biological production of fuels and chemicals are summarized in **Figure 1**. Traditionally people follow a two-step route to firstly collect plant biomass and then convert biomass to fuels by microbial fermentation (Stephanopoulos, 2007); whereas recently interest in harnessing photosynthetic microbes to directly convert CO_2 to fuels has been dramatically increased (Chisti, 2007; Lu, 2010). Compared to crops, the per-hectare oil yield of cyanobacteria or microalgae is about two orders of magnitude higher and the cultivation land needed is around two orders of magnitude less (Chisti, 2007). It is estimated that more than $1 billion has been invested in the algae-to-biofuel research and development since 2007 in US alone (Mascarelli, 2009).

Biodiesel

Cyanobacteria and algae are rich in energy stock compounds, such as diacylglycerol (DAG), triacylglycerol (TAG) and starch, which can be extracted and used for biodiesel production (van de Meene et al., 2006; Chisti, 2007; Radakovits et al., 2010; Sheng et al., 2011). To further increase the oil contents in the cells, effects have been made to block metabolic pathways as well as to overexpress genes of limiting steps. For example, two different starch-deficient strains of *C. reinhardtii*, the *sta6* and *sta7* mutants that carries gene knockout in the ADP-glucose pyrophosphorylase and isoamylase genes, respectively, have been isolated (Mouille et al., 1996; Posewitz et al., 2005); and these mutants accumulated increased levels of TAG during nitrogen deprivation (Wang et al., 2009). Another starchless mutant of *Chlorella pyrenoidosa* has also been reported that the lipid content of this mutant has been elevated by nearly twofold relative to the wild-type under nitrogen limitation culture conditions (Ramazanov and Ramazanov, 2006). It indicated that blocking the starch biosynthesis may be an effective way to increase lipid, and thus potentially biodiesel, production.

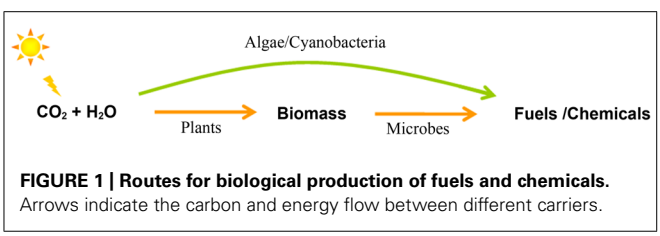


FIGURE 1 | Routes for biological production of fuels and chemicals. Arrows indicate the carbon and energy flow between different carriers.

Nevertheless, lipid extraction process is energy-intensive and significant amount of glycerol as a byproduct have been two of the major hurdles for commercial production of biodiesel (Chisti, 2007; Fernando et al., 2007; Liu and Curtiss, 2009). Efforts have been made from both process engineering and genetic engineering approaches to facilitate the lipid extraction (Liu and Curtiss, 2009; Liu et al., 2011a; Sheng et al., 2011). Specifically, Liu and colleagues have constructed inducible systems to conditionally express phage lysis genes and lipolytic enzyme genes in *Synechocystis* 6803 to trigger the cell lysis upon harvest and thus help lipid extraction from this species (Liu and Curtiss, 2009; Liu et al., 2011a). To produce secretable biofuels from a synthetic biology approach is another way to resolve above issues.

Free fatty acids

Enhanced production of free fatty acid (FFA) has already been achieved in *E. coli* through a series of genetic engineering (Davis et al., 2000; Lu et al., 2008). In a recent study, Liu et al. (2011b) engineered cyanobacterium *Synechocystis* strains to produce and secret FFAs to up to 197 mg/L at a cell density of 1.0×10^9 cells/mL. The acetyl-CoA carboxylase (ACC) was overexpressed to drive the metabolic flux toward FFAs, while the fatty acid activation gene *aas* (*slr1609*) was deleted to inactivate the FFAs degradation. Poly-β-hydroxybutyrate (PHB) synthesis genes (*slr1993* and *slr1994*) and the phosphotransacetylase gene *pta* (*slr2132*) were deleted to block competitive pathways. Particularly, two genetic modifications turned to significantly increase the FFAs production and secretion: overexpression of thioesterases and weakening the polar peptidoglycan layer of the cell wall of *Synechocystis* 6803.

Alkanes and alkenes

Although it was known that some cyanobacteria can synthesize alkanes, the molecular mechanism had been mysterious until recently an alkane/alkene biosynthetic pathways were identified in cyanobacteria (Steen et al., 2010; Mendez-Perez et al., 2011). Steen et al. (2010) identified an alkane/alkene biosynthetic pathway that two successive biochemical reactions catalyzed by an acyl-ACP reductase and an aldehyde decarbonylase, respectively, converts acyl-ACP (intermediates of fatty acid metabolism) to alkanes/alkenes. In order to increase the alkane production in cyanobacteria, heterologous expression of acyl-ACP reductase and aldehyde decarbonylase genes (from *Synechococcus* 7942) has been achieved in *Synechococcus* 7002, which led to a total intracellular accumulation of n-alkane to up to 5% of the dry cell weight (Reppas and Ridley, 2010). In another research, Mendez-Perez

et al. (2011) identified the genes responsible for α-olefin biosynthesis in *Synechococcus* 7002. In addition, overexpression of the *accBCDA* operon (which encodes ACC) in *Synechocystis* was also reported to enhance alkane/alkene production (Tan et al., 2011), consistent with the aforementioned results of FFAs production. Although it is believed there are certain alkane/alkene secretion pathways, the specific mechanisms are still under exploration (Radakovits et al., 2010).

Ethanol

Ethanol production via microbial fermentation has undergone a sharp increasing in the past decade for its utility as supplement in transportation fuel (Stephanopoulos, 2007; Energy Information Administration, 2010). In 1999, photosynthetic production of up to 230 mg/L ethanol has been reported using genetically engineered cyanobacterium *Synechococcus* 7942, in which an artificial operon of *pdc-adh* (genes originally from *Zymomonas mobilis*) was expressed under a P_{lac} and P_{rbc} promoters via a shuttle vector pCB4 (Deng and Coleman, 1999). In a recent study, the *pdc-adh* expression cassette was integrated into the chromosome of *Synechocystis* 6803 at the *psbA2* locus. Driven by the light-inducible strong P_{psbA2} promoter, expression of *pdc/adh* resulted in ∼550 mg/L ethanol production by the engineered *Synechocystis* under high light (∼1000 μE/m^2/s) conditions (Dexter and Fu, 2009). In algae, although many species have fermentative pathways to produce ethanol, the pathways are only functional under dark and anaerobic conditions (Hirayama et al., 1998). Algal ethanol is currently produced via heterotrophic fermentation of algal biomass using heterotrophs such as yeast and *E. coli* (Nguyen et al., 2009; Harun et al., 2010; Wargacki et al., 2012), which follows the two-step route (**Figure 1**). Direct photosynthetic production of ethanol by algae would be possible using a similar approach as being demonstrated in cyanobacteria by expressing foreign ethanol biosynthesis pathways, or by tuning the native regulatory pathways in algae.

Isobutanol and 1-butanol

Compared with ethanol, isobutanol and 1-butanol have much higher energy density. The energy density of butanol reaches 29.2 MJ/L, about 90% of that of gasoline, 32.5 MJ/L, and it is also less volatile and less corrosive than ethanol (Dürre, 2007). Therefore, butanol is regarded as a better gasoline substitute. Recently, significant progress has been achieved for photosynthetic production of butanol. Liao and colleagues introduced an artificial isobutanol biosynthesis pathway into *Synechococcus* 7942 and the engineered strains were able to photosynthetically produce isobutyraldehyde and isobutanol at titers of 1100 and 450 mg/L, respectively (Atsumi et al., 2009). In contrast, photosynthetic production of 1-butanol in oxygenic cyanobacteria or algae has been hard because the intrinsic oxygen-sensitivity and NADH-dependence of the 1-butanol biosynthetic pathway are conflict with the photo-oxygenisis and lack of NADH cofactors in cyanobacteria (Atsumi et al., 2008a; Inui et al., 2008; Lan and Liao, 2011). When a 1-butanol pathway was overexpressed in *Synechococcus* 7942, the 1-butanol was barely detectable (∼1 mg/L) after 2 weeks cultivation under photosynthetic conditions. Up to 14.5 mg/L 1-butanol has been achieved in *Synechococcus* 7942 under an anoxic condition (Lan and Liao, 2011). Further analysis revealed that the reversible acetyl-CoA condensation reaction catalyzed by thiolase (encoded by *atoB*) strongly favors the thiolysis of acetoacetyl-CoA rather than the condensation of two acetyl-CoA molecules, and thus AtoB may be insufficient to drive the flux from acetyl-CoA pool toward 1-butanol biosynthesis under photosynthetic conditions (Lan and Liao, 2012). To this end, an alternate ATP-driven acetoacetyl-CoA biosynthetic pathway was constructed by overexpressing an acetoacetyl-CoA synthase (NphT7) which instead condenses malonyl-CoA and acetyl-CoA in *Synechococcus*. With co-expressing the downstream NADH-dependent 1-butanol biosynthetic pathway, 6.5 mg/L 1-butanol has been produced under photosynthetic conditions. After the NADH-dependent bifunctional aldehyde/alcohol dehydrogenase (AdhE2) was further replaced with separate NADPH-dependent butyraldehyde dehydrogenase (Bldh) and alcohol dehydrogenase (YqhD), the 1-butanol production was increased by fourfold, up to ∼30 mg/L, under the same photosynthetic condition (Lan and Liao, 2012).

Longer carbon chain fatty alcohols

In order to produce long-chain alcohols, Lu and colleagues heterologously expressed fatty acetyl-CoA reductases from different sources in *Synechocystis* and the resultant strains achieved production of fatty alcohols, including hexadecanol (C16) and octadecanol (C18; Tan et al., 2011). Although the titer was very low (about 0.2 mg/L), it is amenable for further improvement via further enhancing upstream pathways and addressing secretion issues as that in the engineering of *Synechocystis* 6803 for enhanced fatty acid production (Liu et al., 2011b). Production of the intermediate-chain alcohols (C5 to C10) in *E. coli* has been well summarized by Lamsen and Atsumi (2012). Briefly, C5 to C10 alcohols have been successfully biosynthesized via the expanded 1-butanol pathway (Dekishima et al., 2011), the engineered reversal of the β-oxidation pathway (Dellomonaco et al., 2011) and the 2-keto acid metabolic pathways (Atsumi et al., 2008b; Zhang et al., 2008). Since cyanobacteria and algae share with *E. coli* the most chassis metabolic pathways required for longer-chain alcohol biosynthesis, it is believed that similar approaches can be used to achieve the biosynthesis of alcohols with carbon chain length >5 in cyanobacteria and algae.

Hydrogen

Besides liquid biofuels, production of hydrogen – a gaseous, carbon-free, and high-energy-content fuel – in algae and cyanobacteria has also gained increasing attention in recent years (Melis et al., 2000; Kruse et al., 2005; Ghirardi et al., 2007, 2009; Hankamer et al., 2007; Hemschemeier et al., 2009; Lee et al., 2010; Srirangan et al., 2011). Many cyanobacteria and algae naturally produce hydrogen as a secondary metabolite to balance the redox energetics. In order to fortify the hydrogen production, endeavor has been made to augment the electron flux, instead of the carbon flux, toward H_2 biosynthesis catalyzed by hydrogenases ($2H^+ + 2e^- \rightarrow H_2$). In alga *C. reinhardtii*, for instance, blocking the cyclic electron transfer around PSI turned to eliminate the possible electron competition for electron with hydrogenase;

as a result, the H_2 evolution rate increased 5–13 times under a range of conditions (Kruse et al., 2005). Hydrogenase has been tethered to the PSI to obtain a much greater electron throughput and thus H_2 evolution rate (Ihara et al., 2006; Schwarze et al., 2010; Lubner et al., 2011). However, to date these experiments were all conducted *in vitro* and efforts need to be made from a synthetic biology approach to validate the concept *in vivo*. In another study, expression of an exogenous ferredoxin from *Clostridium acetobutylicum* in addition to the native ferredoxin could fortify the electron flow toward the hydrogenase HydA via siphoning electrons from the fermentation of internal reducing equivalents (such as glycogen). As a result, the hydrogen production was enhanced by approximately twofold (Ducat et al., 2011a) under light-dependent anoxic conditions. On the other hand, efforts have been made to block pathways competitive for reductant consumption to facilitate the H_2 production. For example, after the *ldhA* gene (which is responsible for NADH consumption in lactate production) was inactivated in *Synechococcus* 7002, the NADH/NAD^+ ratio increased markedly and therefore the hydrogen production by the native bidirectional [NiFe] hydrogenase was increased fivefold under anoxic dark conditions (McNeely et al., 2010). The oxygen-sensitivity of both two major [NiFe] and [FeFe] hydrogenases is the greatest challenge to date which is discussed infra.

OTHER COMMODITY CHEMICALS

Although significant attention has been paid to photosynthetic production of fuels from CO_2, the relative values (in term of USD per photon) of fuels are much lower than those of other commodity chemicals. For example, it is estimated that the relative value of a photon fixed in lactic acid is about 3.5-fold greater than that in octane (Ducat et al., 2011b). Therefore, photosynthetic production of chemicals with higher unit values than fuels is economically more desirable at least in the near term.

Ethylene

Ethylene, the simplest unsaturated alkene, is one of the most important building-blocks in synthetic chemical industry. However, its production almost exclusively relies on petroleum. To make the production sustainable, biosynthesis of ethylene from renewable resources has been explored. Sakai et al. (1997) first demonstrated photosynthetic production of gaseous ethylene from CO_2 in genetically engineered *Synechococcus* by heterologously expressing a single *efe* gene of *Pseudomonas syringae* on a pUC303-derived shuttle vector. Later, by integrating the *efe* gene into the *psbA1* locus of the *Synechococcus* 7942 genome, the research group achieved higher ethylene production with a titer of ∼37 mg/L (Takahama et al., 2003). However, the engineered *Synechococcus* strains were not genetically stable, resulting in declined ethylene production during successive batch cultivations (Takahama et al., 2003). Since production of every two molecules of ethylenes consumes three molecules of 2-oxoglutarate and one molecule of L-arginine (Fukuda et al., 1992), the genetic instability might be attributed to the shortage in the tricarboxylic acid (TCA) cycle intermediates, leading to a severe depression on cell growth (Takahama et al., 2003). In order to sustain the ethylene production in cyanobacteria or algae, metabolic flux toward TCA cycle should be enhanced and alternative ethylene biosynthesis pathways might be considered (Yang and Hoffman, 1984; Fukuda et al., 1989; Kende, 1993; Kosugi et al., 2000).

Isoprene

Isoprene is another important feedstock in the synthetic chemistry and potentially a biofuel. Biosynthesis and emission of isoprene occurs in many plants as a way to cope with heat flecks and reactive oxygen species, and the genetic mechanism has been investigated (Sharkey et al., 2008). Lindberg et al. (2010) cloned the *IspS* gene (encoding isoprene synthase) from *Pueraria montana* and integrated into the *psbA2* locus of the *Synechocystis* genome, conferring heterologous expression of the isoprene synthase under the light-dependent P_{psbA2} promoter in *Synechocystis*. Codon usage turned to be a very important factor for optimal expression of the *IspS* gene. After codon optimization, the *IspS* gene expression was enhanced by about 10-fold. Isoprene was eventually produced at a rate of ∼50 mg/g dry cell/day under high light (∼500 $\mu E/m^2/s$) culture conditions. It is noteworthy that heterologous expression of *IspS* by replacing the *psbA2* gene did not affect photosynthesis significantly and depress the growth of the transformants (Lindberg et al., 2010), which was differed from the aforementioned ethylene-producing cyanobacteria (Sakai et al., 1997; Takahama et al., 2003).

Acetone

Acetone represents the simplest ketone which serves as a solvent and precursor for industrial chemicals (Yurieva et al., 1996). Microbial production of acetone has been achieved in fermentation of *Clostridia* and recombinant *E. coli* using sugar as feedstocks (Bermejo et al., 1998). However, the maximal yield is merely 50% with the other half carbon being released as CO_2 when hexose is the sole carbon source. Recently, through a combination of co-expression of the acetoacetate decarboxylase (*adc*) and coenzyme A transferase (*ctfAB*) and deletion of the PHB polymerase (PhaEC) in *Synechocystis* 6803, 3–5 mg/L acetone has been produced under nitrogen and phosphate deprived, dark and anaerobic culture conditions. After deleting the phosphotransacetylase-encoding gene *pta*, the competitive acetate production was remarkably reduced and the acetone titer has been evidently increased to 36.0 mg/L in the culture (Zhou et al., 2012).

Poly-β-hydroxybutyrates

Cyanobacteria are the natural producers of PHB, a type of polyhydroxyalkanoates (PHAs) that serves as biodegradable plastics (Hein et al., 1998; Taroncher-Oldenburg et al., 2000). However, the yield is very low and nutrient deprivation and acetate addition are usually necessary for accumulation of PHB (Wu et al., 2001). By introducing PHB biosynthesis genes from *Ralstonia eutropha* into *Synechococcus* 7942 coupled with nitrogen starvation and acetate supplementation, the PHB biosynthesis in the recombinant cyanobacteria has reached a maximum of 25.6% of the dry cell weight (Takahashi et al., 1998). Efforts in identifying gene disruptions which might contribute to increase of PHB accumulation were also made and several gene disruptions with positive effects were discovered (Tyo et al., 2009). Nevertheless, similar with other types of macromolecules PHB can not be secreted out of cells;

the required extraction process is energy-intensive and remains as one of the major hurdles for commercial applications (Chisti, 2007; Liu and Curtiss, 2009). As a result, 3-hydroxybutyrate (3HB), the monomer of PHB and a building block molecule for other PHAs, has been successfully produced and secreted by genetically engineered *E. coli* (Lee and Lee, 2003; Liu et al., 2007; Tseng et al., 2009). Hence, photosynthetic production of 3HB in cyanobacteria and algae might be a feasible approach to cope with the secretion problem.

Lactic acid

Lactic acid is another chemical that can serve as a building block for synthesizing biodegradable polyesters with valuable medical properties. It is also used as a preservative and acidulant in food industry, and can serve as an advanced nutrient for neuron cells (Wee et al., 2006). While conventional production of lactic acid relies on microbial fermentation of sugars (Wee et al., 2006), photosynthetic production of lactic acid using CO_2 as carbon source has been recently demonstrated (Niederholtmeyer et al., 2010). Through heterologously expressing three genes, including *ldhA*, *lldP*, and *udhA*, in cyanobacterium *Synechococcus* 7942, Niederholtmeyer et al. (2010) has accomplished production of lactic acid with a titer of ∼56 mg/L under photoautotrophic culture condition. While LdhA catabolizes the conversion of pyruvate to lactate, expression of the lactate transporter gene *lldP* turned to be essential for lactate secretion from the engineered *Synechococcus* strain (Niederholtmeyer et al., 2010). Repletion of NADH, a cofactor for LdhA, through expressing the NADPH/NADH transhydrogenase (encoded by *udhA*) greatly enhanced the lactate production but reduced the growth rate of *Synechococcus* (Niederholtmeyer et al., 2010).

Sugars

Fresh water cyanobacteria accumulate solutes such as glucosylglycerol and sucrose when they are exposed to salt stress (Hagemann, 2011). By knocking out the *agp* gene (which contributes to the biosynthesis of glucosylglycerol) from the *Synechocystis* 6803 genome, Miao et al. (2003) achieved sucrose accumulation of up to 44 mg/L/OD_{730} after 0.9 M salt shock for 96 h. In another study, overexpression of *invA*, *glf*, and *galU* genes in *Synechococcus* 7942 resulted in up to 45 mg/L total hexose production (including glucose and fructose) in the culture supplemented with 200 mM NaCl (Niederholtmeyer et al., 2010). While InvA catalyzes the conversion of sucrose to glucose and fructose, expression of the glucose or fructose transporter GLF (encoded by *glf* gene) was essential for glucose or fructose secretion. Additional expression of GalU enhanced the biosynthesis of intracellular precursors and thus further increased the hexose sugar production by over 30% in the culture (Niederholtmeyer et al., 2010).

CHALLENGES AND OPPORTUNITIES OF SYNTHETIC BIOLOGY IN CYANOBACTERIA AND ALGAE

Despite of promising progresses, there are challenges ahead for synthetic biology to reach its full power in modifying cyanobacteria and algae for biotechnological applications. Here we briefly discuss the challenges and possible strategies.

IMPROVING TOOLS FOR GENETIC MANIPULATION

Effective "BioBricks"

Although a few "BioBricks" have been characterized in cyanobacteria, the limit number of gene expression elements would not fulfill the need of synthetic biology in cyanobacteria. After an initial gene expression, a fine-tuning of the gene expression is usually the next step in order to further optimize the properties of the genetically engineered strains, which requires a good number of "BioBricks." Currently most of the "BioBricks" were collected from *E. coli*, but the *E. coli* "BioBricks" might behave differently in cyanobacteria. For example, tightly regulated IPTG-inducible lacI/P_{tac} gene expression system does not work as well in cyanobacteria as it does in *E. coli* (Huang et al., 2010). Thus, systematic collection and characterization of "BioBricks" in cyanobacteria is necessary. Additionally, in contrast to various commercialized *E. coli* and yeast strains that have been genetically modified to serve as chassis for different purposes, there are few such cyanobacterial or algal species available nowadays. To design and construct a series of chassis strains is thus an urgent task. Moreover, to our knowledge, there has been no study of the performance of a given BioBrick in different cyanobacterial species. We assume that a defined BioBrick might behave differently across cyanobacterial species and the efficiency of the BioBrick need to be characterized for each cyanobacterial species.

Improved transformation efficiency

Standardized transformation vectors/protocols have been established for model cyanobacteria, such as *Synechococcus* and *Synechocystis*, although the transformation efficiency still needs further improvement (Eaton-Rye, 2004; Heidorn et al., 2011). However, the transformation methods for model filamentous cyanobacteria, such as *Anabaena* and *Spirulina*, are still under development (Ducat et al., 2011b), and so far no genetic engineering has been conducted in the marine N_2-fixing cyanobacterium *Trichodesmium* despite significant interest on its ability of peaking the fixation of CO_2 and N_2 simultaneously during the day time (Chen et al., 1998; Berman-Frank et al., 2001). *In vivo* restriction activities have been demonstrated as an important barrier for introducing foreign DNA into cyanobacterial cells (Elhai et al., 1997; Koksharova and Wolk, 2002). Hence, it would be helpful to construct methylation-defect cyanobacterium host strains or to establish *in vitro* systems that can methylate the foreign DNA before transformation. Additionally, since the bacteriophage λ recombination system has greatly improved the *E. coli* transformation efficiency (Yu et al., 2000), we propose that high-efficiency homologous recombination in cyanobacterial cells might be achievable by developing a proper cyanophage recombination system.

In order to improve transformation efficiency of other algal species, endeavor could be made to uncover the mechanism behind the recently discovered truth that highly efficient homologous recombination occurs after electroporation of the industrially relevant oil-producing alga *Nannochloropsis* sp. (Kilian et al., 2011). Recently, through an approach of *ex vivo* assembly of the chloroplast genome before bombarding it into the green alga *C. reinhardtii*, O'Neill et al. (2012) demonstrated that simultaneous and multi-loci genetic modifications of the chloroplast of the green

alga *C. reinhardtii* could occur after one single round of transformation, providing an alternative method to improve the efficiency of multiple-gene transfer.

IMPROVING PHOTOSYNTHESIS EFFICIENCY

Although the solar energy conversion efficiencies of algae and cyanobacteria are 2–3 folds higher than those of crop plants, the efficiencies are still low with yields around 5–7% during the growing season and around 3% in bioreactors on an annual basis (Blankenship et al., 2011). A recent study on *in silico* modeling of the reconstructed photosynthetic process revealed that the regulation of the photosynthesis activity is quite complex and a high degree of cooperativity of nine alternative electron flow pathways is responsible for optimized photosynthesis performance in *Synechocystis* 6803 (Nogales et al., 2012).

Light harvesting

The photosynthetic microorganisms in nature have been selected by the abilities to reproduce but not by the ability to produce a maximal amount of biomass or specific products. In order to thrive in the wild environment, cyanobacteria and algae have maximized their expression of pigments and antenna to compete with the competitors for sunlight. However, when monoculture was employed to produce high-density of biomass or maximal titers of specific products in photo-bioreactors, excessive photon capture by the cells in the surface layer can block the light availability to the cells underneath (Melis, 2009). To address this issue, studies have been conducted on minimizing the size of the photosystem antenna complex through various strategies, such as by expressing truncated light-harvesting antenna complex (LHC) mutants (Blankenship et al., 2011; Ort and Melis, 2011; Work et al., 2012), by down-regulating the expression of LHC through RNA interference (RNAi) and expression of LHC translation repressor in both cyanobacteria and algae (Mussgnug et al., 2007; Work et al., 2012). For example, the photosynthetic activity (measured by oxygen evolution) was about threefold higher in the alga strain *Stm3LR3* (with LHC being down-regulated via RNAi) than in the parent strain *Stm3* (without RNAi) after 100 min of high-light treatment; the cell growth rate also increased under high-light conditions after the LHC was down-regulated via RNAi (Mussgnug et al., 2007). Another bold proposal was to increase the photosynthesis efficiency by extending the light absorption range of the photosystems in cyanobacteria and algae (Blankenship et al., 2011). As the chlorophyll, carotenoids, and other accessory pigments in cyanobacteria and algae capture only visible region of the spectrum of solar radiation (400 to 700 nm), about 50% of the incident solar energy is dissipated and wasted during photosynthesis. Moreover, since the two photosystems compete for light with the same wavelengths, the overall efficiency is significantly reduced. Thus, it was proposed that one of the two photosystems be engineered to extend the absorption maxima to ∼1100 nm, approximately doubling the solar photon capture, by heterologously expressing bacteriochlorophyll *b* (Blankenship et al., 2011).

CO_2 fixation

RuBisCO is an essential enzyme in photosynthetic carbon fixation in Calvin–Benson–Bassham (CBB) cycle, catalyzing the combination of ribulose-1,5-bisphosphate with CO_2. However, the reaction is slow. In addition, RuBisCO can also take O_2 as substrate in addition to CO_2 which further lower the carbon fixation efficiency. A recent study has revealed that despite slow catalytic turnover and confused CO_2/O_2 substrate specificity, RuBisCOs might have been nearly perfectly optimized (Tcherkez et al., 2006). In nature, cyanobacteria and some algae have evolved certain CO_2-concentrating mechanisms (CCMs) to increase the CO_2 fixation efficiencies. In cyanobacteria, RuBisCOs are sequestered together with carbonic anhydrous in carboxysomes, polyhedral microcompartments (MCPs) with proteinaceous shells. Anhydrase catalyzes the conversion of HCO_3^- to CO_2 which is trapped by MCPs for RuBisCOs. Because CCMs can result in much higher CO_2 concentration, and thus higher CO_2 to O_2 ratio, around the RuBisCOs, the carbon fixation efficiency is greatly increased (Espie and Kimber, 2011). It has been found that *Synechococcus* 7942 cells with more carboxysomes exhibited higher CO_2 fixation rates (Savage et al., 2010). Heterologous expression of *Synechococcus* 6301 *rbcLS* (that encodes RuBisCO) in *Synechococcus* 7942 also led to more efficient CO_2 fixation and higher yield of isobutyraldehyde in the genetically modified isobutyraldehyde-producing strain (Atsumi et al., 2009). Besides, overexpression of bicarbonate transporters has also been proposed to improve the photosynthesis efficiency (Price et al., 2011). Alternatively, RuBisCO-independent carbon fixation pathways have been posited. A recent work using *in silico* modeling of the recombination of existing metabolic building blocks showed that some of the proposed carbon fixation cycles have overall higher kinetic rates (Bar-Even et al., 2010). For example, by coupling the phosphoenolpyruvate carboxylase and the core of the natural C4 carbon fixation cycle, the overall CO_2 fixation rate was predicted as 2–3 folds higher than that of the CBB cycle which employs RuBisCO (Bar-Even et al., 2010).

OVERCOMING THE OXIDATIVE STRESS

Since cyanobacteria and algae are oxygenic microorganisms, the abundant oxygen evolved by splitting water during photosynthesis process becomes an issue for expressing oxygen-sensitive enzymes. For example, either [NiFe] or [FeFe] hydrogenase required for biological production of H_2 has low oxygen-tolerance (Lee et al., 2010); and the nitrogenases which fix N_2 into NH_4^+ are also extremely oxygen-sensitive (Fay, 1992). From a broader prospect, this oxygen sensitivity issue could be crucial for successful expression of a large number of pathways from anaerobic microorganisms in oxygenic cyanobacteria and algae. To address the issue, efforts have been made to obtain oxygen-resistant enzymes from nature or through mutagenesis. For example, hydrogenases with better oxygen-tolerance have been found from *Ralstonia eutropha* H16 (Saggu et al., 2009) and *Hydrogenovibrio marinus* (Yoon et al., 2011); and elevated oxygen-tolerance has been made for the hydrogenase of *Desulfovibrio fructosovorans* by a single V74M mutation (Dementin et al., 2009). Alternatively, temporal segregation of oxygenic photosynthesis and hydrogen biosynthesis would be another option. In nature, many cyanobacterial species have evolved the mechanism to photosynthetically fix CO_2 during day time while fix N_2 by the oxygen-sensitive nitrogenases during night (Fay, 1992). Thereby, the solar energy can

be firstly fixed into carbohydrates, such as starch, during oxygenic photosynthesis and then be utilized to power the oxygen-sensitive reactions during dark anoxic conditions. In addition, spatial segregation could be used. Hydrogenases can be localized to certain advantageous space, such as being expressed in heterocysts, to avoid the oxidative stress (Fay, 1992). Recent studies on bacterial MCPs assembling might have provided another opportunity to spatially segregate incompatible oxygenic and oxygen-sensitive processes (Fan et al., 2010; Heinhorst and Cannon, 2010; Bonacci et al., 2012). Moreover, Mehler reaction can be used to overcome the oxidative stress (Mehler, 1951; Asada, 2006). Mehler reaction has been evolved to overcome the intracellular oxidative stress by scavenging reactive oxygen species in cyanobacteria and chloroplasts (Kana, 1993; Asada, 2006). For instance, during N_2-fixation period Mehler reaction consumes ∼75% of gross O_2 production and therefore maintains the O_2 concentration at a low level (Kana, 1993; Milligan et al., 2007). However, Mehler reaction consumes reductant significantly (Asada, 2006); thus, in the future it will be of great interest and of vital importance to maintain the activity locally around the oxygen-sensitive enzymes rather than in the entire cytoplastic environment.

SYSTEMATIC APPROACHES

Functional genomics

Functional genomics, i.e., transcriptomics, proteomics, and metabolomics, would greatly promote the development of synthetic biology in cyanobacteria and algae. Albeit the genomes of some many species of cyanobacteria and algae have been sequenced[1,2], a large portion of the sequenced genomes have not yet been annotated and the regulatory networks are still very poorly understood. The study of cyanobacterial and algal transcriptomes, proteomes, and metabolomes would allow for identification of new genes, pathways and regulatory networks which are essential to expand the size and diversity of the pool of genetic tools for synthetic biology. For example, recent transcriptomics studies on *Synechocystis* 6803 has enhanced the understanding of the transcriptional regulation in this photosynthetic microorganism which revealed that approximate two-thirds of the transcriptional start sites give rise to asRNAs and noncoding RNAs (ncRNAs), indicating that asRNAs and ncRNAs play an important role in cyanobacterial genetic regulation (Mitschke et al., 2011). We prospect that omics may be the key to collect information about the interactions and regulations to develop a sustainable green chemistry industry.

Metabolic modeling

Although most synthetic biology research in cyanobacteria and algae focus on local pathway optimization, comprehensive synthetic biology summons optimization of the genetic network and metabolic flux at the systems level. Genome-scale metabolic modeling allows theoretically evaluating the impact of genetic and environmental perturbations on the biomass yield and metabolic flux distribution and allows predicting the optimal metabolic flux profile to maximize the value of a given objective function (Shastri and Morgan, 2005; Knoop et al., 2010; Yoshikawa et al., 2011). The *in silico* modeling may thus provide a systematic approach to design an optimal metabolic network to maximize the production of the interest biofuel or chemical. Such genome-scale metabolic network models have been constructed for cyanobacteria and algae, and have been utilized to predict new targets to improve product yields and new pathways (Shastri and Morgan, 2005; Knoop et al., 2010; Dal'Molin et al., 2011; Yoshikawa et al., 2011). However, the reconstruction of the global metabolic networks is still in the infancy stage and the simulation results rely significantly on the included pathways. For instance, with ambiguities in metabolic networks in *Synechocystis* 6803, the estimated metabolic fluxes could be significantly different from the experimental results (Yoshikawa et al., 2011). In order to refine the quality of the reconstructed metabolic networks and thus the simulation of metabolic flux, it is inevitable to couple with experimental characterization of the metabolic networks in cyanobacteria and algae (Yoshikawa et al., 2011). As an example, by firstly investigating the *in vitro* activities of the purified relevant enzyme products (heterologously expressed in *E. coli*) and subsequently verifying their *in vivo* activities in the native host *Synechococcus* 7002, Zhang and Bryant (2011) reported that two enzymes could functionally compensate for the missing 2-oxoglutarate dehydrogenase in the TCA cycle. Further database searches indicated that homologs of these two enzymes occur in all cyanobacteria but *Prochlorococcus* and marine *Synechococcus*, which overturned the previously widely accepted assumption that cyanobacteria possess an incomplete TCA cycle (Meeks, 2011; Zhang and Bryant, 2011). Such discoveries would be of utter importance to reconstruct qualified *in silico* models for simulating the metabolic flux in the future.

CONCLUSION

Owing to the relatively simple genetic contents and the ability to capture solar energy, fix CO_2, grow fast and directly synthesize specific products, cyanobacteria and algae have become excellent candidates for building autotrophic cell factories to produce renewable surrogate fuels and chemicals. With a large pool of genome sequences and improved genetic tools being available, application of synthetic biology in these photosynthetic microorganisms are highly desirable. In recent years, exciting results have been achieved not only in understanding of the fundamental molecular mechanisms but also in producing various interest products, such as biofuels and chemicals, utilizing cyanobacteria and algae as the production platforms. Nevertheless, synthetic biology in cyanobacteria and algae is still in its infancy and synthetic biologists are facing great challenges and opportunities in addressing various issues, such as improving the tools for genetic manipulation, enhancing light harvesting, increasing CO_2 fixation efficiency, and overcoming of the intracellular oxidative stress. Systematic approaches, such as functional genomics and metabolic modeling, may also diversify the genetic tools and help the metabolic network design. It is doubtless that synthetic biology would be indispensable for the future success in applying cyanobacteria and algae for various biotechnological purposes.

ACKNOWLEDGMENTS

This work was supported by the NEPTUNE fund granted to Deirdre R. Meldrum at ASU. Weiwen Zhang is currently funded by a grant from National Basic Research Program of China (National "973" program, project No. 2011CBA00803).

REFERENCES

Adhya, S., and Gottesman, M. (1982). Promoter occlusion: transcription through a promoter may inhibit its activity. *Cell* 29, 939–944.

Apt, K. E., Kroth-Pancic, P. G., and Grossman, A. R. (1996). Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Mol. Gen. Genet.* 252, 572–579.

Argueta, C., Yuksek, K., and Summers, M. (2004). Construction and use of GFP reporter vectors for analysis of cell-type-specific gene expression in *Nostoc punctiforme*. *J. Microbiol. Methods* 59, 181–188.

Asada, K. (2006). Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiol.* 141, 391–396.

Atsumi, S., Cann, A. F., Connor, M. R., Shen, C. R., Smith, K. M., Brynildsen, M. P., Chou, K. J., Hanai, T., and Liao, J. C. (2008a). Metabolic engineering of *Escherichia coli* for 1-butanol production. *Metab. Eng.* 10, 305–311.

Atsumi, S., Hanai, T., and Liao, J. C. (2008b). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89.

Atsumi, S., Higashide, W., and Liao, J. C. (2009). Direct photosynthetic recycling of carbon dioxide to isobutyraldehyde. *Nat. Biotechnol.* 27, 1177–1180.

Bar-Even, A., Noor, E., Lewis, N. E., and Milo, R. (2010). Design and analysis of synthetic carbon fixation pathways. *Proc. Natl. Acad. Sci. U.S.A.* 107, 8889–8894.

Bayer, T. S., Widmaier, D. M., Temme, K., Mirsky, E. A., Santi, D. V., and Voigt, C. A. (2009). Synthesis of methyl halides from biomass using engineered microbes. *J. Am. Chem. Soc.* 131, 6508–6515.

Beck, C., Knoop, H., Axmann, I. M., and Steuer, R. (2012). The diversity of cyanobacterial metabolism: genome analysis of multiple phototrophic microorganisms. *BMC Genomics* 13, 56. doi: 10.1186/1471-2164-13-56

Becker, E., and Meyer, R. (1997). Acquisition of resistance genes by the IncQ plasmid R1162 is limited by its high copy number and lack of a partitioning mechanism. *J. Bacteriol.* 179, 5947–5950.

Benfey, P. N., Ren, L., and Chua, N. H. (1990). Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. *EMBO J.* 9, 1677–1684.

Benner, S. (2003). Act natural. *Nature* 421, 118.

Berman-Frank, I., Lundgren, P., Chen, Y. B., Küpper, H., Kolber, Z., Bergman, B., and Falkowski, P. (2001). Segregation of nitrogen fixation and oxygenic photosynthesis in the marine cyanobacterium *Trichodesmium*. *Science* 294, 1534–1537.

Bermejo, L. L., Welker, N. E., and Papoutsakis, E. T. (1998). Expression of *Clostridium acetobutylicum* ATCC 824 genes in *Escherichia coli* for acetone production and acetate detoxification. *Appl. Environ. Microbiol.* 64, 1079–1085.

Blankenship, R. E., Tiede, D. M., Barber, J., Brudvig, G. W., Fleming, G., Ghirardi, M., Gunner, M. R., Junge, W., Kramer, D. M., Melis, A., Moore, T. A., Moser, C. C., Nocera, D. G., Nozik, A. J., Ort, D. R., Parson, W. W., Prince, R. C., and Sayre, R. T. (2011). Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. *Science* 332, 805–809.

Bonacci, W., Teng, P. K., Afonso, B., Niederholtmeyer, H., Grob, P., Silver, P. A., and Savage, D. F. (2012). Modularity of a carbon-fixing protein organelle. *Proc. Natl. Acad. Sci. U.S.A.* 109, 478–483.

Boynton, J. E., and Gillham, N. W. (1993). Chloroplast transformation in *Chlamydomonas*. *Methods Enzymol.* 217, 510–536.

Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., and Sanford, J. C. (1988). Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. *Science* 240, 1534–1538.

Buzby, J. S., Porter, R. D., and Stevens, S. E. Jr. (1985). Expression of the *Escherichia coli* lacZ gene on a plasmid vector in a cyanobacterium. *Science* 230, 805–807.

Campbell, W. H., and Gowri, G. (1990). Codon usage in higher plants, green algae, and cyanobacteria. *Plant Physiol.* 92, 1–11.

Cerutti, H., Ma, X., Msanne, J., and Repas, T. (2011). RNA-mediated silencing in algae: biological roles and tools for analysis of gene function. *Eukaryot. Cell* 10, 1164–1172.

Chen, H. Y., Bjerknes, M., Kumar, R., and Jay, E. (1994). Determination of the optimal aligned spacing between the Shine–Dalgarno sequence and the translation initiation codon of *Escherichia coli* mRNAs. *Nucleic Acids Res.* 22, 4953–4957.

Chen, Y. B., Dominic, B., Mellon, M. T., and Zehr, J. P. (1998). Circadian rhythm of nitrogenase gene expression in the diazotrophic filamentous nonheterocystous cyanobacterium *Trichodesmium sp.* strain IMS101. *J. Bacteriol.* 180, 3598–3605.

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnol. Adv.* 25, 294–306.

Coll, J. M. (2006). Methodologies for transferring DNA into eukaryotic microalgae. *Spanish J. Agric. Res.* 4, 316–330.

Connor, M., and Atsumi, S. (2010). Synthetic biology guides biofuel production. *J. Biomed. Biotechnol.* 2010, 541698.

Dal'Molin, C. G., Quek, L. E., Palfreyman, R. W., and Nielsen, L. K. (2011). AlgaGEM – a genome-scale metabolic reconstruction of algae based on the *Chlamydomonas reinhardtii* genome. *BMC Genomics* 12, S5. doi: 10.1186/1471-2164-12-S4-S5

Davis, M. S., Solbiati, J., and Cronan, J. E. Jr. (2000). Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. *J. Biol. Chem.* 275, 28593–28598.

de Smit, M. H., and van Duin, J. (1990). Secondary structure of the ribosome binding site determines translational efficiency: a quantitative analysis. *Proc. Natl. Acad. Sci. U.S.A.* 87, 7668–7672.

Debuchy, R., Purton, S., and Rochaix, J. D. (1989). The argininosuccinate lyase gene of *Chlamydomonas reinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. *EMBO J.* 8, 2803–2809.

Dekishima, Y., Lan, E. I., Shen, C. R., Cho, K. M., and Liao, J. C. (2011). Extending carbon chain length of 1-butanol pathway for 1-hexanol synthesis from glucose by engineered *Escherichia coli*. *J. Am. Chem. Soc.* 133, 11399–11401.

Dellomonaco, C., Clomburg, J. M., Miller, E. N., and Gonzalez, R. (2011). Engineered reversal of the β-oxidation cycle for the synthesis of fuels and chemicals. *Nature* 476, 355–359.

Dementin, S., Leroux, F., Cournac, L., de Lacey, A. L., Volbeda, A., Léger, C., Burlat, B., Martinez, N., Champ, S., Martin, L., Sanganas, O., Haumann, M., Fernández, V. M., Guigliarelli, B., Fontecilla-Camps, J. C., and Rousset, M. (2009). Introduction of methionines in the gas channel makes [NiFe] hydrogenase aero-tolerant. *J. Am. Chem. Soc.* 131, 10156–10164.

Deng, M. D., and Coleman, J. R. (1999). Ethanol synthesis by genetic engineering in cyanobacteria. *Appl. Environ. Microbiol.* 65, 523–528.

Dexter, J., and Fu, P. (2009). Metabolic engineering of cyanobacteria for ethanol production. *Energ. Environ. Sci.* 2, 857–864.

Ducat, D. C., Sachdeva, G., and Silver, P. A. (2011a). Rewiring hydrogenase-dependent redox circuits in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 108, 3941–3946.

Ducat, D. C., Way, J. C., and Silver, P. A. (2011b). Engineering cyanobacteria to generate high-value products. *Trends Biotechnol.* 29, 95–103.

Dürre, P. (2007). Biobutanol: an attractive biofuel. *Biotechnol. J.* 2, 1525–1534.

Dwyer, D. J., Kohanski, M. A., Hayete, B., and Collins, J. J. (2007). Gyrase inhibitors induce an oxidative damage cellular death pathway in *Escherichia coli*. *Mol. Syst. Biol.* 3, 91.

Eaton-Rye, J. J. (2004). The construction of gene knockouts in the cyanobacterium *Synechocystis* sp. PCC 6803. *Methods Mol. Biol.* 274, 309–324.

Eichler-Stahlberg, A., Weisheit, W., Ruecker, O., and Heitzer, M. (2009). Strategies to facilitate transgene expression in *Chlamydomonas reinhardtii*. *Planta* 229, 873–883.

Elhai, J., Vepritskiy, A., Muro-Pastor, A. M., Flores, E., and Wolk, C. P. (1997). Reduction of conjugal transfer efficiency by three restriction activities of *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 179, 1998–2005.

Elhai, J., and Wolk, C. P. (1988). Conjugal transfer of DNA to cyanobacteria. *Methods Enzymol.* 167, 747–754.

Endy, D. (2005). Foundations for engineering biology. *Nature* 438, 449–453.

Energy Information Administration. (2010). Annual Energy Review 2009, U.S.

Espie, G. S., and Kimber, M. S. (2011). Carboxysomes: cyanobacterial RubisCO comes in small packages. *Photosynth. Res.* 109, 7–20.

Fan, C., Cheng, S., Liu, Y., Escobar, C. M., Crowley, C. S., Jefferson, R. E., Yeates, T. O., and Bobik, T. A. (2010). Short N-terminal sequences package proteins into bacterial microcompartments. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7509–7514.

Fay, P. (1992). Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol. Rev.* 56, 340–373.

Feng, S., Xue, L., Liu, H., and Lu, P. (2009). Improvement of efficiency of genetic transformation for *Dunaliella salina* by glass beads method. *Mol. Biol. Rep.* 36, 1433–1439.

Fernando, G., Adhikari, S., Kota, K., and Bandi, R. (2007). Glycerol based automotive fuels from future biorefineries. *Fuel* 86, 2806–2809.

Fernández, E., Schnell, R., Ranum, L. P., Hussey, S. C., Silflow, C. D., and Lefebvre, P. A. (1989). Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* 86, 6449–6453.

Fukuda, H., Ogawa, T., Tazaki, M., Nagahama, K., Fujii, T., Tanase, S., and Morino, Y. (1992). Two reactions are simultaneously catalyzed by a single enzyme: the arginine-dependent simultaneous formation of two products, ethylene and succinate, from 2-oxoglutarate by an enzyme from Pseudomonas syringae. *Biochem. Biophys. Res. Commun.* 188, 483–489.

Fukuda, H., Takahashi, M., Fujii, T., Tazaki, M., and Ogawa, T. (1989). An NADH:Fe(III)EDTA oxidoreductase from *Cryptococcus albidus*: an enzyme involved in ethylene production *in vivo*? *FEMS Microbiol. Lett.* 51, 107–111.

Furukawa, Y., Moriuchi, T., and Morishima, K. (2006). Design principle and prototyping of a direct photosynthetic/metabolic biofuel cell (DPMFC). *J. Micromech. Microeng.* 16, S220–S225.

Geerts, D., Bovy, A., de Vrieze, G., Borrias, M., and Weisbeek, P. (1995). Inducible expression of heterologous genes targeted to a chromosomal platform in the cyanobacterium *Synechococcus* sp. PCC 7942. *Microbiology* 141, 831–841.

Georg, J., Voss, B., Scholz, I., Mitschke, J., Wilde, A., and Hess, W. R. (2009). Evidence for a major role of antisense RNAs in cyanobacterial gene regulation. *Mol. Syst. Biol.* 5, 305.

Ghirardi, M. L, Dubini, A., Yu, J., and Maness, P. C. (2009). Photobiological hydrogen-producing systems. *Chem. Soc. Rev.* 38, 52–61.

Ghirardi, M. L, Posewitz, M. C., Maness, P. C., Dubini, A., Yu, J., and Seibert, M. (2007). Hydrogenases and hydrogen photoproduction in oxygenic photosynthetic organisms. *Annu. Rev. Plant Biol.* 58, 71–91.

Golden, S. S., Brusslan, J., and Haselkorn, R. (1987). Genetic engineering of the cyanobacterial chromosome. *Methods Enzymol.* 153, 215–231.

Golden, S. S., and Sherman, L. A. (1983). A hybrid plasmid is a stable cloning vector for the cyanobacterium *Anacystis nidulans* R2. *J. Bacteriol.* 155, 966–972.

Grigorieva, G., and Shestakov, S. (1982). Transformation in the cyanobacterium *Synechocystis* sp. 6803. *FEMS Microbiol. Lett.* 13, 367–370.

Hagemann, M. (2011). Molecular biology of cyanobacterial salt acclimation. *FEMS Microbiol. Rev.* 35, 87–123.

Hankamer, B., Lehr, F., Rupprecht, J., Mussgnug, J. H., Posten, C., and Kruse, O. (2007). Photosynthetic biomass and H-2 production by green algae: from bioengineering to bioreactor scale-up. *Physiol. Plant.* 131, 10–21.

Harun, R., Danquah, M. K., and Forde, G. M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* 85, 199–203.

Heidorn, T., Camsund, D., Huang, H. H., Lindberg, P., Oliveira, P., Stensjö, K., and Lindblad, P. (2011). Synthetic biology in cyanobacteria: engineering and analyzing novel functions. *Methods Enzymol.* 497, 539–579.

Hein, S., Tran, H., and Steinbüchel, A. (1998). *Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. *Arch. Microbiol.* 170, 162–170.

Heinhorst, S., and Cannon, G. (2010). Addressing microbial organelles: a short peptide directs enzymes to the interior. *Proc. Natl. Acad. Sci. U.S.A.* 107, 7627–7628.

Heitzer, M., Eckert, A., Fuhrmann, M., and Griesbeck, C. (2007). Influence of codon bias on the expression of foreign genes in microalgae. *Adv. Exp. Med. Biol.* 616, 46–53.

Hemschemeier, A., Melis, A., and Happe, T. (2009). Analytical approaches to photobiological hydrogen production in unicellular green algae. *Photosynth. Res.* 102, 523–540.

Hernández, J. A., Muro-Pastor, A. M., Flores, E., Bes, M. T., Peleato, M. L., and Fillat, M. F. (2006). Identification of a furA cis antisense RNA in the cyanobacterium *Anabaena* sp. PCC 7120. *J. Mol. Biol.* 355, 325–334.

Hirayama, S., Ueda, R., Ogushi, Y., Hirano, A., Samejima, Y., Hon-Nami, K., and Kunito, S. (1998). Ethanol production from carbon dioxide by fermentative microalgae. *Stud. Surf. Sci. Catal.* 114, 657–660.

Huang, H. H, Camsund, D., Lindblad, P., and Heidorn, T. (2010). Design and characterization of molecular tools for a synthetic biology approach towards developing cyanobacterial biotechnology. *Nucleic Acids Res.* 38, 2577–2593.

Ihara, M., Nishihara, H., Yoon, K. S., Lenz, O., Friedrich, B., Nakamoto, H., Kojima, K., Honma, D., Kamachi, T., and Okura, I. (2006). Light-driven hydrogen production by a hybrid complex of a [NiFe]-hydrogenase and the cyanobacterial photosystem I. *Photochem. Photobiol.* 82, 676–682.

Imashimizu, M., Fujiwara, S., Tanigawa, R., Tanaka, K., Hirokawa, T., Nakajima, Y., Higo, J., and Tsuzuki, M. (2003). Thymine at -5 is crucial for cpc promoter activity of *Synechocystis* sp. strain PCC 6714. *J. Bacteriol.* 185, 6477–6480.

Inui, M., Suda, M., Kimura, S., Yasuda, K., Suzuki, H., Toda, H., Yamamoto, S., Okino, S., Suzuki, N., and Yukawa, H. (2008). Expression of *Clostridium acetobutylicum* butanol synthetic genes in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 77, 1305–1316.

Isaacs, F. J., Dwyer, D. J., Ding, C., Pervouchine, D. D., Cantor, C. R., and Collins, J. J. (2004). Engineered riboregulators enable post-transcriptional control of gene expression. *Nat. Biotechnol.* 22, 841–847.

Iwaki, T., Haranoh, K., Inoue, N., Kojima, K., Satoh, R., Nishino, T., Wada, S., Ihara, H., Tsuyama, S., Kobayashi, H., and Wadano, A. (2006). Expression of foreign type I ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) stimulates photosynthesis in cyanobacterium *Synechococcus* PCC7942 cells. *Photosynth. Res.* 88, 287–297.

Kana, T. M. (1993). Rapid oxygen cycling in *Trichodesmium thiebautii*. *Limnol. Oceanogr.* 38, 18–24.

Kane, J. F. (1995). Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Curr. Opin. Biotechnol.* 6, 494–500.

Kaneko, T., Sato, S., Kotani, H., Tanaka, A., Asamizu, E., Nakamura, Y., Miyajima, N., Hirosawa, M., Sugiura, M., Sasamoto, S., Kimura, T., Hosouchi, T., Matsuno, A., Muraki, A., Nakazaki, N., Naruo, K., Okumura, S., Shimpo, S., Takeuchi, C., Wada, T., Watanabe, A., Yamada, M., Yasuda, M., and Tabata, S. (1996). Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. *DNA Res.* 3, 109–136.

Kathiresan, S., Chandrashekar, A., Ravishankar, A., and Sarada, R. (2009). Agrobacterium-mediated transformation in the green alga *Haematococcus pluvialis* (Chlorophyceae, Volvocales). *J. Phycol.* 45, 642–649.

Keasling, J. (2008). Synthetic biology for synthetic chemistry. *ACS Chem. Biol.* 3, 64–76.

Kende, H. (1993). Ethylene biosynthesis. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 44, 283–307.

Kilian, O., Benemann, C. S., Niyogi, K. K., and Vick, B. (2011). High-efficiency homologous recombination in the oil-producing alga *Nannochloropsis* sp. *Proc. Natl. Acad. Sci. U.S.A.* 108, 21265–21269.

Kindle, K. L. (1990). High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. *Proc. Natl. Acad. Sci. U.S.A.* 87, 1228–1232.

Knoop, H., Zilliges, Y., Lockau, W., and Steuer, R. (2010). The metabolic network of *Synechocystis* sp. PCC 6803: systemic properties of autotrophic growth. *Plant Physiol.* 154, 410–422.

Koksharova, O., and Wolk, C. (2002). Genetic tools for cyanobacteria. *Appl. Microbiol. Biotechnol.* 58, 123–137.

Kosugi, Y., Shibuya, K., Tsuruno, N., Iwazaki, Y., Mochizuki, A., Yoshioka, T., Hashiba, T., and Satoh, S. (2000). Expression of genes responsible for ethylene production and wilting are differently regulated in carnation (*Dianthus caryophyllus* L.) petals. *Plant Sci.* 158, 139–145.

Kruse, O., Rupprecht, J., Bader, K. P., Thomas-Hall, S., Schenk, P. M., Finazzi, G., and Hankamer, B. (2005). Improved photobiological H2 production in engineered green algal cells. *J. Biol. Chem.* 280, 34170–34177.

Kuhlemeier, C. J., Thomas, A. A., van der Ende, A., van Leen, R. W., Borrias, W. E., van den Hondel, C. A., and van Arkel, G. A. (1983). A host-vector system for gene cloning in the cyanobacterium *Anacystis nidulans* R2. *Plasmid* 10, 156–163.

Kuhlemeier, C. J., and van Arkel, G. A. (1987). Host-vector systems for gene cloning in cyanobacteria. *Methods Enzymol.* 153, 199–215.

Kumar, S. V., Misquitta, R. W., Reddy, V. S., Rao, B. J., and Rajam, M. V. (2004). Genetic transformation of the green alga – *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. *Plant Sci.* 166, 731–738.

Lambert, G., and Carr, N. (1983). A restriction map of plasmid pDC1 from the filamentous cyanobacterium *Nostoc* sp. MAC PCC 8009. *Plasmid* 10, 196–198.

Lamsen, E. N., and Atsumi, S. (2012). Recent progress in synthetic biology for microbial production of C3-C10

alcohols. *Front. Microbiol.* 3:196. doi: 10.3389/fmicb.2012.00196

Lan, E. I., and Liao, J. C. (2011). Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng.* 13, 353–363.

Lan, E. I., and Liao, J. C. (2012). ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6018–6023.

Lang, J. D., and Haselkorn, R. (1991). A vector for analysis of promoters in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* 173, 2729–2731.

Langridge, P., Brown, J. W. S., Pintortoro, J. A., Feix, G., Neuhaus, G., Neuhausurl, G., and Schweiger, H. G. (1986). Expression of zein genes in *Acetabularia mediterranea. Eur. J. Cell Biol.* 39, 257–264.

Lee, H. S., Vermaas, W. F., and Rittmann, B. E. (2010). Biological hydrogen production: prospects and challenges. *Trends Biotechnol.* 28, 262–271.

Lee S. K., Chou H., Ham T. S., Lee T. S., and Keasling J. D. (2008). Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Curr. Opin. Biotechnol.* 19, 556–563.

Lee, S. Y., and Lee, Y. (2003). Metabolic engineering of *Escherichia coli* for production of enantiomerically pure (R)-(−)-hydroxycarboxylic acids. *Appl. Environ. Microbiol.* 69, 3421–3426.

León, R., and Fernández, E. (2007). Nuclear transformation of eukaryotic microalgae: historical overview, achievements and problems. *Adv. Exp. Med. Biol.* 616, 1–11.

León-Bañares, R., González-Ballester, D., Galván, A., and Fernández, E. (2004). Transgenic microalgae as green cell-factories. *Trends Biotechnol.* 22, 45–52.

Lerche, K., and Hallmann, A. (2009). Stable nuclear transformation of *Gonium pectorale. BMC Biotechnol.* 9, 64. doi: 10.1186/1472-6750-9-64

Li, J., Lu, Y., Xue, L., and Xie, H. (2010). A structurally novel salt-regulated promoter of duplicated carbonic anhydrase gene 1 from *Dunaliella salina. Mol. Biol. Rep.* 37, 1143–1154.

Li, R., and Golden, S. S. (1993). Enhancer activity of light-responsive regulatory elements in the untranslated leader regions of cyanobacterial psbA genes. *Proc. Natl. Acad. Sci. U.S.A.* 90, 11678–11682.

Lindberg, P., Park, S., and Melis, A. (2010). Engineering a platform for photosynthetic isoprene production in cyanobacteria, using *Synechocystis* as the model organism. *Metab. Eng.* 12, 70–79.

Liu, Q., Ouyang, S., Chung, A., Wu, Q., and Chen, G. (2007). Microbial production of R-3-hydroxybutyric acid by recombinant *E. coli* harboring genes of phbA, phbB, and tesB. *Appl. Microbiol. Biotechnol.* 76, 811–818.

Liu, X., and Curtiss, R. III. (2009). Nickel-inducible lysis system in *Synechocystis* sp. PCC 6803. *Proc. Natl. Acad. Sci. U.S.A.* 106, 21550–21554.

Liu, X., Fallon, S., Sheng, J., and Curtiss, R. III. (2011a). CO(2)-limitation-inducible Green Recovery of fatty acids from cyanobacterial biomass. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6905–6908.

Liu, X., Sheng, J., and Curtiss, R. III. (2011b). Fatty acid production in genetically modified cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 108, 6899–6904.

Lu, X. (2010). A perspective: Photosynthetic production of fatty acid-based biofuels in genetically engineered cyanobacteria. *Biotechnol. Adv.* 28, 742–746.

Lu, X., Vora, H., and Khosla, C. (2008). Overproduction of free fatty acids in *E. coli*: implications for biodiesel production. *Metab. Eng.* 10, 333–339.

Lu, Y., Li, J., Xue, L., Yan, H., Yuan, H., and Wang, C. (2011). A duplicated carbonic anhydrase 1 (DCA1) promoter mediates the nitrate reductase gene switch of *Dunaliella salina. J. Appl. Phycol.* 23, 673–680.

Lubner, C. E., Applegate, A. M., Knörzer, P., Ganago, A., Bryant, D. A., Happe, T., and Golbeck, J. H. (2011). Solar hydrogen-producing bionanodevice outperforms natural photosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20988–20991.

Luinenburg, I., and Coleman, J. R. (1993). Expression of *Escherichia coli* phosphoenolpyruvate carboxylase in a cyanobacterium. Functional complementation of *Synechococcus* PCC 7942 ppc. *Plant Physiol.* 101, 121–126.

Ma, J., Campbell, A., and Karlin, S. (2002). Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. *J. Bacteriol.* 184, 5733–5745.

Ma, S. M., Li, J. W., Choi, J. W., Zhou, H., Lee, K. K., Moorthie, V. A., Xie, X., Kealey, J. T., Da Silva, N. A., Vederas, J. C., and Tang, Y. (2009). Complete reconstitution of a highly reducing iterative polyketide synthase. *Science* 326, 589–592.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21, 796–802.

Mascarelli, A. L. (2009). Gold rush for algae. *Nature* 461, 460–461.

McNeely, K., Xu, Y., Bennette, N., Bryant, D. A., and Dismukes, G. C. (2010). Redirecting reductant flux into hydrogen production via metabolic engineering of fermentative carbon metabolism in a cyanobacterium. *Appl. Environ. Microbiol.* 76, 5032–5038.

Meeks, J. C. (2011). Closing the cycle. *Science* 334, 1508–1509.

Mehler, A. H. (1951). Studies on reactions of illuminated chloroplasts: I. Mechanism of the reduction of oxygen and other hill reagents. *Arch. Biochem. Biophys.* 33, 65–77.

Melis, A. (2009). Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. *Plant Sci.* 177, 272–280.

Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L., and Seibert, M. (2000). Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii. Plant Physiol.* 122, 127–135.

Mendez-Perez, D., Begemann, M. B., and Pfleger, B. F. (2011). Modular synthase-encoding gene involved in α-olefin biosynthesis in *Synechococcus* sp. strain PCC 7002. *Appl. Environ. Microbiol.* 77, 4264–4267.

Mermet-Bouvier, P., Cassier-Chauvat, C., Marraccini, P., and Chauvat, F. (1993). Transfer and replication of RSF1010-derived plasmids in several cyanobacteria of the general *Synechocystis* and *Synechococcus. Curr. Microbiol.* 27, 323–327.

Mermet-Bouvier, P., and Chauvat, F. (1994). A conditional expression vector for the cyanobacteria *Synechocystis* sp. strains PCC6803 and PCC6714 or *Synechococcus* sp. strains PCC7942 and PCC6301. *Curr. Microbiol.* 28, 145–148.

Meyer, R. (2009). Replication and conjugative mobilization of broad host-range IncQ plasmids. *Plasmid* 62, 57–70.

Miao, X., Wu, Q., Wu, G., and Zhao, N. (2003). Sucrose accumulation in salt-stressed cells of agp gene deletion-mutant in cyanobacterium *Synechocystis* sp. PCC 6803. *FEMS Microbiol. Lett.* 218, 71–77.

Milligan, A. J., Berman-Frank, I., Gerchman, Y., Dismukes, G. C., and Falkowski, P. G. (2007). Light-dependent oxygen consumption in nitrogen-fixing cyanobacteria plays a key role in nitrogenase protection. *J. Phycol.* 43, 845–852.

Mitschke, J., Georg, J., Scholz, I., Sharma, C. M., Dienst, D., Bantscheff, J., Voss, B., Steglich, C., Wilde, A., Vogel, J., and Hess, W. R. (2011). An experimentally anchored map of transcriptional start sites in the model cyanobacterium *Synechocystis* sp. PCC6803. *Proc. Natl. Acad. Sci. U.S.A.* 108, 2124–2129.

Miyagawa-Yamaguchi, A., Okami, T., Kira, N., Yamaguchi, H., Ohnishi, K., and Adachi, M. (2011). Stable nuclear transformation of the diatom *Chaetoceros* sp. *Phycol. Res.* 59, 113–119.

Mouille, G., Maddelein, M. L., Libessart, N., Talaga, P., Decq, A., Delrue, B., and Ball, S. (1996). Preamylopectin processing: a mandatory step for starch biosynthesis in plants. *Plant Cell* 8, 1353–1366.

Mukherji, S., and van Oudenaarden, A. (2009). Synthetic biology: understanding biological design from synthetic circuits. *Nat. Rev. Genet.* 10, 859–871.

Mulkidjanian, A. Y., Koonin, E. V., Makarova, K. S., Mekhedov, S. L., Sorokin, A., Wolf, Y. I., Dufresne, A., Partensky, F., Burd, H., Kaznadzey, D., Haselkorn, R., and Galperin, M. Y. (2006). The cyanobacterial genome core and the origin of photosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 103, 13126–13131.

Muramatsu, M., and Hihara, Y. (2006). Characterization of high-light-responsive promoters of the psaAB genes in *Synechocystis* sp. PCC 6803. *Plant Cell Physiol.* 47, 878–890.

Muramatsu, M., and Hihara, Y. (2007). Coordinated high-light response of genes encoding subunits of photosystem I is achieved by AT-rich upstream sequences in the cyanobacterium *Synechocystis* sp. strain PCC 6803. *J. Bacteriol.* 189, 2750–2758.

Mussgnug, J. H., Thomas-Hall, S., Rupprecht, J., Foo, A., Klassen, V., McDowall, A., Schenk, P. M., Kruse, O., and Hankamer, B. (2007). Engineering photosynthetic light capture: impacts on improved solar energy to biomass conversion. *Plant Biotechnol. J.* 5, 802–814.

Muto, M., Henry, R. E., and Mayfield, S. P. (2009). Accumulation and processing of a recombinant protein designed as a cleavable fusion to the endogenous Rubisco LSU protein in *Chlamydomonas* chloroplast. *BMC Biotechnol.* 9, 26. doi: 10.1186/1472-6750-9-26

Nakamura, Y., Gojobori, T., and Ikemura, T. (2000). Codon usage tabulated from international DNA

sequence databases: status for the year 2000. *Nucleic Acids Res.* 28, 292.

Neuhaus, G., Neuhaus-Url, G., de Groot, E. J., and Schweiger, H. G. (1986). High yield and stable transformation of the unicellular green alga *Acetabularia* by microinjection of SV40 DNA and pSV2neo. *EMBO J.* 5, 1437–1444.

Ng, W. O., Zentella, R., Wang, Y., Taylor, J. S., and Pakrasi, H. B. (2000). PhrA, the major photoreactivating factor in the cyanobacterium *Synechocystis* sp. strain PCC 6803 codes for a cyclobutane-pyrimidine-dimer-specific DNA photolyase. *Arch. Microbiol.* 173, 412–417.

Nguyen, M. T., Choi, S. P., Lee, J., Lee, J. H., and Sim, S. J. (2009). Hydrothermal acid pretreatment of *Chlamydomonas reinhardtii* biomass for ethanol production. *J. Microbiol. Biotechnol.* 19, 161–166.

Niederholtmeyer, H., Wolfstädter, B. T., Savage, D. F., Silver, P. A., and Way, J. C. (2010). Engineering cyanobacteria to synthesize and export hydrophilic products. *Appl. Environ. Microbiol.* 76, 3462–3466.

Nogales, J., Gudmundsson, S., Knight, E. M., Palsson, B. O., and Thiele, I. (2012). Detailing the optimality of photosynthesis in cyanobacteria through systems biology analysis. *Proc. Natl. Acad. Sci. U.S.A.* 109, 2678–2683.

O'Neill, B. M., Mikkelson, K. L., Gutierrez, N. M., Cunningham, J. L., Wolff, K. L., Szyjka, S. J., Yohn, C. B., Redding, K. E., and Mendez, M. J. (2012). An exogenous chloroplast genome for complex sequence manipulation in algae. *Nucleic Acids Res.* 40, 2782–2792.

Ort, D. R., and Melis, A. (2011). Optimizing antenna size to maximize photosynthetic efficiency. *Plant Physiol.* 155, 79–85.

Pfleger, B. F., Pitera, D. J., Smolke, C. D., and Keasling, J. D. (2006). Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat. Biotechnol.* 24, 1027–1032.

Pisciotta, J. M., Zou, Y., and Baskakov, I. V. (2010). Light-dependent electrogenic activity of cyanobacteria. *PLoS ONE* 5, e10821. doi: 10.1371/journal.pone.0010821

Posewitz, M. C., King, P. W., Smolinski, S. L., Smith, R. D., Ginley, A. R., Ghirardi, M. L., and Seibert, M. (2005). Identification of genes required for hydrogenase activity in *Chlamydomonas reinhardtii*. *Biochem. Soc. Trans.* 33, 102–104.

Potvin, G., and Zhang, Z. (2010). Strategies for high-level recombinant protein expression in transgenic microalgae: a review. *Biotechnol. Adv.* 28, 910–918.

Prather, K. L., and Martin, C. H. (2008). *De novo* biosynthetic pathways: rational design of microbial chemical factories. *Curr. Opin. Biotechnol.* 19, 468–474.

Price, G. D., Badger, M. R., and von Caemmerer, S. (2011). The prospect of using cyanobacterial bicarbonate transporters to improve leaf photosynthesis in C3 crop plants. *Plant Physiol.* 155, 20–26.

Qin, S., Lin, H., and Jiang, P. (2012). Advances in genetic engineering of marine algae. *Biotechnol. Adv.* doi: 10.1016/j.biotechadv.2012.05.004. [Epub ahead of print].

Radakovits, R., Jinkerson, R. E., Darzins, A., and Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell* 9, 486–501.

Ramazanov, A., and Ramazanov, Z. (2006). Isolation and characterization of a starchless mutant of *Chlorella pyrenoidosa* STL-PI with a high growth rate, and high protein and polyunsaturated fatty acid content. *Phycol. Res.* 54, 255–259.

Reaston, J., van den Hondel, C. A., van Arkel, G. A., and Stewart, W. D. (1982). A physical map of plasmid pDU1 from the cyanobacterium *Nostoc* PCC 7524. *Plasmid* 7, 101–104.

Reppas, N. B., and Ridley, C. P. (2010). *Methods and compositions for the recombinant biosynthesis of n-alkanes.* Joule Unlimited, Inc., US Patent No. 7794969.

Rittmann, B. E. (2008). Opportunities for renewable bioenergy using microorganisms. *Biotechnol. Bioeng.* 100, 203–212.

Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., Ho, K. A., Eachus, R. A., Ham, T. S., Kirby, J., Chang, M. C., Withers, S. T., Shiba, Y., Sarpong, R., and Keasling, J. D. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943.

Ruffing, A. M. (2011). Engineering cyanobacteria: teaching and old bug new tricks. *Bioeng. Bugs* 2, 136–149.

Saggu, M., Zebger, I., Ludwig, M., Lenz, O., Friedrich, B., Hildebrandt, P., and Lendzian, F. (2009). Spectroscopic insights into the oxygen-tolerant membrane-associated [NiFe] hydrogenase of *Ralstonia eutropha* H16. *J. Biol. Chem.* 284, 16264–16276.

Sakai, M., Ogawa, T., Matsuoka, M., and Fukuda, H. (1997). Photosynthetic conversion of carbon dioxide to ethylene by the recombinant cyanobacterium, *Synechococcus* sp. PCC 7942, which harbors a gene for the ethylene-forming enzyme of *Pseudomonas syringae*. *J. Ferment. Bioeng.* 84, 434–443.

Salis, H. M., Mirsky, E. A., and Voigt, C. A. (2009). Automated design of synthetic ribosome binding sites to control protein expression. *Nat. Biotechnol.* 27, 946–950.

Savage, D. F., Afonso, B., Chen, A. H., and Silver, P. A. (2010). Spatially ordered dynamics of the bacterial carbon fixation machinery. *Science* 327, 1258–1261.

Schroda, M., Blöcker, D., and Beck, C. F. (2000). The HSP70A promoter as a tool for the improved expression of transgenes in *Chlamydomonas*. *Plant J.* 21, 121–131.

Schwarze, A., Kopczak, M. J., Rögner, M., and Lenz, O. (2010). Requirements for construction of a functional hybrid complex of photosystem I and [NiFe]-hydrogenase. *Appl. Environ. Microbiol.* 76, 2641–2651.

Sharkey, T. D., Wiberley, A. E., and Donohue, A. R. (2008). Isoprene emission from plants: why and how. *Ann. Bot.* 101, 5–18.

Shastri, A. A., and Morgan, J. A. (2005). Flux balance analysis of photoautotrophic metabolism. *Biotechnol. Prog.* 21, 1617–1626.

Sheng, J., Vannela, R., and Rittmann, B. E. (2011). Evaluation of methods to extract and quantify lipids from *Synechocystis* PCC 6803. *Bioresour. Technol.* 102, 1697–1703.

Shestakov, S. V., and Khyen, N. T. (1970). Evidence for genetic transformation in blue-green alga *Anacystis nidulans*. *Mol. Gen. Genet.* 107, 372–375.

Srirangan, K., Pyne, M. E., and Perry Chou, C. (2011). Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. *Bioresour. Technol.* 102, 8589–8604.

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., McClure, A., Del Cardayre, S. B., and Keasling, J. D. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science* 315, 801–804.

Stevens, D. R., Rochaix, J. D., and Purton, S. (1996). The bacterial phleomycin resistance gene ble as a dominant selectable marker in *Chlamydomonas*. *Mol. Gen. Genet.* 251, 23–30.

Summers, M. L., Wallis, J. G., Campbell, E. L., and Meeks, J. C. (1995). Genetic evidence of a major role for glucose-6-phosphate dehydrogenase in nitrogen fixation and dark growth of the cyanobacterium *Nostoc* sp. strain ATCC 29133. *J. Bacteriol.* 177, 6184–6194.

Takahama, K., Matsuoka, M., Nagahama, K., and Ogawa, T. (2003). Construction and analysis of a recombinant cyanobacterium expressing a chromosomally inserted gene for an ethylene-forming enzyme at the psbAI locus. *J. Biosci. Bioeng.* 95, 302–305.

Takahashi, H., Miyake, M., Tokiwa, Y., and Asada, Y. (1998). Improved accumulation of poly-3-hydroxybutyrate by a recombinant cyanobacterium. *Biotechnol. Lett.* 20, 183–186.

Takahashi, M., Uji, T., Saga, N., and Mikami, K. (2010). Isolation and regeneration of transiently transformed protoplasts from gametophytic blades of the marine red alga *Porphyra yezoensis*. *Electron. J. Biotechnol.* 13, 2.

Takeshima, Y., Sugiura, M., and Hagiwara, H. (1994). A novel expression vector for the cyanobacterium, *Synechococcus* PCC 6301. *DNA Res.* 1, 181–189.

Tan, X., Yao, L., Gao, Q., Wang, W., Qi, F., and Lu, X. (2011). Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. *Metab. Eng.* 13, 169–176.

Taroncher-Oldenburg, G., Nishina, K., and Stephanopoulos, G. (2000). Identification and analysis of the polyhydroxyalkanoate-specific beta-ketothiolase and acetoacetyl coenzyme A reductase genes in the cyanobacterium *Synechocystis* sp. strain PCC6803. *Appl. Environ. Microbiol.* 66, 4440–4448.

Tcherkez, G. G., Farquhar, G. D., and Andrews, T. J. (2006). Despite slow catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly perfectly optimized. *Proc. Natl. Acad. Sci. U.S.A.* 103, 7246–7251.

Thiel, T., and Wolk, C. P. (1987). Conjugal transfer of plasmids to cyanobacteria. *Methods Enzymol.* 153, 232–243.

Tseng, H., Martin, C., Nielsen, D., and Prather, K. (2009). Metabolic engineering of *Escherichia coli* for enhanced production of (R)- and (S)-3-hydroxybutyrate. *Appl. Environ. Microb.* 75, 3137–3145.

Tyo, K. E., Jin, Y. S., Espinoza, F. A., and Stephanopoulos, G. (2009). Identification of gene disruptions for increased poly-3-hydroxybutyrate accumulation in *Synechocystis* PCC 6803. *Biotechnol. Prog.* 25, 1236–1243.

van de Meene, A. M., Hohmann-Marriott, M. F., Vermaas, W. F., and Roberson, R. W. (2006). The three-dimensional structure of the cyanobacterium *Synechocystis* sp. PCC 6803. *Arch. Microbiol.* 184, 259–270.

Wang, J., Jiang, P., Cui, Y., Guan, X., and Qin, S. (2010). Gene transfer into conchospores of *Porphyra haitanensis* (Bangiales, Rhodophyta) by glass bead agitation. *Phycologia* 49, 355–360.

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., and Goodenough, U. (2009). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 8, 1856–1868.

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N., Kim, P. B., Cooper, S. R., Raisner, R. M., Herman, A., Sivitz, A. B., Lakshmanaswamy, A., Kashiyama, Y., Baker, D., and Yoshikuni, Y. (2012). An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335, 308–313.

Wee, Y. J., Kim, J. N., and Ryu, H. W. (2006). Biotechnological production of lactic acid and its recent applications. *Food Technol. Biotech.* 44, 163–172.

Wolk, C. P., Elhai, J., Kuritz, T., and Holland, D. (1993). Amplified expression of a transcriptional pattern formed during development of *Anabaena*. *Mol. Microbiol.* 7, 441–445.

Wolk, C. P., Vonshak, A., Kehoe, P., and Elhai, J. (1984). Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 81, 1561–1565.

Work, V. H., D'Adamo, S., Radakovits, R., Jinkerson, R. E., and Posewitz, M. C. (2012). Improving photosynthesis and metabolic networks for the competitive production of phototroph-derived biofuels. *Curr. Opin. Biotechnol.* 23, 290–297.

Wu, G. F., Wu, Q. Y., and Shen, Z. Y. (2001). Accumulation of poly-beta-hydroxybutyrate in cyanobacterium *Synechocystis* sp. PCC6803. *Bioresour. Technol.* 76, 85–90.

Yang, S. F., and Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Ann. Rev. Plant Physiol.* 35, 155–189.

Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., Khandurina, J., Trawick, J. D., Osterhout, R. E., Stephen, R., Estadilla, J., Teisan, S., Schreyer, H. B., Andrae, S., Yang, T. H., Lee, S. Y., Burk, M. J., and Van Dien, S. (2011). Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol.* 7, 445–452.

Yoon, K. S., Fukuda, K., Fujisawa, K., and Nishihara, H. (2011). Purification and characterization of a highly thermostable, oxygen-resistant, respiratory [NiFe]-hydrogenase from a marine, aerobic hydrogen-oxidizing bacterium *Hydrogenovibrio marinus*. *Int. J. Hydrogen Energ.* 36, 7081–7088.

Yoshikawa, K., Kojima, Y., Nakajima, T., Furusawa, C., Hirasawa, T., and Shimizu, H. (2011). Reconstruction and verification of a genome-scale metabolic model for *Synechocystis* sp. PCC6803. *Appl. Microbiol. Biotechnol.* 92, 347–358.

Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G., and Court, D. L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 97, 5978–5983.

Yu, T., Li, J., Yang, Y., Qi, L., Chen, B., Zhao, F., Bao, Q., and Wu, J. (2012). Codon usage patterns and adaptive evolution of marine unicellular cyanobacteria *Synechococcus* and *Prochlorococcus*. *Mol. Phylogenet Evol.* 62, 206–213.

Yurieva, T. M., Plyasova, L. M., Makarova, O. V., and Krieger, T. A. (1996). Mechanisms for hydrogenation of acetone to isopropanol and of carbon oxides to methanol over copper-containing oxide catalysts. *J. Mol. Catal. A Chem.* 113, 455–468.

Zang, X., Liu, B., Liu, S., Arunakumara, K. K., and Zhang, X. (2007). Optimum conditions for transformation of *Synechocystis* sp. PCC 6803. *J. Microbiol.* 45, 241–245.

Zhang, S., and Bryant, D. A. (2011). The tricarboxylic acid cycle in cyanobacteria. *Science* 334, 1551–1553.

Zhang, F., Carothers, J. M., and Keasling, J. D. (2012). Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* 30, 354–359.

Zhang, K., Sawaya, M. R., Eisenberg, D. S., and Liao, J. C. (2008). Expanding metabolism for biosynthesis of non-natural alcohols. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20653–20658.

Zhou, J., Zhang, H., Zhang, Y., Li, Y., and Ma, Y. (2012). Designing and creating a modularized synthetic pathway in cyanobacterium *Synechocystis* enables production of acetone from carbon dioxide. *Metab. Eng.* 14, 394–400.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 01 July 2012; accepted: 05 September 2012; published online: 19 September 2012.

*Citation: Wang B, Wang J, Zhang W and Meldrum DR (2012) Application of synthetic biology in cyanobacteria and algae. Front. Microbio. **3**:344. doi: 10.3389/fmicb.2012.00344*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 26 October 2012
doi: 10.3389/fmicb.2012.00360

Synthetic feedback loop model for increasing microbial biofuel production using a biosensor

Mary E. Harrison and Mary J. Dunlop*

School of Engineering, College of Engineering and Mathematical Sciences, University of Vermont, VT, USA

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Jiangxin Wang, Arizona State University, USA
Gabor Balazsi, The University of Texas MD Anderson Cancer Center, USA

****Correspondence:***
Mary J. Dunlop, School of Engineering, College of Engineering and Mathematical Sciences, University of Vermont, 33 Colchester Avenue, 301 Votey Hall, Burlington, VT 05405, USA.
e-mail: mjdunlop@uvm.edu

Current biofuel production methods use engineered bacteria to break down cellulose and convert it to biofuel. A major challenge in microbial fuel production is that increasing biofuel yields can be limited by the toxicity of the biofuel to the organism that is producing it. Previous research has demonstrated that efflux pumps are effective at increasing tolerance to various biofuels. However, when overexpressed, efflux pumps burden cells, which hinders growth and slows biofuel production. Therefore, the toxicity of the biofuel must be balanced with the toxicity of pump overexpression. We have developed a mathematical model for cell growth and biofuel production that implements a synthetic feedback loop using a biosensor to control efflux pump expression. In this way, the production rate will be maximal when the concentration of biofuel is low because the cell does not expend energy expressing efflux pumps when they are not needed. Additionally, the microbe is able to adapt to toxic conditions by triggering the expression of efflux pumps, which allow it to continue biofuel production. Sensitivity analysis indicates that the feedback sensor model is insensitive to many system parameters, but a few key parameters can influence growth and production. In comparison to systems that express efflux pumps at a constant level, the feedback sensor increases overall biofuel production by delaying pump expression until it is needed. This result is more pronounced when model parameters are variable because the system can use feedback to adjust to the actual rate of biofuel production.

Keywords: biosensor, biofuel, feedback, synthetic biology, MexR

INTRODUCTION

Microbial biofuel production strategies use microorganisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Zymomonas mobilis*, and *Clostridium acetobutylicum* to break down cellulosic biomass and convert it into biofuel through fermentation or similar processes (Fischer et al., 2008). Recent developments allow for the optimization of this process through manipulation of the genetic makeup of these microorganisms. Biofuel production is maximized by focusing the microbe's metabolic processes on the pathways involved in production and eliminating non-essential competing pathways (Stephanopoulos, 2007).

Although previous research has focused on ethanol, next-generation biofuels have gained attention due to their compatibility with existing fuels infrastructure, increased energy density, and low corrosiveness (Fischer et al., 2008; Lee et al., 2008; Shi et al., 2011). However, a major barrier to successful and cost competitive production of these advanced biofuels is the development of an engineered microbe that is able to produce biofuel at high yields. One of the obstacles facing this objective is that many next-generation biofuels are toxic to microbes. Therefore, the concentration of biofuel achieved is directly limited by the susceptibility of the microbes to the produced biofuel (Stephanopoulos, 2007; Dunlop, 2011).

Biofuels may accumulate in the cell membrane, which interferes with multiple vital functions and can ultimately lead to cell death. The presence of biofuel in the membrane increases permeability, which disrupts electrochemical gradients established across the membrane in addition to releasing vital components from the cell. Additionally, biofuels may directly damage biological molecules and trigger an acute stress response (Sikkema et al., 1995; Nicolaou et al., 2010; Dunlop, 2011). However, some microorganisms possess mechanisms that enable them to tolerate higher concentrations of biofuels. These mechanisms include using efflux pumps or membrane vesicles to remove harmful compounds, decreasing membrane permeability, increasing membrane rigidity, and metabolizing the toxic compound. Although many of these mechanisms may be useful in improving microbial tolerance to biofuel, we focus here on efflux pumps because they are known to be present in microbes exhibiting tolerance to hydrocarbons and other compounds structurally similar to biofuels (Ramos et al., 2002).

Efflux pumps are membrane transporters that identify harmful compounds and export them from the cell using the proton motive force (Ramos et al., 2002). Efflux pumps are capable of identifying a diverse range of compounds and have proven effective at exporting biofuel (Dunlop et al., 2011). Although they can be helpful in improving tolerance, if overexpressed, efflux pumps can be detrimental. Efflux pumps may alter membrane composition and tax membrane integration machinery, which ultimately slows growth (Wagner et al., 2007). Consequently, when using efflux pumps as a means to increase tolerance to biofuel, the toxicity of pump expression must be managed in addition to biofuel toxicity.

We propose that using a synthetic feedback loop to control efflux pump expression would balance the toxicity of biofuel

production against the adverse effects of pump expression. This study builds on previous work comparing different control strategies for biofuel export (Dunlop et al., 2010). Here, we focus on a specific transcriptional biosensor mechanism for implementing regulatory control, quantifying the parametric sensitivity, and temporal dynamics of the model. Feedback is a common regulatory mechanism used by bacteria to adjust to changing conditions such as fluctuations in nutrient availability, environmental stressors, and signals from other cells in the population. This regulation is often moderated transcriptionally using proteins that bind to a promoter and alter gene expression (Grkovic et al., 2002; Smits et al., 2006; Alon, 2007).

Biosensors are often transcription factors whose activity is modified by changing conditions (Van Der Meer and Belkin, 2010). Biosensors are capable of responding to a wide range of conditions and compounds, including molecules common to fuels. More specifically, AlkS (Sticher et al., 1997; Canosa et al., 2000; Van Beilen et al., 2001), AlkR (Ratajczak et al., 1998), and TbtR (Jude et al., 2004) respond to alkanes; TtgV (Rojas et al., 2003; Teran et al., 2007), TtgR (Duque et al., 2001; Teran et al., 2003, 2007), TtgT (Teran et al., 2007), XylR (Willardson et al., 1998; Paitan et al., 2004), XylS (Koutinas et al., 2010), SepR (Phoenix et al., 2003), SrpS (Sun and Dennis, 2009; Volkers et al., 2010; Sun et al., 2011), TbmR (Leahy et al., 1997), and IbnR (Selifonova and Eaton, 1996) respond to aromatic substrates; and AcrR (Paulsen et al., 1996) and BmoR (Kurth et al., 2008) respond to alcohols. Additionally, TbuT (Stiner and Halverson, 2002) is sensitive to both alkenes and aromatics and MexR (Paulsen et al., 1996; Li et al., 1998; Evans et al., 2001; Van Hamme et al., 2003; Dunlop et al., 2011) may be linked to alkanes and aromatics based on the successful export of these through its associated efflux pump MexAB-OprM. These biosensors commonly control metabolic pathways or tolerance mechanisms that help the microbe survive in harsh environments. The sensor's activity, activating or repressing a pathway, is in turn controlled by environmental triggers, which alter the sensor's strength. For this model, we have concentrated on MexR, a transcriptional repressor, as a prototypical example of a biosensor.

Many identified sensors have been successfully incorporated into simple genetic circuits for use as whole-cell biosensors, which report the presence or absence of a compound of interest (Sorensen et al., 2006; Van Der Meer and Belkin, 2010). The feedback mechanism we suggest incorporates a biosensor that responds to biofuel by increasing transcription from an efflux pump operon. The ability of a fuel production host to tune pump expression based on the amount of intracellular biofuel present would balance biofuel and pump expression to optimize survival and yields.

An alternative strategy for regulating pump expression would be to use a constant controller (no feedback), such as an inducible promoter. In this way, pump expression could be manually calibrated to the expected biofuel production rate. Potential advantages of this approach include its simple design and the availability of well-characterized components. However, biological systems exhibit noise and variability (Kaern et al., 2005; Raser and O'shea, 2005). Even genetically identical cells can display significant differences in gene expression. A constant pump system is unable to respond to variations in the system, which would require frequent monitoring and adjustments to tune control to maintain optimal biofuel yield. Feedback, in contrast, can adapt with time and mitigate uncertainty caused by gene expression noise (Becskei and Serrano, 2000; Thattai and Van Oudenaarden, 2001; Nevozhay et al., 2009). Therefore a feedback controller, which is able to adapt to changing biofuel production conditions can offer advantages over constant pump expression.

Synthetic feedback mechanisms to control cellular behavior have been developed and implemented. They employ elements such as riboswitches (Topp and Gallivan, 2008), transcription factors (Binder et al., 2012), and genetic toggle switches (Gardner et al., 2000; Kobayashi et al., 2004; Anesiadis et al., 2008) to control gene expression. Others introduce a synthetic pathway that interacts with native cell functions to introduce and regulate a new response to common molecules (Goldberg et al., 2009). Controllers have also been successfully applied to metabolic networks specifically to increase production of metabolites. This has been accomplished through the use of a toggle switch to monitor changing concentrations of metabolites (Anesiadis et al., 2008). Alternatively, biosensors that detect metabolic intermediates have been used to control expression of genes in a production pathway (Farmer and Liao, 2000; Zhang et al., 2012).

MATERIALS AND METHODS

BIOSENSOR AND SYNTHETIC FEEDBACK CONTROL MODEL

The model was adapted from (Dunlop et al., 2010) to include biosensor production and dynamics. It includes a biosensor MexR(R) that represses efflux pump expression until it is deactivated in the presence of biofuel (**Figure 1A**). The biosensor is regulated by an inducible promoter, P_{lac}, which can be controlled by exogenous addition of isopropyl β-D-1-thiogalactopyranoside (IPTG). MexR works to repress efflux pump expression by binding to the promoter region of the efflux pump operon. When biofuel is present, MexR is deactivated so that it is unable to bind to the promoter and control expression.

The model consists of a system of five differential equations describing the growth of the overall culture and the relative concentration of important compounds in the bacterium. The dynamics of the system are described by the following equations:

$$\frac{dn}{dt} = \alpha_n n\left(1 - \frac{n}{n_{\max}}\right) - \delta_n b_i n - \frac{\alpha_n n p}{p + \gamma_p}$$

$$\frac{dR}{dt} = \alpha_R + k_R\left(\frac{I}{I + \gamma_I}\right) - \beta_R R$$

$$\frac{dp}{dt} = \alpha_p + k_p \frac{1}{\frac{R}{1+k_b b_i} + \gamma_R} - \beta_p p$$

$$\frac{db_i}{dt} = \alpha_b n - \delta_b p b_i$$

$$\frac{db_e}{dt} = V\delta_b p b_i n$$

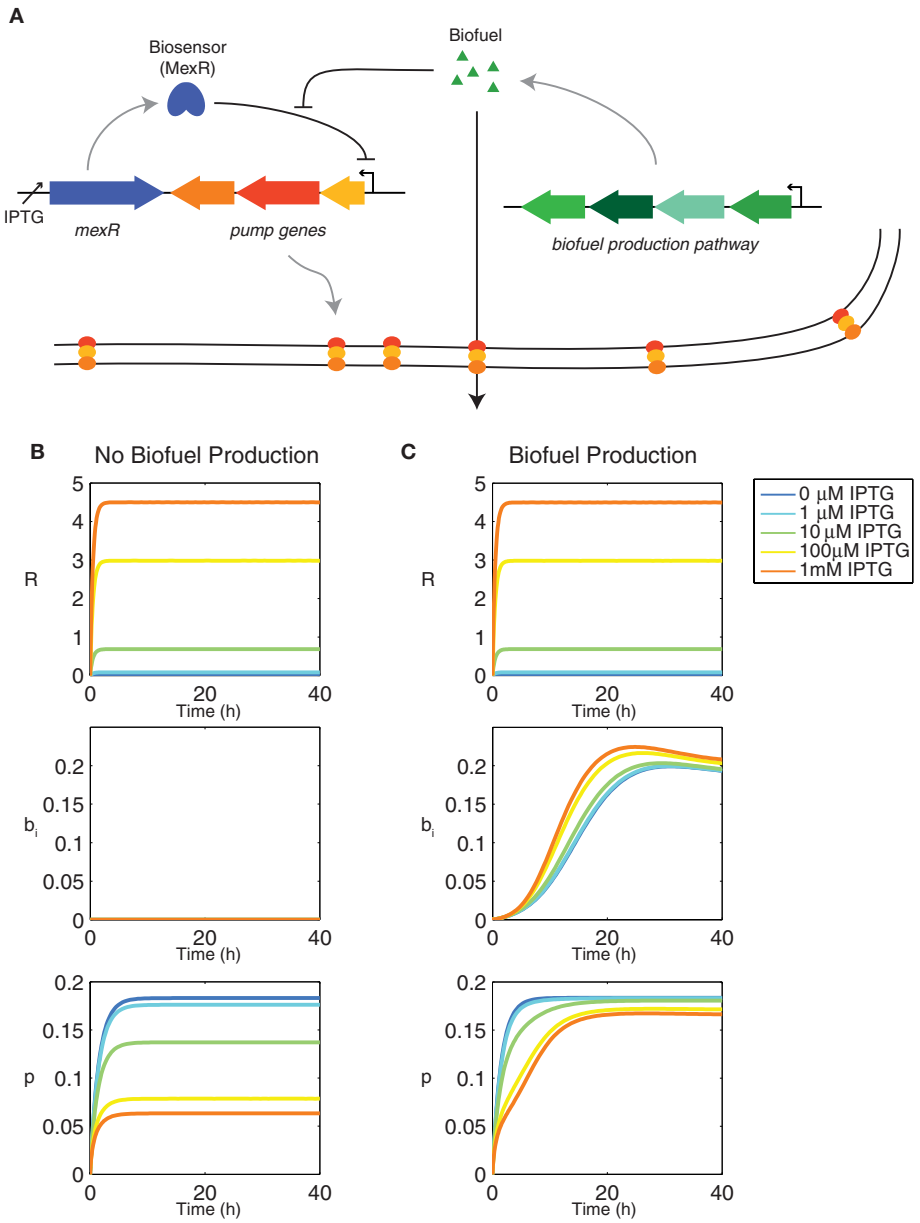


FIGURE 1 | Genetic components of the synthetic feedback loop and dynamics of the biosensor. (A) Gene circuit design for the biosensor and synthetic feedback loop. **(B)** Transient behavior of the feedback model using the biosensor MexR without biofuel production ($\alpha_b = 0\,h^{-1}$) and **(C)** with biofuel production ($\alpha_b = 0.1\,h^{-1}$). All other model parameters are as listed in **Table 1**.

where n is the cell density, R is the concentration of repressor proteins, p is the concentration of pumps, b_i is the concentration of intracellular biofuel, and b_e is the concentration of extracellular biofuel.

The dynamics for cell growth, n, model lag, exponential, and stationary phases. Growth is hindered by biofuel toxicity ($\delta_n b_i n$) and pump toxicity ($\alpha_n np/(p+\gamma_p)$), as determined experimentally in (Dunlop et al., 2010); the maximum population size is set by n_{max}. Basal production of R and p, given by α_R and α_p, represent the low level of expression that occurs when the promoter is not activated. The degradation rates are given by β_R and β_p. The production rates k_R and k_p represent the strength of expression for R and p, respectively. Repressor activation by the inducer IPTG is modeled as $I/(I+\gamma_I)$, where γ_I indicates the inducer value that corresponds to half maximal activation of repressor. This term models a rise in repressor concentration as the amount of inducer is increased. Repression of efflux pump expression is described as $1/(R/(1+k_b b_i)+\gamma_R)$ where k_b is the equilibrium constant for the deactivation of R and $R/(1+k_b b_i)$ represents the amount of active R in the system. Although this model assumes that the repressor binds at a single site, as in promoter designs from Zhang et al. (2012), alternative models with higher Hill coefficients give similar

Table 1 | Parameter values for feedback control model.

Parameter	Description	Value
α_n	Growth rate	0.66 h^{-1}
α_R	Basal repressor production rate	0.01 h^{-1}
α_p	Basal pump production rate	0.01 h^{-1}
α_b	Biofuel production rate	0.1 h^{-1}
β_R	Repressor degradation rate	2.1 h^{-1}
β_p	Pump degradation rate	0.66 h^{-1}
δ_n	Biofuel toxicity coefficient	0.91 M^{-1} h^{-1}
δ_b	Biofuel export rate per pump	0.5 M^{-1} h^{-1}
γ_p	Pump toxicity threshold	0.14
γ_I	Inducer saturation threshold	60 μM
γ_R	Repressor saturation threshold	1.8
k_R	Repressor activation constant	10 h^{-1}
k_p	Pump activation constant	0.2 h^{-1}
k_b	Repressor deactivation constant	100 M^{-1}
n_{max}	Maximum population size	1.0
V	Ratio of intra to extracellular volume	0.01

results (data not shown). Biofuel is produced at a rate α_b, which is scaled by n to model the impact of cell viability on biofuel production (dead cells produce no biofuel). Once biofuel is produced intracellularly, we make the simplifying assumption that it may only exit the cell via the action of efflux pumps ($\delta_b p b_i$). Upon export this quantity is scaled to correct for the number of cells and differences in the intra and extracellular volumes to give the extracellular biofuel concentration.

All model parameters are shown in **Table 1**. The growth rate α_n, biofuel production rate α_b, biofuel toxicity coefficient δ_n, pump protein degradation rate β_p, biofuel export rate δ_b, and pump toxicity threshold γ_p values from (Dunlop et al., 2010) were used in this model, where δ_n and γ_p were derived from experimental results. The inducer saturation threshold was estimated from the P_{lac} promoter IPTG induction curve (Lutz and Bujard, 1997). The repressor and pump dynamics are based on MexR's repression of MexAB (Poole et al., 1996; Narita et al., 2003).

SENSITIVITY ANALYSIS

Single parameter and two-parameter sensitivity analyses were conducted by varying the value of each parameter by 20% above and below the nominal values given in **Table 1**. Sensitivity was determined by the percent change in population size caused by altering the variable or combination of variables, as measured by cell density n at 40 h. For the two-parameter test, all four combinations of increasing and decreasing each parameter were considered. We define the maximum change as the greatest change resulting from each combination of parameters. Similarly, the minimum change is the smallest change resulting from the combination of parameters. When a parameter was paired with itself, the change caused by altering that single parameter was used.

CONSTANT PUMP MODEL

The constant pump system is able to express efflux pumps, but unlike the sensor model, its expression is fixed at a constant level. The model utilizes an inducer to control pump expression as follows: $dp/dt = \alpha_p + k_p\,(I/(I+\gamma_I)) - \beta_p p$. The repressor equation is removed from the system and the growth, intracellular biofuel concentration, and extracellular biofuel concentration equations remain the same as in the biosensor model. The inducer saturation threshold γ_I, degradation rate β_p, and basal production α_p are the same as used in the biosensor model, but the pump activation constant k_p is set to k_R since the constant pump model uses the same IPTG-inducible promoter. The constant pump model was optimized by varying IPTG levels (I) from 0 to 1 mM. The value of I selected is the one that produced the greatest amount of extracellular biofuel to allow for a controlled comparison against the feedback loop system.

VARIABILITY IN MODEL PARAMETERS

The effect of parametric variability on the system was tested by running simulations with parameter values drawn from a normal distribution. For 1000 simulations, all model parameters were chosen randomly from normal distributions with means of the nominal values (given in **Table 1**) and SD of 25% of the nominal value. The biofuel produced at 40 h was then averaged for all simulations. The fully induced sensor model (1 mM IPTG) was compared to the constant pump model.

SIMULATIONS

Simulations were performed in Matlab (Mathworks, Inc.) using the ode45 solver and custom analysis software.

RESULTS

SENSOR DYNAMICS

The feedback system includes a repressor MexR (R) that inhibits efflux pump expression until it is deactivated by biofuel. When this occurs, efflux pumps are produced, biofuel is exported, and cells continue to grow and produce biofuel. Transcription of the repressor is activated by an inducer, IPTG, which sets the amount of repressor in the system as well as baseline pump expression (**Figure 1B**). It is important to note that the feedback loop design does not require an inducible promoter; this is simply used to tune the system, but could be replaced with a constitutive promoter (Alper et al., 2005). When the cells produce biofuel, some of the repressor in the system is deactivated, which inhibits its ability to bind to the efflux pump promoter and repress transcription of the efflux pump operon (**Figure 1C**). The total amount of repressor includes activated and unactivated forms and therefore does not change when the cells produce biofuel. Pump expression, however, increases when biofuel is produced as a result of repressor deactivation. The most induced form of the system exhibits the greatest change because it contains the most repressor. The most induced form is also the slowest to reach steady state pump expression. The amount of repressor in the system directly contributes to the sensor's ability to both repress pump expression initially as well as adapt to changing biofuel concentrations. Therefore, the most induced form of the sensor, which exhibits the highest concentration of repressor, is the most responsive.

SENSITIVITY

Single parameter sensitivity analysis (**Figure 2A**) shows that the system is robust to variation in many of the model parameters,

however a set of six influential parameters do impact cell viability (greater than 5% changes). These six parameters – biofuel export rate δ_b, biofuel toxicity coefficient δ_n, biofuel production rate α_b, growth rate α_n, pump toxicity threshold γ_p, and maximum cell density $n_{\max}$ – have the greatest impact on the system when they are varied. The growth rate, maximum cell density, pump toxicity threshold, and biofuel toxicity coefficient are based directly on experimental data, but are likely to vary if the bacterial host, efflux pump system, or type of biofuel produced are altered. In contrast to the importance of these six influential parameters, the remaining parameters account for only small changes in cell viability.

Single parameter studies can miss important constructive or destructive effects from the simultaneous variation of parameters. To address this, we conducted a two-parameter sensitivity analysis, which shows that altering parameters in combination can augment (**Figure 2B**) or negate (**Figure 2C**) the effects of altering a single influential parameter. When two of the influential parameters are altered so that cell growth is decreased or increased, the effect is additive. Similarly if influential parameters are changed so that their effects on growth are opposite, the total change in growth is minimized. This result is not observed for combinations with less influential parameters. The less influential parameters do not alter the change caused by a major parameter, nor do they produce a considerable change when combined with another minor parameter. This conclusion reinforces the finding from the single parameter analysis that the sensor model is most dependent on a small subset of influential parameters.

CONSTANT PUMP VERSUS FEEDBACK CONTROL

Theoretically, in the absence of dynamics and variability, a constant pump system can be tuned so that it performs as well as a controller that incorporates feedback. In fact, constant controllers

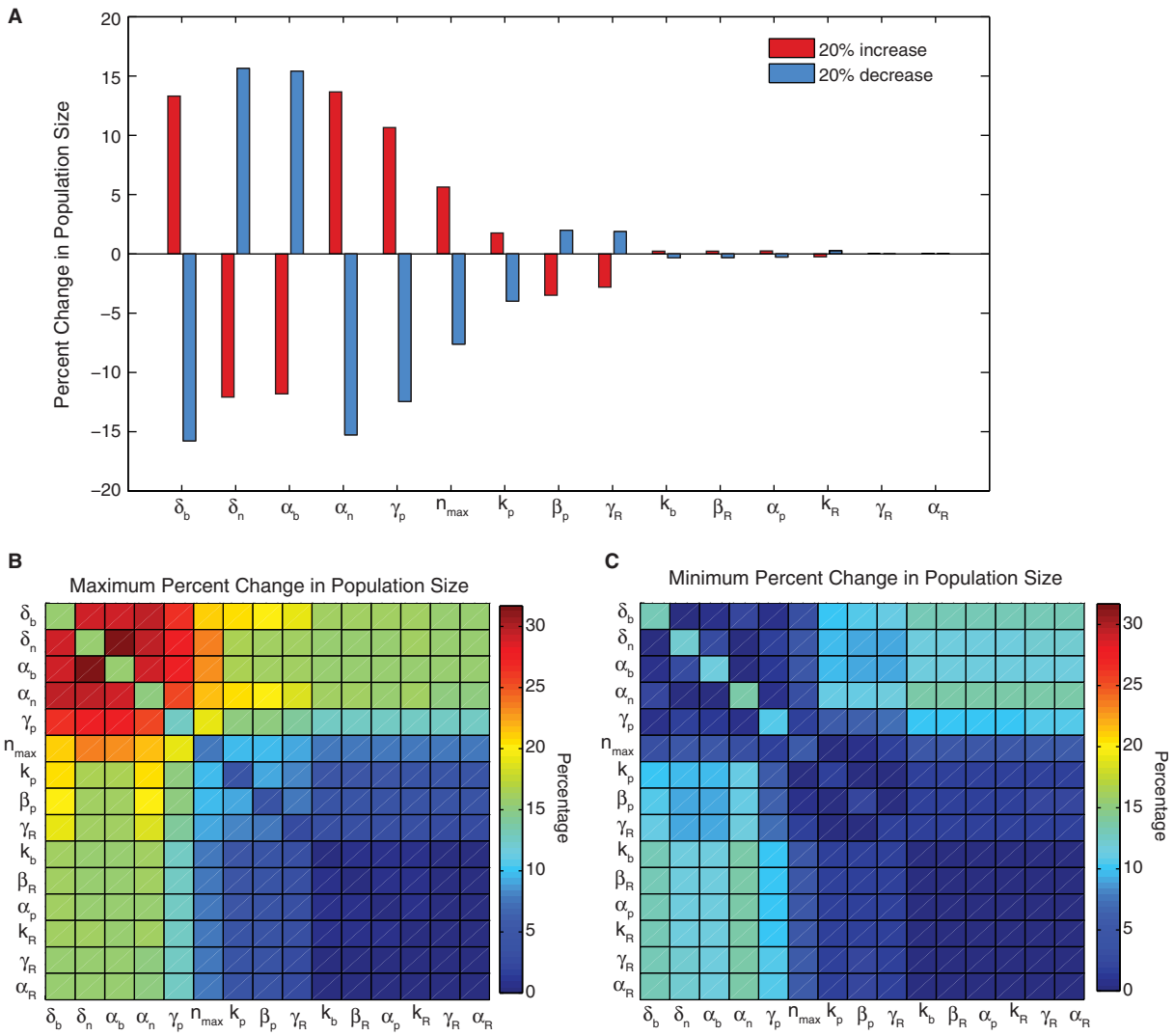


FIGURE 2 | Sensitivity analysis. (A) The percent change in population size for a 20% increase or decrease in a single parameter. **(B)** The maximum change and **(C)** minimum change observed for all four combinations of 20% increases and decreases in parameter values for every two-parameter pair. When a parameter is combined with itself, the single parameter change is shown.

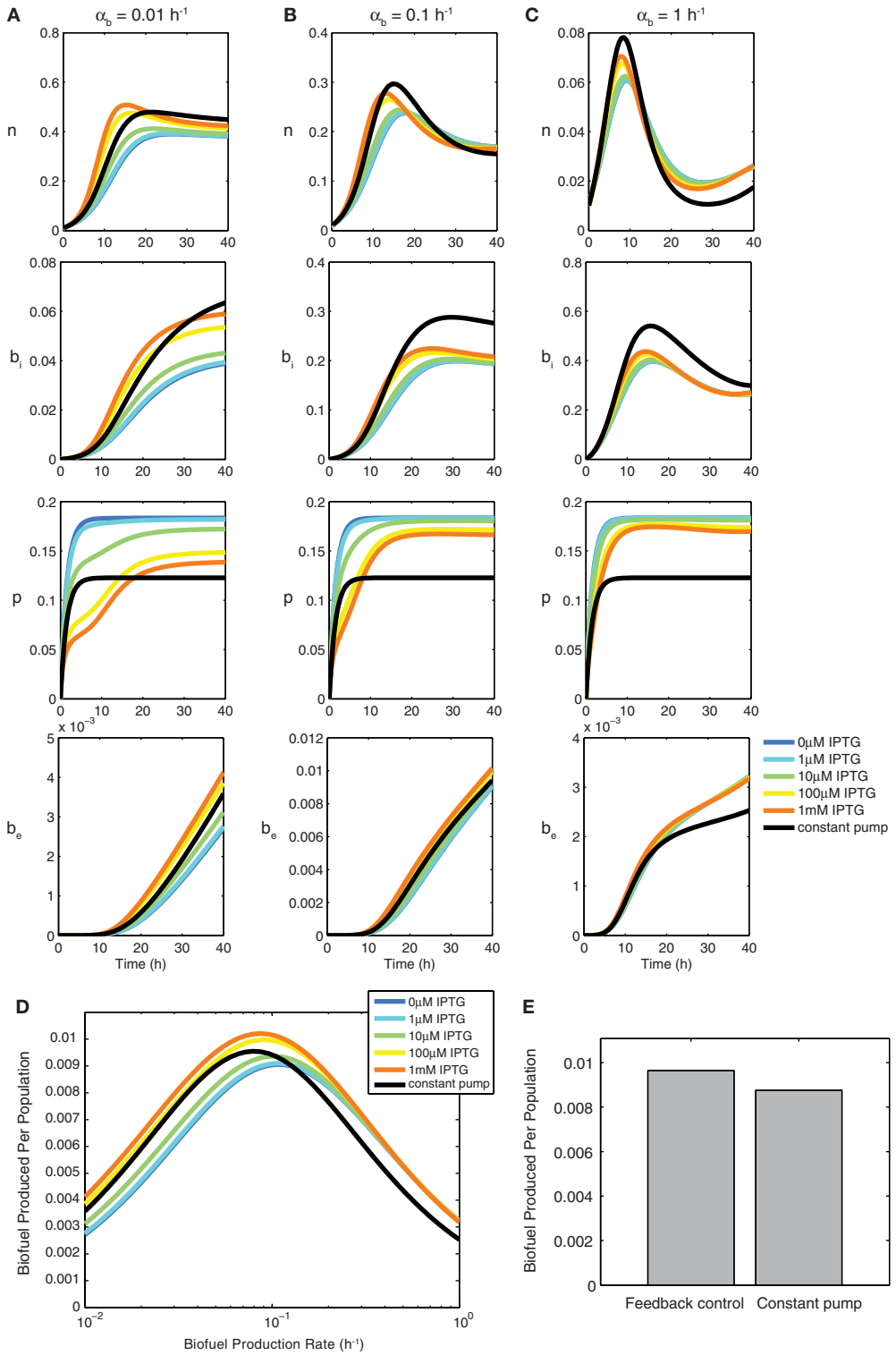


FIGURE 3 | Constant pump versus feedback control model using a biosensor. Transient behavior for growth *n*, intracellular biofuel b_i, pump expression *p*, and extracellular biofuel b_e for biofuel production rates α_b of **(A)** 0.01 h^{-1}, **(B)** 0.1 h^{-1}, and **(C)** 1 h^{-1}. Note the differences in *y*-axis scales. **(D)** Relative biofuel produced per population as a function of biofuel production rate. **(E)** Relative biofuel produced per population when the model parameters are variable.

have several potential advantages over feedback controllers. They are simpler to build and it is easier to predict behavior because they require fewer components. Additionally, they may be tuned using inducible promoters, which are well characterized and readily available. In practice, however, systems exhibit dynamic behavior as well as noise, which make perfect tuning of a constant controller impossible (Kaern et al., 2005; Raser and O'shea, 2005). Therefore a feedback controller that is able to tune itself would be advantageous in realistic production systems.

Figure 3 compares the feedback model dynamics to the constant pump model. For all biofuel production rates, the most highly induced sensor model produces the most biofuel. The feedback model's high biofuel production is due to the system's ability to delay efflux pump expression until intracellular biofuel has reached a toxic level. This delay allows the cells to grow, reach a higher population density, and have more cells producing biofuel at a maximal rate because energy is not wasted expressing efflux pumps before they are needed.

As the biofuel production rate is increased (**Figures 3A–C**), the delay in pump expression displayed by the most induced form of the sensor decreases because intracellular biofuel accumulates more quickly and efflux pumps are needed earlier. Additionally, pump expression by the sensor increases to accommodate the higher biofuel production rate, while pump expression in the constant pump model remains steady. As the biofuel production rate α_b is increased, the feedback model produces the most biofuel by balancing the toxicity of biofuel with the detrimental effects of pump expression. Increasing pump expression aids overall production by decreasing toxicity, which enables cells to grow, balancing production, and export. The constant pump model is unable to adapt export levels.

Figure 3D shows how the feedback model compares to the constant pump model as a function of the biofuel production rate α_b. The increased overall production due to faster growth rate caused by delayed pump expression is observed by comparing the most induced form of the sensor model to the constant pump model at $0.1\ h^{-1}$, which, by design, is the optimal production rate for the constant pump model. The constant pump model is not able to do as well as the feedback model once the biofuel production rate for which it is tuned is surpassed, nor is it able to capture the benefits of delayed pump expression that the feedback model exploits at lower biofuel production rates.

Next we tested how cell-to-cell variability in model parameters influences biofuel yields. Studies have shown that substantial variability in gene expression exists at the single-cell level (Kaern et al., 2005; Raser and O'shea, 2005), suggesting that biofuel production is unlikely to be uniform across a population of cells. **Figure 3E** shows that the feedback model is better suited than the constant pump when the parameters are variable. The biofuel produced for the feedback model is higher, on average, when parameters are variable, which shows that the feedback model's ability to adapt to changing biofuel production is more pronounced when a system is noisy. Importantly, these results compare the optimized constant controller against the feedback system. Deviation from optimal induction levels in the constant controller result in dramatically decreased yields, thus the results presented here show the best-case scenario for the constant controller.

DISCUSSION

We present a model of a synthetic biosensor and feedback control system to increase cell viability and biofuel production and quantify parametric sensitivity and the effect of variability in the model's parameters. Our model implements a realistic mechanism of efflux pump control that utilizes a biosensor; the biosensor we chose represses efflux pump expression until it is deactivated by biofuel, which is a common type of regulation in bacterial transport systems (Grkovic et al., 2002; Ramos et al., 2002). This regulation mechanism assures that efflux pumps are repressed until biofuel is present, which minimizes the negative effects of efflux pump overexpression while ensuring that their expression is initiated when needed (Isken et al., 1999; Wagner et al., 2007).

The feedback model we developed demonstrates that a subset of model parameters can influence the system's behavior, but most have minor effects. The influential parameters relate to the amount of biofuel produced, efficiency of pump export, toxicity thresholds for efflux pump expression and biofuel produced, and growth rate. For the system presented, many of these terms are based on experimental values. However, these parameter values, and the subsequent behavior of the system may change significantly if the biofuel produced, efflux system, or biosensor are altered. By considering multiple parameters, we show that if one variable is altered, it is possible to negate a detrimental effect by appropriately varying another influential parameter. It would be interesting to test the same biosensor with different efflux pumps or hosts to study the tunability of the system.

Even when optimized for maximal production, the constant pump model consistently produced less extracellular biofuel than the feedback model. This is due to the feedback sensor's ability to delay pump expression until it is necessary, which minimizes the negative effects of pump expression by allowing cells to grow well early on, and reduces energy requirements within the cell so that more biofuel can be produced. The advantages of a feedback control system are apparent when the parameters are variable, as is likely to be the case in a production setting. Therefore, the feedback model would prove useful in real-life applications where variability and noise are typical.

There are several possible extensions to this work. For example, diffusion was omitted here for simplicity, but could be incorporated into a model using this system to control tolerance mechanisms. Additionally, simulating different biosensors or tolerance mechanisms would test the modularity of the system, as well as how much initial tuning is required each time a component is modified. Similarly, by altering the biofuel production rate and toxicity coefficient, the applicability of the sensor to various potential biofuels could be determined. Stochastic simulations modeling the temporal dynamics of all components could further explore the impact of system variability on biofuel yields. Feedback control represents a valuable contribution to synthetic biology designs for optimizing biofuel yields and will be an important area for future experimental studies.

ACKNOWLEDGMENTS

This research was supported by the Office of Science (BER), US Department of Energy and funding from the University of Vermont.

REFERENCES

Alon, U. (2007). Network motifs: theory and experimental approaches. *Nat. Rev. Genet.* 8, 450–461.

Alper, H., Fischer, C., Nevoigt, E., and Stephanopoulos, G. (2005). Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12678–12683.

Anesiadis, N., Cluett, W. R., and Mahadevan, R. (2008). Dynamic metabolic engineering for increasing bioprocess productivity. *Metab. Eng.* 10, 255–266.

Becskei, A., and Serrano, L. (2000). Engineering stability in gene networks by autoregulation. *Nature* 405, 590–593.

Binder, S., Schendzielorz, G., Stabler, N., Krumbach, K., Hoffmann, K., Bott, M. et al. (2012). A high-throughput approach to identify genomic variants of bacterial metabolite producers at the single-cell level. *Genome Biol.* 13, R40.

Canosa, I., Sanchez-Romero, J. M., Yuste, L., and Rojo, F. (2000). A positive feedback mechanism controls expression of AlkS, the transcriptional regulator of the Pseudomonas oleovorans alkane degradation pathway. *Mol. Microbiol.* 35, 791–799.

Dunlop, M. J. (2011). Engineering microbes for tolerance to next-generation biofuels. *Biotechnol. Biofuels* 4, 32.

Dunlop, M. J., Dossani, Z. Y., Szmidt, H. L., Chu, H. C., Lee, T. S., Keasling, J. D., Hadi, M. Z., and Mukhopadhyay, A. (2011). Engineering microbial biofuel tolerance and export using efflux pumps. *Mol. Syst. Biol.* 7, 487.

Dunlop, M. J., Keasling, J. D., and Mukhopadhyay, A. (2010). A model for improving microbial biofuel production using a synthetic feedback loop. *Syst. Synth. Biol.* 4, 95–104.

Duque, E., Segura, A., Mosqueda, G., and Ramos, J. L. (2001). Global and cognate regulators control the expression of the organic solvent efflux pumps TtgABC and TtgDEF of Pseudomonas putida. *Mol. Microbiol.* 39, 1100–1106.

Evans, K., Adewoye, L., and Poole, K. (2001). MexR repressor of the mexAB-oprM multidrug efflux operon of Pseudomonas aeruginosa: identification of MexR binding sites in the mexA-mexR intergenic region. *J. Bacteriol.* 183, 807–812.

Farmer, W. R., and Liao, J. C. (2000). Improving lycopene production in Escherichia coli by engineering metabolic control. *Nat. Biotechnol.* 18, 533–537.

Fischer, C. R., Klein-Marcuschamer, D., and Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metab. Eng.* 10, 295–304.

Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000). Construction of a genetic toggle switch in Escherichia coli. *Nature* 403, 339–342.

Goldberg, S. D., Derr, P., Degrado, W. F., and Goulian, M. (2009). Engineered single- and multi-cell chemotaxis pathways in E. coli. *Mol. Syst. Biol.* 5, 283.

Grkovic, S., Brown, M. H., and Skurray, R. A. (2002). Regulation of bacterial drug export systems. *Microbiol. Mol. Biol. Rev.* 66, 671–701, table of contents.

Isken, S., Derks, A., Wolffs, P. F., and De Bont, J. A. (1999). Effect of organic solvents on the yield of solvent-tolerant Pseudomonas putida S12. *Appl. Environ. Microbiol.* 65, 2631–2635.

Jude, F., Arpin, C., Brachet-Castang, C., Capdepuy, M., Caumette, P., and Quentin, C. (2004). TbtABM, a multidrug efflux pump associated with tributyltin resistance in Pseudomonas stutzeri. *FEMS Microbiol. Lett.* 232, 7–14.

Kaern, M., Elston, T. C., Blake, W. J., and Collins, J. J. (2005). Stochasticity in gene expression: from theories to phenotypes. *Nat. Rev. Genet.* 6, 451–464.

Kobayashi, H., Kaern, M., Araki, M., Chung, K., Gardner, T. S., Cantor, C. R. et al. (2004). Programmable cells: interfacing natural and engineered gene networks. *Proc. Natl. Acad. Sci. U.S.A.* 101, 8414–8419.

Koutinas, M., Lam, M. C., Kiparissides, A., Silva-Rocha, R., Godinho, M., Livingston, A. G. et al. (2010). The regulatory logic of m-xylene biodegradation by Pseudomonas putida mt-2 exposed by dynamic modelling of the principal node Ps/Pr of the TOL plasmid. *Environ. Microbiol.* 12, 1705–1718.

Kurth, E. G., Doughty, D. M., Bottomley, P. J., Arp, D. J., and Sayavedra-Soto, L. A. (2008). Involvement of BmoR and BmoG in n-alkane metabolism in "Pseudomonas butanovora." *Microbiology* 154, 139–147.

Leahy, J. G., Johnson, G. R., and Olsen, R. H. (1997). Cross-regulation of toluene monooxygenases by the transcriptional activators TbmR and TbuT. *Appl. Environ. Microbiol.* 63, 3736–3739.

Lee, S. K., Chou, H., Ham, T. S., Lee, T. S., and Keasling, J. D. (2008). Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels. *Curr. Opin. Biotechnol.* 19, 556–563.

Li, X. Z., Zhang, L., and Poole, K. (1998). Role of the multidrug efflux systems of Pseudomonas aeruginosa in organic solvent tolerance. *J. Bacteriol.* 180, 2987–2991.

Lutz, R., and Bujard, H. (1997). Independent and tight regulation of transcriptional units in Escherichia coli via the LacR/O, the TetR/O and AraC/I1-I2 regulatory elements. *Nucleic Acids Res.* 25, 1203–1210.

Narita, S., Eda, S., Yoshihara, E., and Nakae, T. (2003). Linkage of the efflux-pump expression level with substrate extrusion rate in the MexAB-OprM efflux pump of Pseudomonas aeruginosa. *Biochem. Biophys. Res. Commun.* 308, 922–926.

Nevozhay, D., Adams, R. M., Murphy, K. F., Josic, K., and Balazsi, G. (2009). Negative autoregulation linearizes the dose-response and suppresses the heterogeneity of gene expression. *Proc. Natl. Acad. Sci. U.S.A.* 106, 5123–5128.

Nicolaou, S. A., Gaida, S. M., and Papoutsakis, E. T. (2010). A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: From biofuels and chemicals, to biocatalysis and bioremediation. *Metab. Eng.* 12, 307–331.

Paitan, Y., Biran, I., Shechter, N., Biran, D., Rishpon, J., and Ron, E. Z. (2004). Monitoring aromatic hydrocarbons by whole cell electrochemical biosensors. *Anal. Biochem.* 335, 175–183.

Paulsen, I. T., Brown, M. H., and Skurray, R. A. (1996). Proton-dependent multidrug efflux systems. *Microbiol. Rev.* 60, 575–608.

Phoenix, P., Keane, A., Patel, A., Bergeron, H., Ghoshal, S., and Lau, P. C. (2003). Characterization of a new solvent-responsive gene locus in Pseudomonas putida F1 and its functionalization as a versatile biosensor. *Environ. Microbiol.* 5, 1309–1327.

Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D. E., and Bianco, N. (1996). Expression of the multidrug resistance operon mexA-mexB-oprM in Pseudomonas aeruginosa: mexR encodes a regulator of operon expression. *Antimicrob. Agents Chemother.* 40, 2021–2028.

Ramos, J. L., Duque, E., Gallegos, M. T., Godoy, P., Ramos-Gonzalez, M. I., Rojas, A. et al. (2002). Mechanisms of solvent tolerance in gram-negative bacteria. *Annu. Rev. Microbiol.* 56, 743–768.

Raser, J. M., and O'shea, E. K. (2005). Noise in gene expression: origins, consequences, and control. *Science* 309, 2010–2013.

Ratajczak, A., Geissdorfer, W., and Hillen, W. (1998). Expression of alkane hydroxylase from Acinetobacter sp. Strain ADP1 is induced by a broad range of n-alkanes and requires the transcriptional activator AlkR. *J. Bacteriol.* 180, 5822–5827.

Rojas, A., Segura, A., Guazzaroni, M. E., Teran, W., Hurtado, A., Gallegos, M. T. et al. (2003). In vivo and in vitro evidence that TtgV is the specific regulator of the TtgGHI multidrug and solvent efflux pump of Pseudomonas putida. *J. Bacteriol.* 185, 4755–4763.

Selifonova, O. V., and Eaton, R. W. (1996). Use of an ipb-lux fusion to study regulation of the isopropylbenzene catabolism operon of Pseudomonas putida RE204 and to detect hydrophobic pollutants in the environment. *Appl. Environ. Microbiol.* 62, 778–783.

Shi, S., Valle-Rodriguez, J. O., Siewers, V., and Nielsen, J. (2011). Prospects for microbial biodiesel production. *Biotechnol. J.* 6, 277–285.

Sikkema, J., De Bont, J. A., and Poolman, B. (1995). Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* 59, 201–222.

Smits, W. K., Kuipers, O. P., and Veening, J. W. (2006). Phenotypic variation in bacteria: the role of feedback regulation. *Nat. Rev. Microbiol.* 4, 259–271.

Sorensen, S. J., Burmolle, M., and Hansen, L. H. (2006). Making biosense of toxicity: new developments in whole-cell biosensors. *Curr. Opin. Biotechnol.* 17, 11–16.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science* 315, 801–804.

Sticher, P., Jaspers, M. C., Stemmler, K., Harms, H., Zehnder, A. J., and Van Der Meer, J. R. (1997). Development and characterization of a whole-cell bioluminescent sensor for bioavailable middle-chain alkanes in contaminated groundwater samples. *Appl. Environ. Microbiol.* 63, 4053–4060.

Stiner, L., and Halverson, L. J. (2002). Development and characterization of a green fluorescent protein-based bacterial biosensor for bioavailable toluene and related compounds. *Appl. Environ. Microbiol.* 68, 1962–1971.

Sun, X., and Dennis, J. J. (2009). A novel insertion sequence derepresses efflux pump expression and preadapts Pseudomonas putida S12 for extreme solvent stress. *J. Bacteriol.* 191, 6773–6777.

Sun, X., Zahir, Z., Lynch, K. H., and Dennis, J. J. (2011). An antirepressor, SrpR, is involved in transcriptional regulation of the SrpABC solvent tolerance efflux pump of Pseudomonas putida S12. *J. Bacteriol.* 193, 2717–2725.

Teran, W., Felipe, A., Fillet, S., Guazzaroni, M. E., Krell, T., and Ruiz, R. et al. (2007). Complexity in efflux pump control: cross-regulation by the paralogues TtgV and TtgT. *Mol. Microbiol.* 66, 1416–1428.

Teran, W., Felipe, A., Segura, A., Rojas, A., Ramos, J. L., and Gallegos, M. T. (2003). Antibiotic-dependent induction of Pseudomonas putida DOT-T1E TtgABC efflux pump is mediated by the drug binding repressor TtgR. *Antimicrob. Agents Chemother.* 47, 3067–3072.

Thattai, M., and Van Oudenaarden, A. (2001). Intrinsic noise in gene regulatory networks. *Proc. Natl. Acad. Sci. U.S.A.* 98, 8614–8619.

Topp, S., and Gallivan, J. P. (2008). Riboswitches in unexpected places – a synthetic riboswitch in a protein coding region. *RNA* 14, 2498–2503.

Van Beilen, J. B., Panke, S., Lucchini, S., Franchini, A. G., Rothlisberger, M., and Witholt, B. (2001). Analysis of Pseudomonas putida alkane-degradation gene clusters and flanking insertion sequences: evolution and regulation of the alk genes. *Microbiology* 147, 1621–1630.

Van Der Meer, J. R., and Belkin, S. (2010). Where microbiology meets microengineering: design and applications of reporter bacteria. *Nat. Rev. Microbiol.* 8, 511–522.

Van Hamme, J. D., Singh, A., and Ward, O. P. (2003). Recent advances in petroleum microbiology. *Microbiol. Mol. Biol. Rev.* 67, 503–549.

Volkers, R. J. M., Ballerstedt, H., De Winde, J. H., and Ruijssenaars, H. J. (2010). Isolation and genetic characterization of an improved benzene-tolerant mutant of Pseudomonas putida S12. *Environ. Microbiol. Rep.* 2, 456–460.

Wagner, S., Baars, L., Ytterberg, A. J., Klussmeier, A., Wagner, C. S., Nord, O. et al. (2007). Consequences of membrane protein overexpression in Escherichia coli. *Mol. Cell Proteomics* 6, 1527–1550.

Willardson, B. M., Wilkins, J. F., Rand, T. A., Schupp, J. M., Hill, K. K., and Keim, P. et al. (1998). Development and testing of a bacterial biosensor for toluene-based environmental contaminants. *Appl. Environ. Microbiol.* 64, 1006–1012.

Zhang, F., Carothers, J. M., and Keasling, J. D. (2012). Design of a dynamic sensor-regulator system for production of chemicals and fuels derived from fatty acids. *Nat. Biotechnol.* 30, 354–359.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 20 July 2012; accepted: 24 September 2012; published online: 26 October 2012.

*Citation: Harrison ME and Dunlop MJ (2012) Synthetic feedback loop model for increasing microbial biofuel production using a biosensor. Front. Microbio. **3**:360. doi: 10.3389/fmicb.2012.00360*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 October 2012
doi: 10.3389/fmicb.2012.00355

Balance of *XYL1* and *XYL2* expression in different yeast chassis for improved xylose fermentation

Jian Zha[1], Meng-Long Hu[1], Ming-Hua Shen[1], Bing-Zhi Li[1], Jing-Yu Wang[2] and Ying-Jin Yuan[1]*

[1] *Key Laboratory of Systems Bioengineering (Tianjin University), Ministry of Education, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin, P. R. China*
[2] *Department of Biochemical Engineering, School of Chemical Engineering and Technology, Tianjin University, Tianjin, P. R. China*

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Matthew W. Fields, Montana State University, USA
Xiao Wang, Arizona State University, USA
Hao Song, Nanyang Technological University, Singapore

***Correspondence:**
Ying-Jin Yuan, Department of Pharmaceutical Engineering, School of Chemical Engineering and Technology, Tianjin University, Weijin Street 92, Tianjin 300072, P. R. China.
e-mail: yjyuan@tju.edu.cn

Reducing xylitol formation is necessary in engineering xylose utilization in recombinant *Saccharomyces cerevisiae* for ethanol production through xylose reductase/xylitol dehydrogenase pathway. To balance the expression of *XYL1* and mutant *XYL2* encoding xylose reductase (XR) and NADP$^+$-dependent xylitol dehydrogenase (XDH), respectively, we utilized a strategy combining chassis selection and direct fine-tuning of *XYL1* and *XYL2* expression in this study. A *XYL1* gene under the control of various promoters of *ADH1*, truncated *ADH1* and *PGK1*, and a mutated *XYL2* with different copy numbers were constructed into different xylose-utilizing modules, which were then expressed in two yeast chassises W303a and L2612. The strategy enabled an improved L2612-derived recombinant strain with *XYL1* controlled by promoter *PGK1* and with two copies of *XYL2*. The strain exhibited a 21.3% lower xylitol yield and a 40.0% higher ethanol yield. The results demonstrate the feasibility of the combinatorial strategy for construction of an efficient xylose-fermenting *S. cerevisiae*.

Keywords: pathway balance, chassis, xylose reductase, xylitol dehydrogenase, xylose, ethanol

INTRODUCTION

Efficient utilization of xylose is important for economic fermentation of biomass to fuels and chemicals (Jeffries, 2006; Chu and Lee, 2007; Stephanopoulos, 2007; Fischer et al., 2008). *Saccharomyces cerevisiae* (*S. cerevisiae*) is commonly used for industrial ethanol fermentation. However, because *S. cerevisiae* is not able to assimilate xylose naturally, engineering *S. cerevisiae* for efficient xylose utilization by introducing a xylose pathway from xylose-fermenting yeasts such as *Scheffersomyces stipitis* has attracted a great interest in recent years (Chu and Lee, 2007; Fischer et al., 2008; Matsushika et al., 2009a). Through this pathway, xylose is reduced to xylitol by NADPH-dependent xylose reductase (XR) encoded by *XYL1* and then xylitol is converted into xylulose, which can be converted by NAD$^+$-dependent xylitol dehydrogenase (XDH) encoded by *XYL2*. Xylulose can be phosphorylated into xylulose-5-phosphate (X-5P) by xylulokinase (XK) for metabolism into the non-oxidative pentose phosphate pathway (PPP) and the glycolysis pathway. Another pathway to convert xylose into xylulose in one step is the xylose isomerase pathway (Chu and Lee, 2007; Matsushika et al., 2009a). Functional expression of a xylose isomerase in yeast was achieved after efforts of a few decades (Chu and Lee, 2007). Pronk and co-workers successfully cloned a xylose isomerase from fungi *Pyromyces sp.* (ATCC 76762) (Kuyper et al., 2003). However, the ethanol productivity of the xylose isomerase pathway was 1.6-fold lower than that of XR-XDH pathway although the overall ethanol yield (0.43 g/g) was 30.0% higher (Karhumaa et al., 2007). Recently more research attention was paid on the XR-XDH pathway for improved ethanol yield and productivity. However, the ethanol yield from xylose fermentation is very low due to the fact that a large fraction of consumed xylose is secreted as its reduction product xylitol (Jeppsson et al., 2006; Chu and Lee, 2007; Matsushika et al., 2009a).

Formation of xylitol is mainly ascribed to the difference in co-factor preference between XR and XDH, which causes intracellular redox imbalance and leads to xylitol accumulation (Jeffries, 2006; Chu and Lee, 2007). This hypothesis has been supported by the observation that alteration of cofactor dependence of XR or XDH by directed evolution decreased xylitol production and increased ethanol yield (Jeppsson et al., 2006; Matsushika et al., 2008; Petschacher and Nidetzky, 2008; Bengtsson et al., 2009). Genome-scale modeling of the xylose pathway with NADP$^+$-dependent XDH simulated an increased ethanol production by 24.7% and reduced fermentation time by 70% (Kao et al., 2011).

Besides the approach to alter the coenzyme preference of XR or XDH, balance of the XR/XDH activity can also improve xylose utilization (Walfridsson et al., 1997; Karhumaa et al., 2006). Walfridsson and co-workers observed that a strain with an XR/XDH activity ratio of 17.5 produced 0.82 g xylitol/g xylose while a strain having a ratio of 0.06 didn't produce xylitol (Walfridsson et al., 1997). Proper balance of enzymatic reactions of the pathway is required for high productivity to avoid accumulation of toxic intermediates (Dueber et al., 2009; Ajikumar et al., 2010; Bond-Watts et al., 2011). Production of xylitol consumes NADPH and destroys the balance of reductive hydrogen pool (Jeppsson et al., 2002). Therefore, balance of *XYL1* and *XYL2* in xylose conversion pathway can maximize pathway flux, recycle NADPH generation and improve ethanol production. Tuning the promoter strengths or plasmid copy number is a commonly used

strategy to balance pathway flux. Lu and Jeffries (2007) shuffled two promoters for key genes *TKL1*, *TAL1*, and *PYK1* in xylose metabolic pathway to optimize the xylose fermentation. The optimal version of *GND2p-TAL1-HXK2p-TKL1-HXK2p-PYK1* was identified by calculation of volumetric ethanol production (Lu and Jeffries, 2007). In this study, we applied the similar strategy to optimize the initial xylose metabolic pathway. Three promoters in *S. cerevisiae* were used to manipulate the expression level of *XYL1* and two plasmids of different gene copy numbers to modulate the expression level of *XYL2* for balance of *XYL1* and *XYL2*.

Chassis is also a factor that should be taken into consideration in improving xylose fermentation (Boghigian et al., 2012). The enzymes in non-oxidative PPP have distinct activities in different chassises, causing varied capacities to metabolize xylulose. Consequently, the different xylulose metabolism resulted in various xylose metabolism styles, as reported that different chassises carrying the identical xylose pathways differed in xylose fermentation (Matsushika et al., 2009b,c; Hector et al., 2011). So far no parameter has been defined to measure the genetic fitness of a chassis for expression of a heterologous xylose pathway. Thus it is plausible that evaluation of different chassises could be part of the avenues to improve xylose fermentation.

In the present study, we reported the construction and optimization of a xylose-utilizing module containing *XYL1* and mutated *XYL2* (D207A/I208R/F209S/N211R) (*mXYL2*) in *S. cerevisiae*. Different parts of the xylose-utilizing module were balanced by fine-tuning the expression levels of *XYL1* and *mXYL2* through various promoters for controlling *XYL1* and different copy numbers for *mXYL2*. Two yeast chassises were selected for functional expression of the xylose-utilizing modules. The results showed that the combined strategy has improved xylose-fermentation to ethanol in *S. cerevisiae*.

METHODS

STRAINS AND MEDIA

Yeast *S. cerevisiae* strain W303a (MATa, leu2-3,112 his3-11, 15 ura3-1 ade2-1 trp1-1 can1-100 rad5-535) and L2612 (MATalpha, leu2-3, leu2-112, ura3-52, trp1-298 can1 cyn1 gal+), a gift from Prof. Thomas Jeffries at University of Wisconsin–Madison, were used as host strains. *E. coli* DH5α was used for common genetic manipulation. *E. coli* DH5α was grown in LB medium (10 g/l tryptone, 5 g/l yeast extract, 10 g/l sodium chloride) supplemented with 100 mg/l ampicillin when used for plasmid construction. Yeast cells were routinely cultured in yeast extract peptone dextrose (YPD) medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose). To select transformants using ura3 or leu2 auxotrophic marker, synthetic component (SC) medium was used, which contained 6.7 g/l YNB, 20 g/l glucose, 20 g/l agar, and 2 g/l amino acid dropout mixture missing uracil or leucine when necessary. Aerobic growth or anaerobic fermentation was performed in YPX medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l xylose).

CONSTRUCTION OF RECOMBINANT PLASMIDS

Plasmids and primers used in the study are described in **Tables 1** and **2**, respectively. Genes *XYL1* and *mXYL2* were codon-optimized and chemically synthesized by Geneart AG (Regensurg, Germany). The *XYL1* sequence contained the optimized ORF sequence of XR from *S. Stipitis* CBS6054 and *PGK1* terminator sequence with *Pst I* at the 5′ end and *Hind III*, *Spe I* at the 3′ end, respectively. Similarly, gene *mXYL2* contained the ORF sequence of mutant XDH and *PGK1* terminator sequence. Restriction site *Pst I* was added at the 5′ end and *BamH I*, *Hind III* at the 3′ end. The *XKS1* including its ORF and native terminator, *ADH1* promoter (*ADH1*), truncated *ADH1* promoter (*tADH1*) and *PGK1* promoter were amplified from genomic DNA of strain L2612 and checked by sequencing.

Plasmids YIplac211-I, YIplac211-II, and YIplac211-III were constructed as follows. First, the three promoters were cloned into vector pTA2 using primers described in **Table 2**. The 1.26 kb *Pst I-Spe I* fragment of gene *XYL1* was inserted into pTA2 to form three types of *XYL1* expression cassettes. The cassettes were released by *Hind III* digestion and inserted into *Hind III* site in plasmid YIplac211 resulting in plasmids YIplac211-XR (*tADH1*), YIplac211-XR (*ADH1*), YIplac211-XR (*PGK1*). The orientations of cassettes were checked by PCR using primers M13R and Xyl1R.

On the basis of serial YIplac211-XR plasmids, *mXYL2,* and *XKS1* expression cassettes were inserted sequentially. The *mXYL2* expression cassette was constructed in pUC18 as follows. The *PGK1* promoter was amplified from genomic DNA using primers *PGK1*pF2 and *PGK1*pR2 and inserted into *BamH I* and *Pst I* sites in pUC18. Next, the DNA fragment of *mXYL2* was inserted into *Pst I* and *Hind III*. After that, the *mXYL2* cassette sequence was released by *BamH I* and cloned into *BamH I* site in plasmids YIplac211-XR (*tADH1*), YIplac211-XR (*ADH1*), and YIplac211-XR (*PGK1*). The correct clones were verified by enzymatic digestion and PCR test using primers Xy12F and M13F, generating plasmids YIplac211-XR (*tADH1*) XDH, YIplac211-XR (*ADH1*) XDH and YIplac211-XR (*PGK1*) XDH.

To clone *XKS1* expression cassette into the above YIplac211-XRXDH plasmids, *XKS1* sequence with restriction sites was amplified from genomic DNA and cloned into *BamH I* and *Pst I* sites of pUC18, yielding plasmid pUC18-XKS1. The *PGK1* promoter sequence amplified from genomic DNA using primers *PGK1*pF3 and *PGK1*pR3 was cloned into *EcoR I* and *BamH I* sites in plasmid pUC18-XKS1 to form the *XKS1* expression cassette. The cassette was then subcloned into *EcoR I* site in analogue YIplac211-XRXDH plasmids. The clones with the correct orientation were checked by enzymatic digestion and PCR test using primers XKS1F and M13F. The resultant plasmids were designated as YIplac211-I, YIplac211-II, YIplac211-III (**Figure 1**).

PRS305-XDH and pRS425-XDH were constructed by inserting *mXYL2* cassette mentioned above into *BamH I* site of pRS305 (Sikorski and Hieter, 1989) and pRS425, respectively.

YEAST TRANSFORMATION

Yeast transformation was carried out by the lithium acetate method (Gietz et al., 1995). Plasmids of YIplac211, YIplac211-I, YIplac211-II, and YIplac211-III were linearized by *ApaI* before transformation. Plasmids pRS305 and pRS305-XDH were

Table 1 | Strains and plasmids used in the study.

Strains/plasmids	Relevant genotype	Source or references
STRAINS		
W303a	*MATa,leu2,his3,ura3,ade2,trp1,can1,rad5*	Jeppsson et al., 2003
L2612	*MATalpha,leu2,ura3, trp*1	Jin and Jeffries, 2003
W303tAR	W303a, Yiplac211-I	This study
W303AR	W303a, Yiplac211-II	This study
W303PR	W303a, Yiplac211-III	This study
W303C	W303a, Yiplac211	This study
L2612C	L2612, Yiplac211	This study
L2612tAR	L2612, Yiplac211-I	This study
L2612AR	L2612, Yiplac211-II	This study
L2612PR	L2612, Yiplac211-III	This study
L2612PR-MD	L2612PR, pRS425-XDH	This study
L2612PR-D	L2612PR, pRS305-XDH	This study
L2612PR-MC	L2612PR, pRS425	This study
L2612PR-C	L2612PR, pRS305	This study
PLASMIDS		
pUC18	Gene cloning	Takara
pTA2	Gene cloning	TOYOBO
Yiplac211	*URA3*, an integrative plasmid	ATCC87593
pRS305	*LEU2*, an integrative plasmid	Sikorski and Hieter, 1989
pRS425	*LEU2*,a multicopy plasmid	ATCC77106
Yiplac211-I	Yiplac211,*tADH1p-XYL1-PGK1t,PGK1p-mXYL2-PGK1t,PGK1p-XKS1-XKS1t*	This study
Yiplac211-II	Yiplac211,*ADH1p-XYL1-PGK1t,PGK1p-mXYL2-PGK1t,PGK1p-XKS1-XKS1t*	This study
Yiplac211-III	Yiplac211,*PGK1p-XYL1-PGK1t,PGKp-mXYL2-PGKt,PGKp-XKS1-XKS1t*	This study
pRS305-XDH	pRS305,*PGK1p-mXYL2-PGK1t*	This study
pRS425-XDH	pRS425, *PGK1p-mXYL2-PGK1t*	This study

Table 2 | Primers used in the study.

Primer name	Sequences
tADH1pF	gggAAGCTTACACTGCCTCATTGATGGTG
ADH1pF	gggAAGCTTAAGAAATGATGGTAAATGAAATA
ADH1pR	gggCTGCAGTGTATATGAGATAGTTGATT
PGK1pF1	gggAAGCTTGATTCCTGACTTCAACTCAAGACG
PGK1pR1	gggCTGCAG TGTTTTATATTTGTTGTAAA
PGK1pF2	gggGGATCCGATTCCTGACTTCAACTCAAGACG
PGK1pR2	gggCTGCAG TGTTTTATATTTGTTGTAAA
PGK1pF3	gggGAATTCGATTCCTGACTTCAACTCAAGACG
PGK1pR3	gggGGATCCTGTTTTATATTTGTTGTAAA
XKS1F	gggGGATCCATGTTGTGTTCAGTAATTCAGAGACAG
XKS1R	gggCTGCAGGAATTCGAGCTCGAGATGATTTAACAATAAC
M13R	TGTAAAACGACGGCCAGTG
Xyl1R	GAGCAAATTCGATCAATCTAGGT
Xyl2F	GCTCCAGGTGGTAGATTTGTC
Xks1F	CGGATGCCTGTGGTATGAA
M13F	CAGGAAACAGCTATGACC

Underlined sequences are restriction enzyme recognition sites.

linearized by *AflII* prior to integration into yeast genome. Transformants were selected on SC medium containing 20 g/l glucose. Amino acids and nucleotides were added when necessary.

FERMENTATION

For inocula preparation, cells were cultivated in YPD medium or SC medium to maintain the plasmids when necessary at 30°C under aerobic conditions. Cells at mid-exponential phase were harvested by centrifugation at 1432×g for 5 min. For aerobic growth, cells were grown in 50 ml YPX medium in 250 ml shake-flasks with an initial optimal density at 600 nm (OD_{600}) of 0.5 (Model 722 grating spectrometer, Shanghai No. 3 Analysis Equipment Factory, Shanghai, China). For anaerobic fermentation, harvested cells were inoculated to an OD_{600} of 2.0 in 100 ml YPX medium in 250 ml shake-flasks sealed by a rubber stopper with a needle to release CO_2 produced during the fermentation process. Both fermentations were conducted at 30°C at 150 rpm unless noted specifically. All the experiments were repeated independently.

MEASUREMENT OF CELL GROWTH AND ANALYSIS OF FERMENTATION PRODUCTS

Cell density was monitored by measuring the absorbance of the culture at 600 nm with a spectrometer (Model 722 grating spectrometer, Shanghai No. 3 Analysis Equipment Factory, Shanghai, China). Samples were taken periodically from cultures and centrifuged at 9600×g for 5 min. Supernatant was collected for analysis on a HPLC system consisting of a HPLC pump (Waters 1515), a Bio-Rad HPX-87H column (Bio-Rad, Hercules, CA) and a refractive index detector (Waters 2414) (Ding et al., 2012). The

column was eluted at 65°C with 5 mM sulfuric acid at a flow rate of 0.6 ml/min.

RESULTS

EFFECTS OF DIFFERENT LEVELS OF XYL1 ON AEROBIC XYLOSE CONSUMPTION

To probe the appropriate expression level of *XYL1* for matching *mXYL2*, three promoters were applied to express *XYL1* along with *PGK1* promoter expressing *mXYL2*. Two versions of *ADH1* promoters, full-length *ADH1* (*ADH1*) and truncated *ADH1* (*tADH1*), were tested here (Liu and Hu, 2010). The constructed xylose conversion pathways were expressed in chassis W303a, resulting in strains W303tAR, W303AR, and W303PR of those the *XYL1* was expressed by the promoters *tADH1*, *ADH1*, and *PGK1*, respectively. Aerobic growth was conducted to characterize these strains compared with the control strain W303C (**Figure 2**). Strains W303tAR, W303AR, and W303PR consumed 1.92, 2.71 and 17.42 g/l xylose, respectively, corresponding to 74.5%, 146.3 %, and 14.8-fold increase than strain W303C which consumed 1.10 g/l xylose (**Table 3**). The xylitol yield in W303tAR and W303C was nearly the same, whereas the xylitol yield of W303AR and W303PR was 73.5% and 30.6% higher than that of W303C. The glycerol yield in W303tAR, W303AR, and W303PR was lower than that of the control strain W303C. The biomass yield from W303tAR and W303PR were 3.86, and 2.58 folds of that in W303C. Although strain W303AR consumed more xylose than W303tAR and W303C, nearly no biomass was produced because most of the assimilated xylose was secreted as xylitol during the growing process, indicating severe imbalance of the xylose metabolic pathway with *XYL1* promoted by *ADH1* (**Table 3**). In summary, only promoter *PGK1* facilitated xylose uptake for strain W303a, while the other promoters failed.

To investigate the capacity to produce ethanol from xylose, xylose fermentation by strain W303PR was conducted with xylose concentration up to 50 g/l at different aeration rate ranging from 0 to 0.556 vvm (volumes of air per volume of liquid per min) in a fermentor (Baoxin Biotech Ltd., Shanghai, shanghai, China). However, ethanol was not produced while a large amount of xylitol was formed. The reason for this may be that metabolic flux was channeled into TCA cycle induced by xylose (Jin et al., 2004), consistent with cell growth in xylose fermentation (**Figure 2A**). These results indicate that W303a is not an appropriate chassis

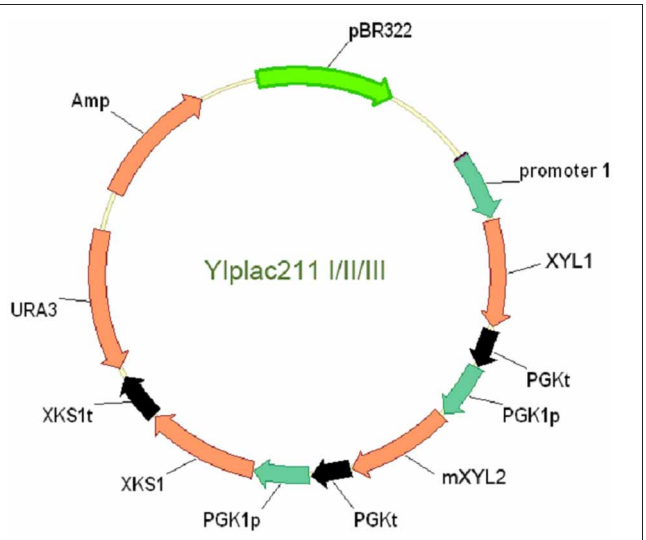


FIGURE 1 | The physical map of the plasmids Ylplac211-I, II, and III for co-expression of *XYL1*, *mXYL2* and *XKS1* in yeast W303a and L2612. Promoter 1 represents *tADH1*, *ADH1*, and *PGK1* promoters in Ylplac211-I, II and III. The transcription orientations of the three genes were the same in the plasmids.

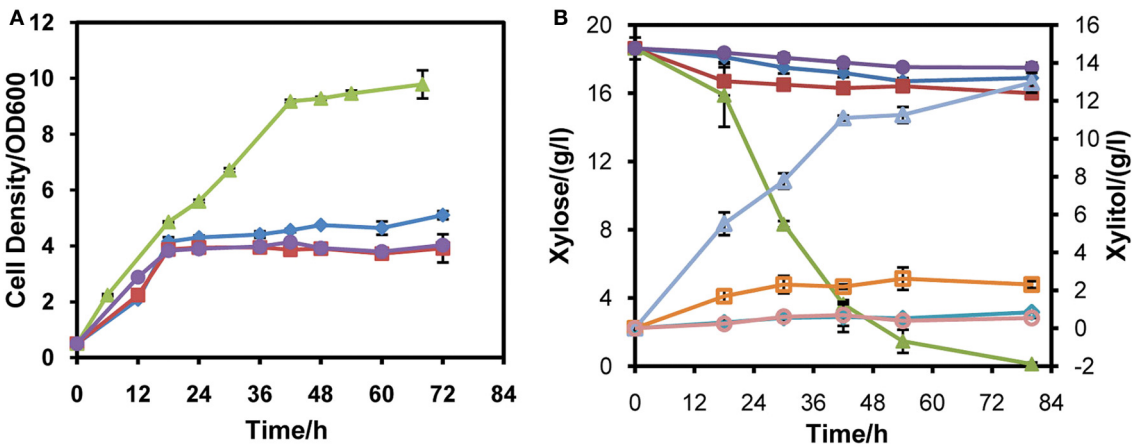


FIGURE 2 | Aerobic xylose consumption of strains W303tAR, W303AR, W303PR, and W303C in YPX medium. (A) growth profiles, **(B)** xylose consumption and xylitol production. Three promoters were applied to express *XYL1* for balance of *XYL1* and *XYL2*. W303tAR, W303AR, and W303PR harbored truncated *ADH1* promoter, intact *ADH1* promoter and *PGK1* promoter, respectively. W303C was the control strain. The aerobic cell growth was conducted in 50 ml YPX medium containing 20 g/l xylose. Symbols: diamonds, W303tAR; squares, W303AR; triangles, W303PR; circles, W303C. In panel **(B)**, closed symbols represent xylose concentration and open symbols represent xylitol concentration. The concentration of glycerol is not presented (see **Table 3**). Ethanol was not detected in the media. The results shown were the mean value of duplicate experiments.

for xylose pathway expression, which leads us to chassis yeast L2612.

COMPARISON OF TWO CHASSIS FOR EXPRESSING XR/XDH PATHWAY

Another yeast chassis L2612 was examined to express the three xylose pathways and compared with chassis W303a. Similar to the results obtained in W303a, the xylose pathways in which *XYL1* was under the control of *ADH1* or *tADH1* did not work in strain L2612 (**Figure 3A**). Only the strain L2612PR harboring the strongest promoter *PGK1* to express *XYL1* grew rapidly under aerobic conditions. Consistently, L2612tAR, L2612AR, and parent strain L2612C consumed 15.7, 17.3, and 8.4% of the total xylose, respectively (**Figure 3B**). In contrast strain L2612PR consumed all the xylose.

The performances of L2612PR and W303PR were compared under conditions of different oxygen supply. As shown in **Table 4**, L2612PR produced less byproduct xylitol than W303PR under different oxygen supply, indicating XR and XDH are more balanced in L2612 than W303PR. Additionally, the glycerol yield in L2612PR was approximately half of that in W303PR under aerobic conditions. Accompanying decreased oxygen supply, the xylose consumption rates in W303PR and L2612PR decreased dramatically by 20.3% and 44.8%, respectively, indicating the xylose metabolism of L2612PR is more dependent on oxygen (**Table 4**). A previous microarray study in the xylose-fermenting strain YSX3 derived from L2612 also supports the xylose metabolism style that xylose metabolic flux is prone to respiratory pathway. Under oxygen-limited conditions, respiratory pathway is blocked leading to decreased xylose metabolism rate (Jin et al., 2004).

Table 3 | Xylose consumption of *S. cerevisiae* strains W303tAR, W303AR, W303PR, and W303C under aerobic conditions.

Strain	Consumed xylose (g/l)[a]	Yield (g/g consumed xylose)[a]		
		Xylitol	Glycerol	Biomass
W303TAR	1.92 ± 0.18	0.44 ± 0.03	0.046 ± 0.004	0.27 ± 0.09
W303AR	2.71 ± 0.52	0.85 ± 0.05	0.018 ± 0.002	0.18 ± 0.01
W303PR	17.42 ± 1.02	0.64 ± 0.01	0.006 ± 0.000	0.01 ± 0.00
W303C	1.10 ± 0.05	0.49 ± 0.01	0.068 ± 0.005	0.07 ± 0.01

Strains W303tAR, W303AR, W303PR were integrated with xylose conversion pathway with XYL1 controlled by truncated ADH1 promoter, intact ADH1 promoter and PGK1 promoter, respectively. W303C was the control strain. The growth was conducted in 50 ml YPX medium containing 20 g/l xylose under aerobic conditions. The initial cell density was adjusted to 0.5 (OD_{600}).

[a] The results were calculated based on metabolites concentration measured at 80 h of the fermentation.

Table 4 | Comparison of xylose consumption performances of W303PR and L2612PR under conditions of different oxygen supply.

	W303PR		L2612PR	
	Oxygen-limited (100 rpm)	Aerobic (150 rpm)	Oxygen-limited (100 rpm)	Aerobic (150 rpm)
r^a_{xylose}	0.247 ± 0.00	0.310 ± 0.03	0.170 ± 0.03	0.308 ± 0.01
$Y^b_{xylitol}$	0.59 ± 0.03	0.60 ± 0.03	0.47 ± 0.02	0.27 ± 0.02
$Y^c_{glycerol}$	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.01	0.04 ± 0.01

W303PR and L2612PR had the same xylose pathway PGK1p-XYL1-PGKp-mXYL2-PGKp-XKS1 except the host strains. The host strains of W303PR and L212PR are W303a and L2612, respectively. The different oxygen supply was obtained by changing the shaking speed. The initial cell density was adjusted to 0.5 (OD_{600}). The results are the average of two independent experiments after 72 h fermentation.

[a] Volumetric xylose consumption rate is given in g/l/h.

[b] Xylitol yield is given in g/g consumed xylose.

[c] Glycerol yield is given in g/g consumed xylose.

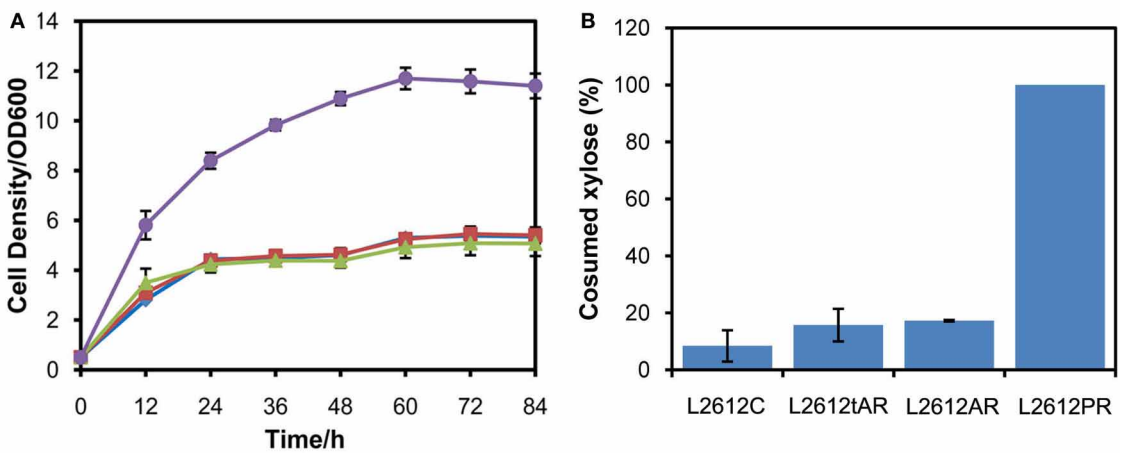


FIGURE 3 | Comparison of growth profiles (A) and xylose uptake capacities (B) of three recombinant strains derived from yeast strain L2612 and control strain L2612C in YPX medium under aerobic conditions. Symbols: squares, L2612C; diamonds, L2612tAR; triangles, L2612AR; circles, L2612PR. Strains L2612tAR, L2612AR, L2612PR were integrated with xylose conversion pathway with *XYL1* controlled by truncated *ADH1* promoter, full-length *ADH1* promoter and *PGK1* promoter in yeast L2612. L2612C was the control strain. Xylose uptake capacities are presented as the percentage of consumed xylose after 72 h. The results shown were the mean value of duplicate experiments.

Considering byproducts xylitol and glycerol production, it is concluded that yeast L2612 is a better chassis to express xylose pathway for ethanol production. L2612PR could be a starting strain for further modification to decrease xylitol production and increase metabolic flux to ethanol under anaerobic conditions.

EFFECTS OF DIFFERENT LEVELS OF *XYL2* ON XYLOSE FERMENTATION

Because XDH is directly associated with the conversion of xylitol to xylulose which can be metabolized by the non-oxidative PPP, we postulated that enhancement in *mXYL2* expression could reduce xylitol secretion, increase the carbon flux to the non-oxidative PPP and consequently increase ethanol production. To verify the postulation, we introduced different copies of *mXYL2* into strain L2612PR. In addition, a parallel investigation of the effects of different expression levels of *mXYL2* on xylose fermentation was also conducted.

We first compared the performances of the constructed strains under aerobic conditions. L2612PR-MD harboring multiple copies of *mXYL2* showed small differences in growth and product distribution compared with the control strain L2612PR-MC (**Table 5**). Different from multiple copies of *mXYL2*, overexpression of *mXYL2* by genomic integration had a significant effect on xylose consumption. Compared with the control strain L2612PR-C, strain L2612PR-D assimilated xylose faster but not significantly ($P = 0.058$) (**Figure 4**). The average xylose consumption rate of L2612PR-D was 10% higher of that in L2612PR-C (**Table 5**). However, the xylitol yield, glycerol yield, and biomass yield in L2612PR-D stayed nearly the same as that in L2612PR-C (**Table 5**).

To verify whether enhanced *mXYL2* expression had any positive effects on xylose fermentation under anaerobic conditions, the performances of the four strains were compared. As shown in **Figure 5**, excessive overexpression of *mXYL2* improved xylose uptake in L2612PR-MD and L2612PR-D compared with their control strains. The overall xylose consumption rate and maximal specific xylose uptake rate improved a bit for L2612PR-MD (**Table 6**). Additionally, L2612PR-MD produced 5.80 g/l xylitol, much less than L2612PR-MC (7.26 g/l) at the end of fermentation (**Figure 5A**, **Table 6**). The xylitol yield decreased by 21.7% from 0.46 g xylitol g consumed xylose^{-1} in L2612PR-MC to 0.36 g xylitol g consumed xylose^{-1} in L2612PR-MD. Consistent with reduced xylitol, ethanol production elevated from 2.60 g/l in L2612PR-MC to 3.65 g/l in L2612PR-MD (**Figure 5A**, **Table 6**), which was a 35.2% increase. Similarly, the xylitol yield reduced by 21.3% from 0.48 g xylitol g consumed xylose^{-1} in L2612PR-C to 0.37 g xylitol g consumed xylose^{-1} in L2612PR-D (**Table 6**, **Figure 5B**). L2612PR-D produced 50.0% more ethanol than L2612PR-C. Correspondingly, the ethanol yield increased from 0.15 g ethanol g consumed xylose^{-1} in L2612PR-C to 0.21 g ethanol g consumed xylose^{-1} in L2612PR-D, elevated by 40.0%. The average xylose consumption rate increased by 13.0% in L2612PR-D than L2612PR-C. Glycerol yield and biomass yield was the same for L2612PR-D and L2612PR-C (**Table 6**). However, the glycerol doubled in L2612PR-MD from L2612PR-MC, which may reflect increased flux towards glyceraldehyde

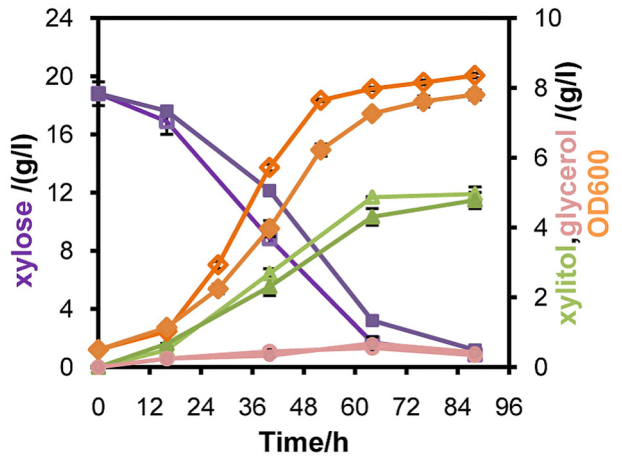


FIGURE 4 | Comparison of the performances of recombinant strain L2612PR-D (Open symbols) and its control strain L2612PR-C (Closed symbols) in xylose under aerobic conditions. L2612PR-D was integrated with one additional copy of *mXYL2* in the genome. The aerobic growth was carried out in 50 ml YPX medium with 20 g/l xylose. The initial OD_{600} of the culture was 0.5. Symbols: diamonds, OD_{600}; squares, xylose consumption; triangles, xylitol concentration; circles, glycerol concentration. The results shown were the average value of two independent experiments.

Table 5 | Aerobic xylose consumption of L2612PR-MD, L2612PR-MC, L2612PR-D, and L2612PR-C.

Strain	r_{xylose}[a]	Xylitol (g/l)[b]	Glycerol (g/l)[b]	Yield (g/g consumed xylose)[b]		
				Xylitol	Glycerol	Biomass
L2612PR-MD	0.240 ± 0.002	4.84 ± 0.20	0.42 ± 0.03	0.27 ± 0.01	0.02 ± 0.00	0.22 ± 0.00
L2612PR-MC	0.257 ± 0.002	5.16 ± 0.04	0.45 ± 0.01	0.29 ± 0.00	0.03 ± 0.00	0.22 ± 0.00
L2612PR-D	0.268 ± 0.001	4.96 ± 0.20	0.34 ± 0.02	0.28 ± 0.01	0.02 ± 0.00	0.22 ± 0.00
L2612PR-C	0.244 ± 0.001	4.78 ± 0.23	0.42 ± 0.02	0.27 ± 0.01	0.02 ± 0.00	0.21 ± 0.00

mXYL2 are overexpressed in strains L2612PR-MD and L2612PR-D in the form of a multicopy plasmid and genomic integration, respectively. L2612PR-MC and L2612PR-C were the corresponding control strain. The experiment was conducted in 50 ml YPX medium containing 20 g/l xylose under aerobic conditions. The initial cell density was adjusted to 0.5 (OD_{600}).

[a] *r_{xylose} the volumetric xylose uptake rate (g xylose/l/h) over 64 h.*

[b] *The values were calculated after 88 h of fermentation.*

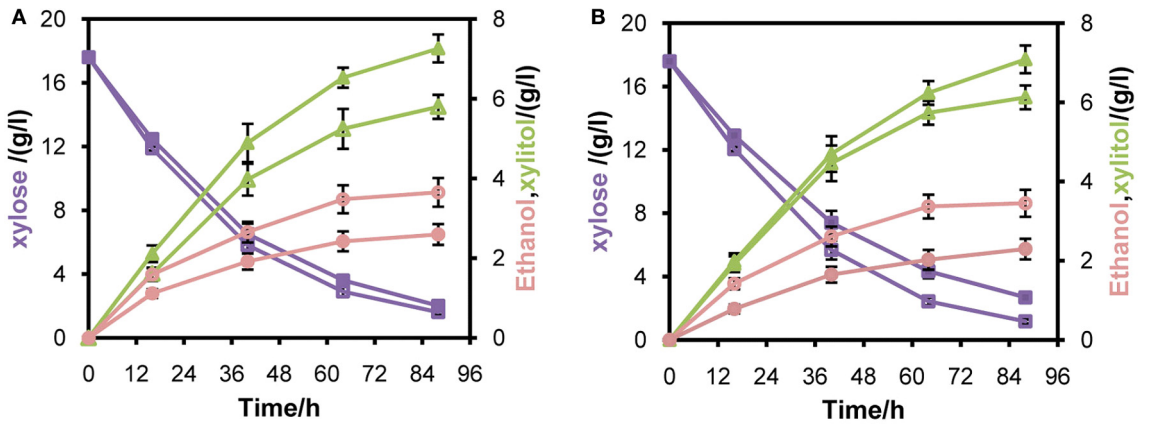


FIGURE 5 | Anaerobic batch fermentation of L2612PR-MD (open symbols), L2612PR-MC (closed symbols) (A), L2612PR-D (open symbols) and L2612PR-C (closed symbols) (B) in YPX medium. L2612PR-MD, L2612PR-D had a multicopy plasmid and an integrative plasmid with *mXYL2* based on L2612PR, respectively. L2612PR-MC and L2612PR-C were the corresponding control strains. Figure legends: squares, xylose consumption; triangles, xylitol concentration; circles, ethanol concentration. The concentration of glycerol is not presented (see **Table 6**). The results shown were the average value of two independent experiments.

Table 6 | Summary of anaerobic batch fermentation of L2612PR-MD, L2612PR-MC, L2612PR-D, and L2612PR-C in xylose medium.

Strain	r_{xylose}[a]	Ethanol (g/l)[b]	Xylitol (g/l)[b]	Glycerol (g/l)[b]	Yield (g product/consumed xylose)[b]			
					Ethanol	Xylitol	Glycerol	Biomass
L2612PR-MD	0.230 ± 0.001	3.65 ± 0.25	5.80 ± 0.40	0.23 ± 0.04	0.23 ± 0.01	0.36 ± 0.02	0.02 ± 0.00	0.06 ± 0.00
L2612PR-MC	0.221 ± 0.001	2.60 ± 0.54	7.26 ± 0.34	0.14 ± 0.01	0.17 ± 0.01	0.46 ± 0.02	0.01 ± 0.00	0.07 ± 0.01
L2612PR-D	0.234 ± 0.002	3.45 ± 0.14	6.13 ± 0.26	0.19 ± 0.01	0.21 ± 0.01	0.37 ± 0.02	0.01 ± 0.00	0.07 ± 0.01
L2612PR-C	0.207 ± 0.001	2.30 ± 0.16	7.09 ± 0.38	0.15 ± 0.02	0.15 ± 0.01	0.48 ± 0.02	0.01 ± 0.00	0.06 ± 0.01

The fermentation was carried out in 100 ml YPX medium with 20 g/l xylose under anaerobic conditions. The beginning cell density was OD_{600} 1.0 for all strains. The fermentation continued for 88 h.

[a] *r_{xylose} the volumetric xylose consumption rate (g xylose/l/h) over 64 h.*

[b] *Ethanol, xylitol and glycerol concentrations were determined at 88 h of fermentation.*

3-phosphate from non-oxidative PPP. Taken together, additional overexpression of *mXYL2* in L2612PR increased the ethanol yield and reduced the xylitol production from xylose, indicating its importance in regulating xylose metabolism by XR/XDH pathway.

DISCUSSION

In the present study, we applied the combined strategy of pathway balance and chassis optimization for improved xylose fermentation by XR/XDH pathway in *S. cerevisiae*. This approach provided a practical way to optimize xylose metabolic pathway for ethanol production and could be applied in other chemical production schemes.

In optimizing the expression of *XYL1*, it was observed that strains with *XYL1* under the control of *tADH1* or *ADH1* promoter had almost no ability to utilize xylose. Since XR determines the entry of xylose into the xylose pathway in the first step, the poor abilities to assimilate xylose may be ascribed to insufficient activity of XR. After increasing activity of XR by using *PGK1* promoter, the strain was able to assimilate and metabolize xylose faster. The result demonstrates that high activity of XR is necessary for rapid xylose metabolism and ethanol fermentation. In a previous study, an additional copy of XR increased xylose consumption rate by 1.7 fold and resulted in a 55% lower xylitol yield (Jeppsson et al., 2003). It was also observed that strain INVSc1 with *XYL1* controlled by the promoter *ADH1* consumed 9% less xylose than the strain harboring the promoter *PGK1* (Matsushika and Sawayama, 2008). The results reported in the literature and our experiments further verify the conclusion that XR activity largely determines the rate of xylose consumption and has important effects on products distribution. Additionally, in our study it was observed that the constitutive strong promoter *ADH1* was almost unable to initiate xylose metabolism. This may be ascribed to the requirement of a much higher expression of *XYL1* in our strains than strains used elsewhere. Moreover, we placed the same pathway in a multicopy plasmid and the resulting recombinant strains obtained the ability to utilize xylose and grow in xylose medium (**Figure 6**). It further indicates that *ADH1* promoter is not able to facilitate enough XR activity for rapid xylose metabolism in W303a. Employment of stronger promoters such as *TDH3* for

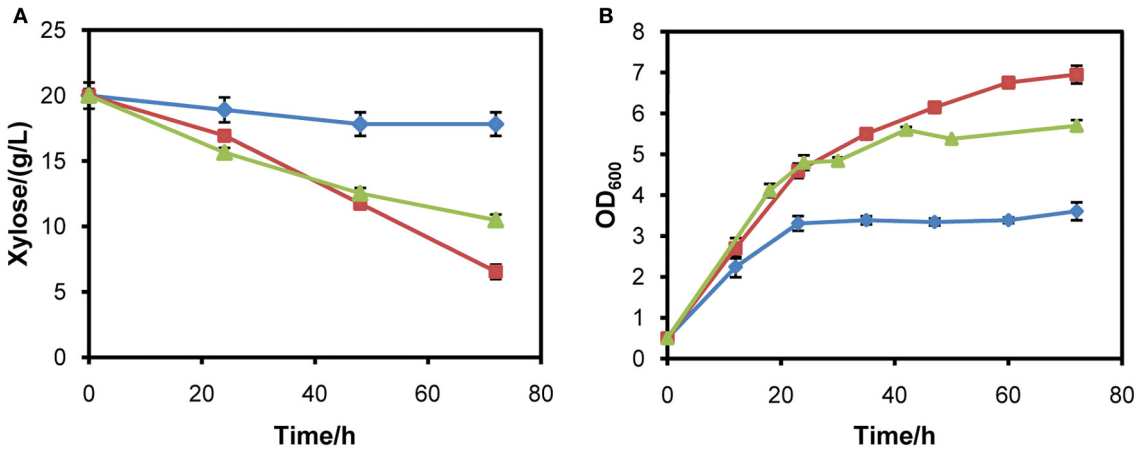


FIGURE 6 | Aerobic growth of strains W303a-C, W303a-I, and W303a-II in YPX medium. (A) Xylose consumption. **(B)** Growth profiles. Symbols: diamonds, W303a-C; squares, W303a-I; triangles, W303a-II. W303a-C is the W303a strain harboring a multicopy plasmid of pRS426. In contrast, W303a-I and W303a-II are strains harboring multiple copies of *tADH1p-XYL1-PGK1t* and *ADH1p-XYL1-PGK1t* coupling *XYL2* and *XKS1* on the backbone of plasmid pRS426, respectively. The initial cell density was adjusted to 0.5 (OD_{600}).

XYL1 expression might further enhance xylose consumption rate and shorten the fermentation time.

Besides high expression of *XYL1*, high expression of *XYL2* is also necessary to decrease xylitol production. Overexpression of *mXYL2* by a multicopy plasmid or genomic integration in strain L2612PR optimized the xylose metabolic pathway for improved ethanol production. These results strongly support the conclusion that high activity of XDH is required for efficient xylose metabolism, in agreement with previous research (Jin and Jeffries, 2003; Karhumaa et al., 2006; Krahulec et al., 2009; Kim et al., 2012). Higher XDH activity may increase the flux from xylitol to xylulose and thereafter flux to central metabolism through phosphorylation and non-oxidative PPP (Karhumaa et al., 2006). In Krahulec and co-workers' research, the xylose pathway with mutant XDH with three-fold higher specificity on $NADP^+$ than NAD^+ did not improve ethanol production or decrease xylitol accumulation (Krahulec et al., 2009). The authors suggested the failure resulted from low activity of XDH since cofactor balance had been attempted. Jin's work also confirmed the importance of XDH by observing that overexpression of XDH did reduce xylitol secretion (Jin and Jeffries, 2003). The 11.25-fold increase in XDH activity caused a 50% reduction in xylitol formation by using a multicopy of *XYL2* instead of genomic integration of *XYL2*. Different from his work without overexpression of *XKS1*, we also over-expressed *XKS1* to enhance XK activity and significant increase in ethanol production was obtained. In his work, as much as 3 g/l xylulose was produced during xylose fermentation and the highest ethanol yield reached about 0.04 g/g, much lower than that in L2612PR-D and L2612PR-MD which originated from the same yeast chassis L2612, implying that the low XK activity limited the conversion of xylulose to non-oxdative PPP and ethanol production. This result indicates that sufficient XK activity contributes to efficient ethanol production from xylose.

Moreover, overexpression of *XYL2* by a multicopy plasmid did not reduce xylitol secretion and biomass formation in aerobic conditions. However, overexpression of *XYL2* by integration of one additional copy accelerated xylose uptake rate despite of no improvement in xylitol formation. Adverse effect of grave overexpression of *XYL2* may be responsible for this. A previous study supports the hypothesis that introduction of a multicopy plasmid pRS424-PGK1p-XYL2 in the xylose-fermenting yeast YSX3 slows down xylose uptake while genomic integration of PGK1p-XYL2 accelerates xylose uptake rate by 36.5% (Kim et al., 2012). "Just enough" overexpression of *XYL2* is essential for efficient xylose fermentation. Further optimization of *XYL2* by probing promoters of different strengths possibly achieves a better xylose-fermenting strain in our case.

As for the high xylitol yield in L2612PR-D, this may be ascribed to the limited flux of the non-oxidative pathway which compelled increased carbon flux to channel into xylitol. In a previous study, upregulation of PPP genes increased the specific growth rate by 2-fold (Karhumaa et al., 2005). Under aerobic conditions, the carbon flux was enlarged compared to anaerobic conditions, which has been observed in other studies (Jouhten et al., 2008). The limited activities of non-oxidative PPP enzymes restrict the flux of carbon source toward glycolytic pathway and excessive carbon source leaks out in the form of xylitol. A metabolic flux analysis and proteomic assay of key enzymes in PPP and glycolitic pathway could verify the hypothesis and provide new clues for further engineering.

Chassis optimization for expression of XR/XDH pathway is another approach for efficient xylose fermentation besides direct regulation of heterogeneous metabolic pathway. The performances of different yeast strains having identical xylose pathways vary in xylose fermentation. Matsushika and co-workers compared the xylulose fermentation of nine industrial diploid strains and the best chassis IR-2 consumed xylulose faster than other candidate strains (Matsushika et al., 2009c). The expression of

XR/XDH pathway in IR-2 allowed an efficient xylose-fermenting strain MA-R5. The comparison indicated that the downstream pathway or xylulose transport system for xylulose metabolism differed largely in these strains. Another example of the inconsistency is that strain TMB3066 grows slower than one of the best xylose-fermenting strain RWB217, both of which have identical xylose isomerase pathways and originate from CEN.PK laboratory strains (Karhumaa et al., 2007). The authors claimed that the differences may result from higher activities of XK or the PPP enzymes in RWB217. Likely, differences in enzymatic activities of the related pathway such as PPP and xylose transportation system, and different regulatory modification over metabolic pathway may contribute to the distinct behavior of W303PR and L2612PR. Systematic comparison between W303PR and L2612PR can give rise to new target genes or networks for improving xylose fermentation further (Wahlbom et al., 2003; Karhumaa et al., 2009).

In optimizing the expression level of *XYL1*, we used different promoters with varied strengths. However, this process was also complemented by applying a promoter library composed of promoter mutants. The mutants with various strengths were assembled into a genetic network and the desired function was obtained by sampling the continuum of gene expression at a series of discrete points or by model-guided rational design (Alper et al., 2005; Hammer et al., 2006; Ellis et al., 2009; Du et al., 2012). The use of such a promoter library made the optimization faster and easier due to the sequence identity or similarity of promoter mutants and thus the construction of genetic network could be accomplished by common cloning manipulation or DNA assembler method (Shao et al., 2009). Furthermore, the introduction of promoters stemming from the same source would avoid unpredicted regulation disorders caused by using promoters of different sources. In future work, such a promoter library can be applied for optimizing the xylose metabolic pathway with additional efforts.

CONCLUSION

A xylose pathway composed of *XYL1*, m*XYL2,* and *XKS1* was constructed. Three promoters *tADH1, ADH1,* and *PGK1* were used to modulate the relative expression levels of *XYL1* and m*XYL2*. The results showed that only the strongest promoter *PGK1* facilitated xylose uptake and metabolism in the constructed strains, demonstrating that it is necessary for the high activity of XR in xylose fermentation. Comparison of the fermentation performances between the constructed strains from chassis W303a and L2612 led to a more efficient xylose-fermenting strain L2612PR, which derived from strain L2612. To enhance the expression of m*XYL2*, an extra copy or multiple copies of *mXYL2* was introduced, leading to the generation of strains L2612PR-MD and L2612PR-D, which exhibited 21% lower xylitol production and 35–40% higher ethanol production. The results indicated the importance of XDH in reducing xylitol accumulation and maximizing the flux to downstream xylose metabolic pathway. In summary, our results have demonstrated that it is effective to combine chassis optimization and heterogeneous pathway balance in constructing ethanolic xylose-fermenting *S. cerevisiae*.

ACKNOWLEDGMENTS

This work was funded by the National High Technology Research and Development Program ("863" Program: 2012AA02A701), the National Natural Science Foundation of China (Major International Joint Research Project: 21020102040), the National Basic Research Program of China ("973" Program: 2013CB733601), International Joint Research Project of Tianjin (11ZCGHHZ00500). We thank Professor Thomas Jeffries at University of Wisconsin–Madison for providing yeast strain L2612.

REFERENCES

Ajikumar, P. K., Xiao, W. H., Tyo, K. E. J., Wang, Y., Simeon, F., Leonard, E., et al. (2010). Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330, 70–74.

Alper, H., Fischer, C., Nevoigt, E., and Stephanopoulos, G. (2005). Tuning genetic control through promoter engineering. *Proc. Natl. Acad. Sci. U.S.A.* 102, 12678–12683.

Bengtsson, O., Hahn-Hagerdal, B., and Gorwa-Grauslund, M. F. (2009). Xylose reductase from *Pichia stipitis* with altered coenzyme preference improves ethanolic xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Biotechnol. Biofuels* 2, 9.

Bond-Watts, B. B., Bellerose, R. J., and Chang, M. C. Y. (2011). Enzyme mechanism as a kinetic control element for designing synthetic biofuel pathways. *Nat. Chem. Biol.* 7, 222–227.

Boghigian, B. A., Salas, D., Ajikumar, P. K., Stephanopoulos, G., and Pfeifer, B. A. (2012). Analysis of heterologous taxadiene production in K- and B-derived *Escherichia coli*. *Appl. Microbiol. Biotechnol.* 93, 1651–1661.

Chu, B. C., and Lee, H. (2007). Genetic improvement of *Saccharomyces cerevisiae* for xylose fermentation. *Biotechnol. Adv.* 25, 425–441.

Ding, M.-Z., Wang, X., Yang, Y., and Yuan, Y.-J. (2012). Comparative metabolic profiling of parental and inhibitors-tolerant yeasts during lignocellulosic ethanol fermentation. *Metabolomics* 8, 232–243.

Du, J., Yuan, Y., Si, T., Lian, J., and Zhao, H. (2012). Customized optimization of metabolic pathways by combinatorial transcriptional engineering. *Nucleic Acids Res*. doi: 10.1093/nar/gks549. [Epub of ahead of print].

Dueber, J. E., Wu, G. C., Malmirchegini, G. R., Moon, T. S., Petzold, C. J., Ullal, A. V., et al. (2009). Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* 27, U753–U759.

Ellis, T., Wang, X., and Collins, J. J. (2009). Diversity-based, model-guided construction of synthetic gene networks with predicted functions. *Nat. Biotechnol.* 27, 465–471.

Fischer, C. R., Klein-Marcuschamer, D., and Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metab. Eng.* 10, 295–304.

Gietz, R. D., Schiestl, R. H., Willems, A. R., and Woods, R. A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. *Yeast* 11, 355–360.

Hammer, K., Mijakovic, I., and Jensen, P. R. (2006). Synthetic promoter libraries–tuning of gene expression. *Trends Biotechnol.* 24, 53–55.

Hector, R. E., Dien, B. S., Cotta, M. A., and Qureshi, N. (2011). Engineering industrial *Saccharomyces cerevisiae* strains for xylose fermentation and comparison for switchgrass conversion. *J. Ind. Microbiol. Biotechnol.* 38, 1193–1202.

Jeffries, T. W. (2006). Engineering yeasts for xylose metabolism. *Curr. Opin. Biotechnol.* 17, 320–326.

Jeppsson, M., Bengtsson, O., Franke, K., Lee, H., Hahn-Hagerdal, R., and Gorwa-Grauslund, M. F. (2006). The expression of a *Pichia stipitis* xylose reductase mutant with higher K-M for NADPH increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 93, 665–673.

Jeppsson, M., Johansson, B., Hahn-Hagerdal, B., and Gorwa-Grauslund, M. F. (2002). Reduced oxidative pentose phosphate pathway flux in recombinant xylose-utilizing *Saccharomyces cerevisiae* strains improves the ethanol yield from xylose. *Appl. Environ. Microbiol.* 68, 1604–1609.

Jeppsson, M., Traff, K., Johansson, B., Hahnhagerdal, B., and Gorwagrauslund, M. (2003). Effect of enhanced xylose reductase activity on xylose consumption and product distribution in xylose-fermenting recombinant. *FEMS Yeast Res.* 3, 167–175.

Jin, Y. S., and Jeffries, T. W. (2003). Changing flux of xylose metabolites by altering expression of xylose reductase and xylitol dehydrogenase in recombinant *Saccharomyces cerevisiae*. *Appl. Biochem. Biotechnol.* 105, 277–285.

Jin, Y. S., Laplaza, J. M., and Jeffries, T. W. (2004). *Saccharomyces cerevisiae* engineered for xylose metabolism exhibits a respiratory response. *Appl. Environ. Microbiol.* 70, 6816–6825.

Jouhten, P., Rintala, E., Huuskonen, A., Tamminen, A., Toivari, M., Wiebe, M., et al. (2008). Oxygen dependence of metabolic fluxes and energy generation of *Saccharomyces cerevisiae* CEN.PK113-1A. *BMC Syst. Biol.* 2, 60.

Kao, K. C., Ghosh, A., Zhao, H., and Price, N. D. (2011). Genome-scale consequences of cofactor balancing in engineered pentose utilization pathways in *Saccharomyces cerevisiae*. *PLoS ONE* 6:e27316. doi: 10.1371/journal.pone.0027316

Karhumaa, K., Fromanger, R., Hahn-Hägerdal, B., and Gorwa-Grauslund, M.-F. (2006). High activity of xylose reductase and xylitol dehydrogenase improves xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 73, 1039–1046.

Karhumaa, K., Garcia Sanchez, R., Hahn-Hagerdal, B., and Gorwa-Grauslund, M. F. (2007). Comparison of the xylose reductase-xylitol dehydrogenase and the xylose isomerase pathways for xylose fermentation by recombinant *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 6, 5.

Karhumaa, K., Hahn-Hagerdal, B., and Gorwa-Grauslund, M. F. (2005). Investigation of limiting metabolic steps in the utilization of xylose by recombinant *Saccharomyces cerevisiae* using metabolic engineering. *Yeast* 22, 359–368.

Karhumaa, K., Pahlman, A. K., Hahn-Hagerdal, B., Levander, F., and Gorwa-Grauslund, M. F. (2009). Proteome analysis of the xylose-fermenting mutant yeast strain TMB (3400). *Yeast* 26, 371–382.

Kim, S. R., Ha, S. J., Kong, I. I., and Jin, Y. S. (2012). High expression of XYL2 coding for xylitol dehydrogenase is necessary for efficient xylose fermentation by engineered *Saccharomyces cerevisiae*. *Metab. Eng.* 14, 336–343.

Krahulec, S., Klimacek, M., and Nidetzky, B. (2009). Engineering of a matched pair of xylose reductase and xylitol dehydrogenase for xylose fermentation by *Saccharomyces cerevisiae*. *Biotechnol. J.* 4, 684–694.

Kuyper, M., Harhangi, H. R., Stave, A. K., Winkler, A. A., Jetten, M. S., De Laat, W. T., et al. (2003). High-level functional expression of a fungal xylose isomerase: the key to efficient ethanolic fermentation of xylose by *Saccharomyces cerevisiae*? *FEMS Yeast Res.* 4, 69–78.

Liu, E. K., and Hu, Y. (2010). Construction of a xylose-fermenting *Saccharomyces cerevisiae* strain by combined approaches of genetic engineering, chemical mutagenesis and evolutionary adaptation. *Biochem. Eng. J.* 48, 204–210.

Lu, C., and Jeffries, T. (2007). Shuffling of promoters for multiple genes to optimize xylose fermentation in an engineered *Saccharomyces cerevisiae* strain. *Appl. Environ. Microbiol.* 73, 6072–6077.

Matsushika, A., Inoue, H., Kodaki, T., and Sawayama, S. (2009a). Ethanol production from xylose in engineered *Saccharomyces cerevisiae* strains: current state and perspectives. *Appl. Microbiol. Biotechnol.* 84, 37–53.

Matsushika, A., Inoue, H., Murakami, K., Takimura, O., and Sawayama, S. (2009b). Bioethanol production performance of five recombinant strains of laboratory and industrial xylose-fermenting *Saccharomyces cerevisiae*. *Bioresour. Technol.* 100, 2392–2398.

Matsushika, A., Inoue, H., Watanabe, S., Kodaki, T., Makino, K., and Sawayama, S. (2009c). Efficient bioethanol production by a recombinant flocculent *Saccharomyces cerevisiae* strain with a genome-integrated NADP(+)-dependent xylitol dehydrogenase gene. *Appl. Environ. Microbiol.* 75, 3818–3822.

Matsushika, A., and Sawayama, S. (2008). Efficient bioethanol production from xylose by recombinant *Saccharomyces cerevisiae* requires high activity of xylose reductase and moderate xylulokinase activity. *J. Biosci. Bioeng.* 106, 306–309.

Matsushika, A., Watanabe, S., Kodaki, T., Makino, K., Inoue, H., Murakami, K., et al. (2008). Expression of protein engineered NADP plus-dependent xylitol dehydrogenase increases ethanol production from xylose in recombinant *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* 81, 243–255.

Petschacher, B., and Nidetzky, B. (2008). Altering the coenzyme preference of xylose reductase to favor utilization of NADH enhances ethanol yield from xylose in a metabolically engineered strain of *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 7, 9.

Shao, Z. Y., Zhao, H., and Zhao, H. M. (2009). DNA assembler, an *in vivo* genetic method for rapid construction of biochemical pathways. *Nucleic Acids Res.* 37, 10.

Sikorski, R. S., and Hieter, P. (1989). A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122, 19–27.

Stephanopoulos, G. (2007). Challenges in engineering microbes for biofuels production. *Science* 315, 801–804.

Wahlbom, C. F., Cordero Otero, R. R., Van Zyl, W. H., Hahn-Hagerdal, B., and Jonsson, L. J. (2003). Molecular analysis of a *Saccharomyces cerevisiae* mutant with improved ability to utilize xylose shows enhanced expression of proteins involved in transport, initial xylose metabolism, and the pentose phosphate pathway. *Appl. Environ. Microbiol.* 69, 740–746.

Walfridsson, M., Anderlund, M., Bao, X., and Hahn-Hagerdal, B. (1997). Expression of different levels of enzymes from the *Pichia stipitis* XYL1 and XYL2 genes in *Saccharomyces cerevisiae* and its effects on product formation during xylose utilisation. *Appl. Microbiol. Biotechnol.* 48, 218–224.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 July 2012; accepted: 19 September 2012; published online: 05 October 2012.

Citation: Zha J, Hu M-L, Shen M-H, Li B-Z, Wang J-Y and Yuan Y-J (2012) Balance of XYL1 and XYL2 expression in different yeast chassis for improved xylose fermentation. Front. Microbio. ***3****:355. doi: 10.3389/fmicb.2012.00355*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 25 January 2013
doi: 10.3389/fmicb.2013.00005

Preparing synthetic biology for the world

Gerd H. G. Moe-Behrens[1], Rene Davis[2] and Karmella A. Haynes[2]*

[1] *Leukippos Institute, Berlin, Germany*
[2] *School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ, USA*

Edited by:
David Nielsen, Arizona State University, USA

Reviewed by:
Lei Chen, Tianjin University, China
Tae Seok Moon, Washington University in St. Louis, USA

***Correspondence:**
Karmella A. Haynes, School of Biological and Health Systems Engineering, Arizona State University, 501 E Tyler Mall, 9709 Tempe, AZ 85287, USA.
e-mail: karmella.haynes@asu.edu

Synthetic Biology promises low-cost, exponentially scalable products and global health solutions in the form of self-replicating organisms, or "living devices." As these promises are realized, proof-of-concept systems will gradually migrate from tightly regulated laboratory or industrial environments into private spaces as, for instance, probiotic health products, food, and even do-it-yourself bioengineered systems. What additional steps, if any, should be taken before releasing engineered self-replicating organisms into a broader user space? In this review, we explain how studies of genetically modified organisms lay groundwork for the future landscape of biosafety. Early in the design process, biological engineers are anticipating potential hazards and developing innovative tools to mitigate risk. Here, we survey lessons learned, ongoing efforts to engineer intrinsic biocontainment, and how different stakeholders in synthetic biology can act to accomplish best practices for biosafety.

Keywords: synthetic biology, biosafety research, containment of biohazards, risk assessment

BEYOND THE LAB—WHERE SYNTHETIC ORGANISMS MAY APPEAR IN THE FUTURE

Synthetic biologists aim to create living systems that serve immediate human needs, rather than waiting for evolution to produce a useful biological function. Reverse-engineered organisms are currently being used in closed industrial settings to produce fuels (e.g., Chromatin Inc., Ginkgo Bioworks, LS9 Inc., Solazyme, Verdezyne, and Synthetic Genomics), generate renewable chemicals of commercial value (e.g., Genencor, Genomatica Sustainable Chemicals, and Verdezyne), and reduce the cost of pharmaceutical production (e.g., Ambrx and Amyris). In these cases, preventing accidental release is straightforward. Closed industrial settings use synthetic organisms where physical containment and proper waste management can be monitored and enforced by regulatory bodies (e.g., Environmental Health and Safety groups and the Environmental Protection Agency in the U.S.). Since accidental release is still a possibility, containment mechanisms that are built into the synthetic organism could be used to enhance safe use. In contrast to closed settings, open systems (e.g., bioremediation, agriculture, and healthcare applications) distribute synthetic organisms across broader spaces in an unpredictable manner, and thus require intrinsic containment mechanisms.

Recent reports of clinical applications and anticipated uses of synthetic organisms show that the appearance of synthetic organisms in broader spaces is on the horizon. Groups in Europe have tested engineered microbes to treat human illnesses such as Crohn's disease (Braat et al., 2006) and oral inflammation (mucositis) (Caluwaerts et al., 2010). Some anticipate the use of engineered organisms in future space travel, taking engineered organisms beyond our planet ("The Initiative | Synthetic Biology," last accessed October 21, 2012, http://syntheticbiology.arc.nasa.gov/node/1). Recent bio-fiction video projects paint intriguing pictures of engineered synthetic organisms operating in personalized contexts as consumer goods ("E. chromi," last accessed October 22, 2012, http://vimeo.com/19759432; "Tuur van Balen—Hacking Yoghurt," last accessed October 22, 2012, http://www.youtube.com/watch?v=Co8NOnErrPU), as living, evolving therapeutics ("Cellularity," last accessed October 22, 2012, http://vimeo.com/10274649), and even as recreational drugs ("Compound 74," last accessed October 22, 2012, http://www.youtube.com/watch?v=lQjF8ir4SKs). These pieces are styled to provoke the viewer with conceptual yet plausible scenarios, and to make us question where the technology may lead. Recent and speculative synthetic biology applications have catalyzed discussions of releasing synthetic organisms into the public sphere. The synthetic biology research community should respond by making visible efforts to address safe use and containment.

AWARENESS, CONCERNS, AND PUBLIC SAFETY CHALLENGES

Synthetic biology is unique because of its ethos—to use design principles from nature for the rational design and construction of molecular systems with novel, reliable functions (Heinemann and Panke, 2006). Synthetic biology uses the same molecular biology practices as genetic engineering. Therefore, the techniques employed by synthetic biology do not pose any unique safety threats. Technologies that made genetic engineering a reality in the early 1970's spurred the organization of the Asilomar Conference on Recombinant DNA (rDNA) to discuss biosafety. Over the subsequent decades, concerns have waned. Now that synthetic biology has gained substantial attention and popularity, concerns about rDNA have re-emerged.

Synthetic biology has been admonished as an extreme form of genetic engineering by watch groups ("111 Organizations Call for Synthetic Biology Moratorium," last accessed October 22, 2012, http://news.sciencemag.org/scienceinsider/2012/03/111-organizations-call-for-synth.html). Catchphrases such as

"extreme genetic engineering" or "playing God," which cast synthetic biology as a threat to human well-being, diminish the fact that the core ethos of synthetic biology, engineering (Heinemann and Panke, 2006), is a design process that aims to make human inventions reliable, predictable, and safe. Policies based on the precautionary principle could stunt the development of synthetic biology. Ironically, synthetic organisms might turn out to be the best solution for global health challenges and ecological problems such as accessible healthcare and carbon emission.

One recent study concluded that the fears that synthetic biologists are tampering with nature or "playing God" are not sufficient to establish a strong argument to restrict synthetic biology research for the sake of human well-being (Link, 2012). Along the same vein, if harnessing electricity had been restricted before the industrial revolution, mankind may never have experienced the benefits of modern electronic technologies. Still, overly optimistic promises of the benefits of synthetic biology are as unsound as than fearful perceptions. If synthetic organisms and their derivatives are to become as ubiquitous as electronic devices, then synthetic biologists must openly address the responsible and safe use of synthetic biological systems.

We can assuage fear and foster familiarity with synthetic biology through effective efforts to inform the public of the actual risks of synthetic biology research, the steps we can take to address the risks, and how this technology can be harnessed to meet society's needs. Since the 1970's, attempts have been made to address public concerns regarding the safety of genetically modified microbes (Schmidt and de Lorenzo, 2012). In 2009, the US Department of Health and Human Services released a finalized list of guidelines for identifying hazardous synthetic agents based on DNA sequence homology ("Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA," November 19, 2010, available at http://www.phe.gov/preparedness/legal/guidance/syndna/Pages/default.aspx). However, scientists have expressed doubt about the usefulness of an approach that focuses only on DNA sequences (Eisenstein, 2010). The biosafety information is enveloped in very technical language that is not accessible to non-specialists. There is little evidence that these efforts have swayed public perceptions ("Awareness and Impressions of Synthetic Biology," September 9, 2010, available at http://www.synbioproject.org/library/publications/archive/6456/). A request for a synthetic biology moratorium released by 111 organizations including ETC Group and Friends of the Earth is an example of how the public may react when coordinated efforts toward executing containment and control strategies are not highly visible ("111 Organizations Call for Synthetic Biology Moratorium," last accessed October 22, 2012, http://news.sciencemag.org/scienceinsider/2012/03/111-organizations-call-for-synth.html).

The Woodrow Wilson Synthetic Biology Project has recently developed a public web portal to present developments and biosafety activities in the field to non-specialists ("Synthetic Biology Project," last accessed October 21, 2012, http://www.synbioproject.org/). In addition, the Woodrow Wilson group has proposed a framework for risk research that addresses four public safety issues (Dana et al., 2012). First, how might synthetic organisms interact with natural ones? Second, how well will they survive in receiving environments? Third, how might they evolve and adapt to fill new ecological niches? Lastly, what is the potential for gene transfer into unmodified organisms? The synthetic biology community can address these questions through designing, building, and testing synthetic systems.

GENETIC SAFEGUARDS: BUILDING CONTAINMENT MECHANISMS INTO SYNTHETIC LIFE

Decades of work in closed settings, such as research labs, might suggest that engineered organisms pose little threat. So far, no bio-hazardous incidents have been traced back to engineered organisms (Schmidt and de Lorenzo, 2012). Furthermore plasmids, the small circular pieces of DNA that encode engineered functions, persist poorly in host cells over time. Reduced viability in plasmid-carrying microbes compared to non-engineered parent strains has been observed (Betenbaugh et al., 1989). Nonetheless, if speculations correctly predict the future use of synthetic biology, the technology will scale to large industrial volumes, introduce large numbers of synthetic organisms into the environment for bioremediation, and be used in private spaces where dispersal and disposal are difficult to monitor. Innovative containment mechanisms will improve safety in open synthetic systems. Genetic safeguards operate within the synthetic organisms themselves to prevent escaped microbes from proliferating unchecked and to prevent the spread of engineered genetic material into unintended host cells.

CONTAINMENT THROUGH ENGINEERED AUXOTROPHY

One method for biocontainment is to engineer auxotrophic organisms that are unable to synthesize an essential compound required for their survival. Once auxotrophic microbes escape the controlled environment where the compound is supplied, they rapidly die (**Figures 1A,B**). The first active genetic containment system, reported in 1987, used engineered auxotrophy (Molin et al., 1987). Prior to this innovation, genetically compromised bacteria were used for industrial applications. These weakened microbes may be safe to use, but this approach reduces industrial productivity and increases product cost. Molin and colleagues designed a DNA cassette that could function as a conditional suicide system in any healthy bacterial strain (Molin et al., 1987). In the absence of an artificially supplied growth supplement, the cassette produced Hok, a toxic protein that damages bacterial cell membranes (Gerdes et al., 1986) and kills the cells. Another version of this system used stochastic activation of Hok to kill a predetermined fraction of cells per unit of time (Molin et al., 1987). Stochastic activation could help to tune the level of lethality so that an optimal level of bioproduction is achieved.

A pioneering containment system for bioremediation applications was published in 1991 by Contreras et al. (1991). They designed a genetic switch to kill microbes once a mission was completed (e.g., after degrading an environmental pollutant). Cells engineered to destroy the pollutant compound benzoate remained alive in the presence of that compound. Benzoate depletion activated an artificial *xylS* gene switch, which produced Gef, a toxic protein that functions in a similar manner as Hok (Poulsen et al., 1989). Later, Jensen et al. showed that two copies of the *xylS-gef* switch improved killing of benzoate-depleted cells (Jensen et al., 1993). Further improvements were pursued by testing other

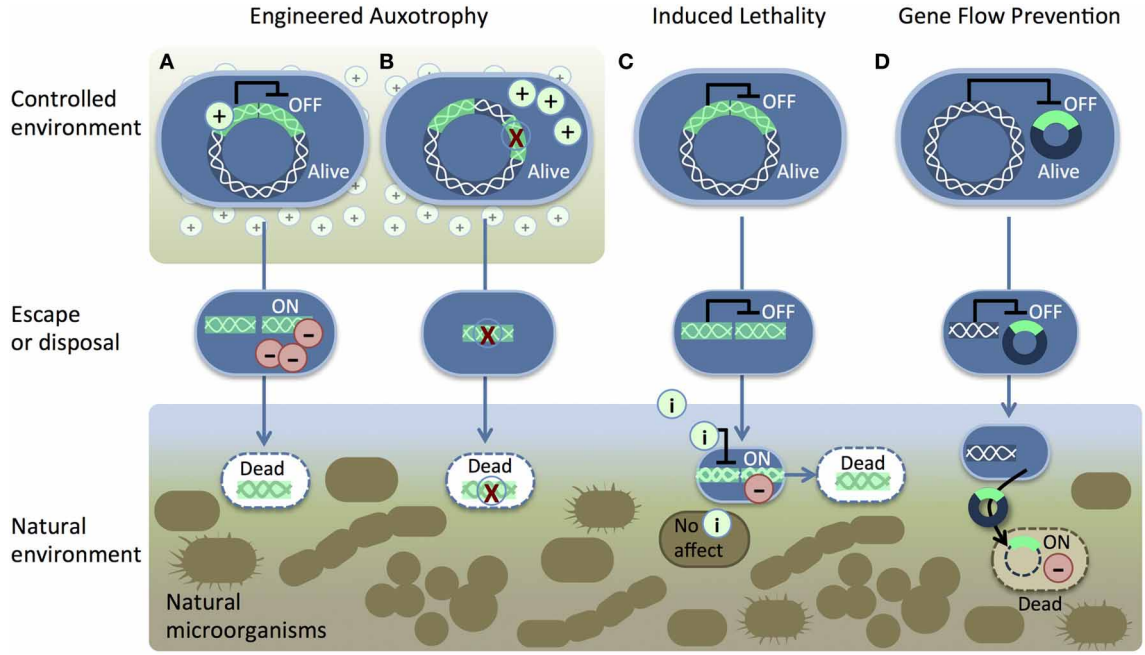


FIGURE 1 | Genetic safeguard strategies. Recombinant DNA (bright green) is introduced into the host chromosome (white wavy lines). Two pathways for engineered auxotrophy **(A,B)** kill synthetic organisms (blue) once they lose access to a supplement (+) in a controlled environment. The supplement either **(A)** suppresses a toxic gene product (–) or **(B)** provides nutrition to compensate for a genetic deletion (red X). The induced lethality system **(C)** produces a toxic gene product (–) in response to an inducer (i) such as IPTG, sucrose, arabinose, or heat. Gene-flow prevention **(D)** is accomplished by placing a toxic gene into the recombinant DNA (dark blue/bright green circle) in an immune host. Transfer of the recombinant plasmid kills unintended host cells.

toxic proteins, including streptavidin (Szafranski et al., 1997). A different gene switch has been designed for trophic containment of engineered yeast. In the absence of high glucose concentrations, the yeast express either toxic RelF or Serratia NucA DNase (Kristoffersen et al., 2000; Balan and Schenberg, 2005).

Deletions of essential genes have been used to improve the efficacy of genetic containment. Ronchel and colleagues placed a dual system in cells where the aspartate-β-semialdehyde dehydrogenase gene (*asd*) was deleted (Ronchel and Ramos, 2001). The *asd* deletion renders Pseudomonas *putida* dependent upon diaminopimelic acid, methionine, lysine, and threonine supplements. An engineered *xylS*-controlled *asd* gene was introduced into cells along with the *xylS-gef* system, so that benzoate depletion caused both production of Gef and deactivation of the growth-promoting gene *asd*. Recently, interleukin 10-secreting auxotrophic *Lactococcus lactis* (Steidler et al., 2003) has been used to treat Crohn's Disease (Braat et al., 2006). In order to prevent uncontrolled proliferation, auxotrophy was created by eliminating thymidylate synthase (*thyA*) (Steidler et al., 2003). The population of engineered bacteria fell below detection limits in the absence of thymidine and did not acquire functional thymidylate synthase from other bacteria in controlled experiments in pigs. A thorough review of biosafety practices for genetically modified *L. lactis* has been recently published (Bahey-El-Din, 2012). Engineered auxotrophy is also highly effective in eukaryotes, such as the aquatic plant *Lemna*. In *Lemna* engineered to produce therapeutic proteins and vaccines, isoleucine auxotrophy was created by using RNA interference (RNAi) to silence threonine deaminase (Nguyen et al., 2012). Engineered auxotrophy via gene knock-out or silencing can remain effective as long as gene transfer does not compensate for the mutations and as long as the nutrient that is required for survival is not available outside of the target environment.

ACTIVE CONTAINMENT THROUGH INDUCED LETHALITY

Induced lethality (**Figure 1C**), or "kill switch" mechanisms have been engineered as genetic safeguards. The engineered organisms survive normally until an inducer signal (e.g., IPTG) is added. Induced lethality could be used clean up synthetic microbe spills without harming other cells in the environment. An early proof of concept switch was created by placing the toxic *hok* gene under the control of the strong and inducible *lac* promoter (Bej et al., 1988). Later, other toxic proteins that are homologous to Hok (Poulsen et al., 1989), such as RelF (Knudsen and Karlström, 1991) and Gef (Bej et al., 1992), were tested in *lac*-controlled kill switches. In microcosm studies, Knudsen and colleagues demonstrated effective IPTG-induced kill switch activation of engineered microbes in soil, seawater, and an animal model (rat intestine) (Knudsen et al., 1995). Other inducers such as heat (Ahrenholtz et al., 1994), sucrose (Recorbet et al., 1993), and arabinose (Li and Wu, 2009) have been used to activate death in engineered cells.

Recent developments in artificial cell division counters have brought us closer to timed, automatic death of synthetic cells. A set of synthetic genetic components that includes a riboregulated transcriptional cascade and a recombinase-based cascade of memory units can count up to three events (Friedland et al.,

2009). These counting circuits could be designed to limit the life span of synthetic cells by linking the circuit to intracellular cell cycle-cues. Genes such as *hok*, *relF*, or *gef* could be added so that a toxic protein is produced after a certain number of cell cycles (Lu et al., 2009).

GENE-FLOW BARRIERS

In the absence of prohibitive mechanisms, plasmids are frequently transferred between microbes through conjugation (Heuer and Smalla, 2007). Furthermore, the death of an engineered organism is not necessarily accompanied by the disappearance of its rDNA. Cell-free DNA can remain functional and transferable even after exposure to harsh conditions (Lyon et al., 2010). Thus, scientists have developed systems to prevent the uptake and inheritance of engineered genetic material.

Gene-flow barriers are created by including a killer gene in the rDNA and placing the rDNA into an immune host. Immunity from the killer gene is provided by a repressor protein that blocks killer gene expression. If unintended hosts take up the engineered DNA, the lethal gene is decoupled from immunity and the new host cell dies (**Figure 1D**). RNA cleaving by colicin E3 reduces survival of recipient cells (Díaz et al., 1994; Munthali et al., 1996). Other systems include an additional safety measure that uses nucleases, such as *EcoRI*, to destroy DNA in recipient cells (Torres et al., 2000). Torres and colleagues created a reinforced barrier by combining colicin E3 and the *Eco*RI DNA endonuclease in a single system (Torres, 2003).

Stable integration of the rDNA may be a simpler way to effectively prevent gene-flow. For instance, integration of rDNA into an engineered microbe's chromosomes reduces transmissibility of the synthetic genetic material (Ronchel et al., 1995; Munthali et al., 1996; Panke et al., 1998; Martínez-García et al., 2011). In plants, rDNA can be inserted into chloroplast DNA instead of chromosomal DNA. Thus, the rDNA remains in stationary plant tissues more often than transmissible pollen granules (Svab and Maliga, 2007).

OBSERVED FAILURES OF ENGINEERED SAFEGUARDS

Unfortunately, not all genetic safeguards are completely failproof. Occasionally, an engineered microbe's DNA may undergo a spontaneous mutation that destroys the genetic switch (Knudsen and Karlström, 1991) or bestows immunity against the lethal gene (Bej et al., 1988), enabling the engineered cells to propagate outside of their contained environment. Are laboratory-measured failure rates high enough to warrant serious concern? How do failure rates scale with population size? We surveyed laboratory studies of genetic safeguard systems and calculated the expected number of survivors per 2 liters, the volume of the familiar soft drink container (**Figure 2**). A proliferating culture of *Escherichia coli* that contains about 100 million (1×10^8) cells per milliliter of culture medium [BioNumbers record ID 10985 (Milo et al., 2009)] would consist of 100 billion cells in a 2-liter volume. **Table 1** shows the projected number of microbes that would survive after the activation of a genetic safeguard. These numbers are based on escape rates reported for various systems tested under laboratory conditions and in environmental microcosm models (i.e., soil, water, rat intestine). The recommended limit of engineered microbe survival or engineered DNA transmission is less than 1 cell per 10^8 cells (Wilson, 1993), or less than 1000 cells per 2 liters, according to the National Institutes of Health. So far, only a few of the genetic safeguards meet this limit. Synthetic biologists should consider the difficulty in meeting this standard when designing genetically-contained synthetic organisms.

IMPROVING GENETIC SAFEGUARDS

SOLUTIONS FOR KILL SWITCH FAILURE

Toxic gene cassettes are attractive because they enable scientists to potentially add a biocontainment mechanism to any synthetic

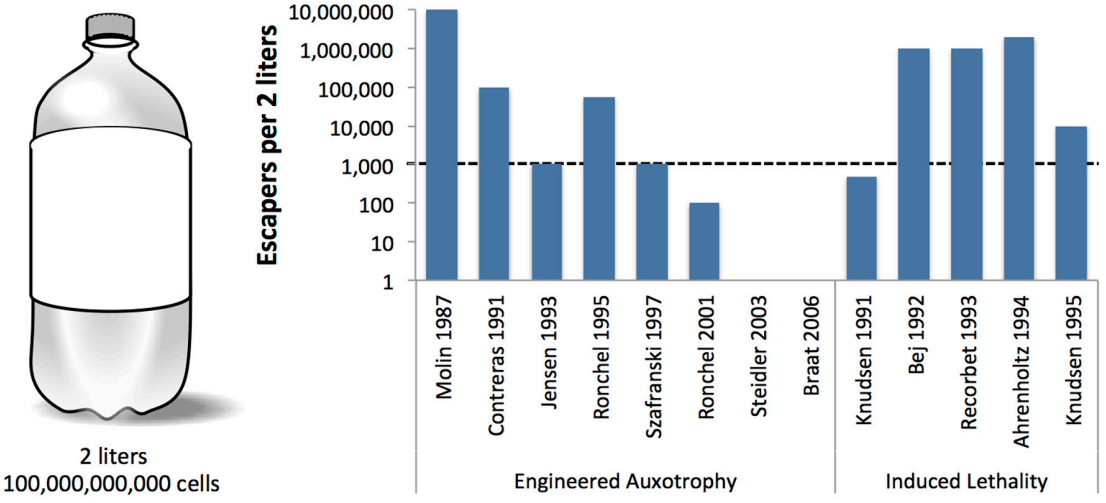


FIGURE 2 | Reported frequencies of engineered bacteria that escape various genetic safeguard systems. A 2-liter volume is represented here as a standard soft drink container (left). Lowest reported frequencies (shown on the y-axis, log scale) were multiplied by the estimated number of cells in 2-liters at 1×10^8 cells/mL, where OD600 = 0.1 [BioNumbers record ID 10985 (Milo et al., 2009)]. The dashed line indicates the maximum survival limit (1000 cells per 2 liters) recommended by the National Institutes of Health (Wilson, 1993).

Table 1 | Lowest reported frequencies of microbes that escape engineered auxotrophy and induced lethality safeguard systems.

Safeguard type	References	Microbe	Mechanism	Reported survival rate
Engineered auxotrophy	Molin et al., 1987	*B. subtilis, E. coli, P. putida*	Tryptophan *hok* switch or stochastic *hok* expression	1.00E-4
	Contreras et al., 1991	*E. coli*	*xylS-gef* switch	1.00E-6
	Jensen et al., 1993	*E. coli, P. putida*	*xylS-gef* switch (2 copies)	1.00E-8
	Ronchel et al., 1995	*P. putida*	*xylS-gef* switch (chromosome insert)	5.70E-7
	Szafranski et al., 1997	*P. putida*	*xylS-streptavidin* switch	1.00xE-8
	Kristoffersen et al., 2000	*S. cerevisiae*	*GAL1-relE* switch	Not reported
	Ronchel and Ramos, 2001	*P. putida* (in soil)	*xylS-gef* switch, gene deletion (*asd*)	1.00E-9
	Steidler et al., 2003	*L. lactis*	Gene deletion (*thyA*)	Below detection limits
	Balan and Schenberg, 2005	*S. cerevisiae* (in soil)	*ADH2-nucA* switch	1.00E-6
	Braat et al., 2006	*L. lactis* (in human patients)	Gene deletion (*thyA*)	Below detection limits
Induced lethality	Bej et al., 1988	*E. coli* (in soil)	IPTG-inducible *hok*	Not reported
	Knudsen and Karlström, 1991	*E. coli*	IPTG-inducible *relF*, 2 copies	5.00E-9
	Bej et al., 1992	*P. putida*	IPTG-inducible *gef*	1.00E-5
	Recorbet et al., 1993	*E. coli*	Sucrose-inducible *sacB*	1.00E-5
	Ahrenholtz et al., 1994	*E. coli*	Heat-inducible *nucA*	2.00E-5
	Knudsen et al., 1995	*E. coli* (in soil, seawater, rats)	IPTG-inducible *relF*, 2 copies	1.00E-7
	Li and Wu, 2009	*E. coli*	Arabinose-inducible *nucA*	Not reported

organism. Thus, lethal genes are the most widely used feature of genetic safeguards. Unfortunately, the lethal gene is a central cause of safeguard failure. Under certain conditions, both deactivation and activation of lethal gene expression may exacerbate the failure of biocontainment. As engineered cells are passaged in the laboratory, or as they propagate in large bioreactors, broken genetic safeguards can gradually accumulate in the population. If the utility of the biocontainment mechanism is lost, then the synthetic organisms might survive in the environment after disposal or accidental release.

Lethal gene expression can be deactivated by spontaneous genetic mutations that arise from DNA replication error (i.e., when newly replicated DNA is not identical to its template) and DNA rearrangements (i.e., transposon mobilizations or chromosome breakage and repair) as a population of synthetic cells increases through many rounds of cell division. As a result, the population becomes non-responsive to the genetic safeguard. Knudsen and Karlström applied a classic Nobel Prize-winning approach (Luria and Delbrück, 1943) to measure the rate of spontaneous mutation of a *relF* kill switch (Knudsen and Karlström, 1991). In several trials, cells were grown for roughly 14 divisions and treated with IPTG to activate the toxic *relF* gene. Up to 49 cells survived in each experiment. Poisson distribution of survival showed that spontaneous mutations deactivated *relF* at various time points during population growth. In a population of synthetic organisms, cells carrying a mutated kill switch might gain a growth advantage and overwhelm the population (**Figure 3**). Experiments have demonstrated that slowing down growth by maintaining cells in a suboptimal medium and at a lowered incubation temperature prevented the accumulation of mutations that damage the lethal gene (Knudsen and Karlström, 1991). Presumably, these measures reduce the number of mutations by preventing rapid cell division.

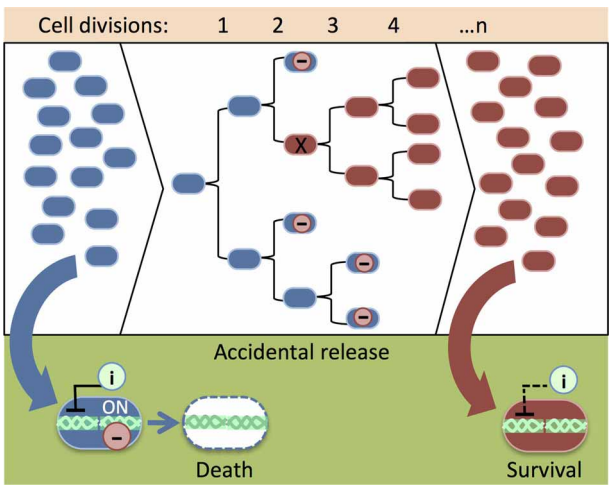


FIGURE 3 | An illustration of the accumulation of damaged genetic safeguards in a population of synthetic organisms. When cells with intact safeguards (blue) escape physical containment (e.g., an accidental spill), an inducer (i) can be added to remove them from the environment (see **Figure 1C**). As the population grows, leaky expression of the lethal protein (–) reduces the viability of cells that carry functional safeguards. Mutation (X) of the lethal gene provides a growth advantage, thus cells that carry damaged safeguards (red) overwhelm the population. Cells with mutated safeguards do not respond to the cell death inducer (i). Consequently, it is difficult to remove the cells from the environment after an accidental release.

The obvious approach for designing an effective kill switch is by expressing high levels of a lethal gene, (e.g., placing *relF*

under the control of a strong promoter). What may be less obvious is that strong promoters have higher basal expression levels, which can lead to genetic safeguard failure in a microbial population. When the kill switch is in the off-state, leaky expression of the toxic gene product will lead to decreased survival of cells that have functional lethal genes. Therefore, tight repression of the lethal gene's promoter can substantially increase the survival of cells that carry a functional genetic safeguard (Knudsen and Karlström, 1991).

MINIMAL GENOMES AND ORTHOGONAL LIFE

Scientists are developing creative new strategies that might address the shortcomings of genetic safeguards, such as the ones described in **Figures 2**, **3**, and **Tables 1**, **2**. Minimal genomes (**Box 1**) that contain only the genes that are necessary to sustain life could make many random DNA mutations lethal. Thus the likelihood of unexpected evolution and unpredictable behavior after the microbe is released into the environment would be reduced (DeWall and Cheng, 2011). Minimal synthetic chromosomes may 1 day be routinely produced by emerging technologies such as whole genome synthesis (Gibson et al., 2010) and large-scale genome editing (MAGE/CAGE; **Box 1**) (Isaacs et al., 2011).

Orthogonal (**Box 1**) life forms that use artificial genetic languages are a proposed genetic firewall that prevents the transfer of synthetic traits to natural biological systems (Schmidt and de Lorenzo, 2012). The orthogonal life form approach uses biochemical building blocks (i.e., nucleic acids and amino acids) that are incompatible with natural cells (for reviews see Liu and Schultz, 2010; Schmidt, 2010). Developments in the field of xeno (**Box 1**) nucleic acids (XNA) have yielded foreign genetic alphabets (Hirao et al., 2012), DNA double helix geometries, and nucleic acid backbones. Artificial bases including Ds, Px (Yamashige et al., 2012), dSICS, dMMO2 (Leconte et al., 2008; Lavergne et al., 2011), and dP, dZ (Sismour, 2004; Yang et al., 2006) preferentially bond as unnatural pairs instead of with the natural A, T, C, and G bases. Alternative DNA geometries such as expanded DNA (xDNA) and wide DNA (yDNA) (Krueger et al., 2007) are too large to fit into natural helices. Alternative nucleic acid backbones including threose (TNA), hexose (HNA), and glycol nucleic acid (GNA) (Pinheiro and Holliger, 2012) replace the natural poly-P-ribose and poly-P-deoxyribose backbones with molecular chains that cannot be replicated by natural polymerases. Recently, practical applications of orthogonal nucleic acids have been reported. XNA has been replicated in cell-free systems (e.g., PCR) (Yang et al., 2011; Betz et al., 2012; Malyshev et al., 2012), XNA has been used to express functional green fluorescent protein (GFP) (Krueger et al., 2011), and living bacteria have been evolved to use chlorouracil as a substitute for thymine (Marlière et al., 2011).

Orthogonal systems have also been engineered at the protein production level. Orthogonal amino acids have been incorporated into proteins in *E. coli* and yeast by matching an artificial transfer RNA (tRNA)/aminoacyl tRNA synthetase pair with an otherwise unused messenger RNA (mRNA) codon (the amber nonsense codon, TAG) (Wang et al., 2001; Chin, 2003) or a completely novel quadruplet codon AGGA (Anderson et al., 2004). The repertoire of functional quadruplet codons was expanded by artificially evolving a new *E. coli* ribosome (Neumann et al., 2010). Synthetic mRNA codons, tRNAs, and ribosomes make up a completely orthogonal protein translation system that may be used to create useful synthetic organisms that do not interfere with natural systems.

We are still a long way from robust orthogonal systems that can be used for practical applications. System-specific replication machinery needs to be developed to truly insulate XNA from DNA-based life forms. Current working orthogonal systems are natural-xeno hybrids. In the long term, fully orthogonal organisms could lead to a new method of engineered auxotrophy. Synthetic microbial survival would decline without a constant supply of orthogonal building blocks (XNA and xeno amino acids) from a controlled environment.

However, whether orthogonal systems can be completely insulated from the natural world is a question that is open for debate. Nucleic acids with alternative backbones (TNA, HNA, and GNA) can bond with natural DNA and RNA (Pinheiro and Holliger, 2012). This bonding could act as a toxin by interfering with DNA replication and proper gene expression. Scientists must still consider how escaped orthogonal organisms might impact natural environments.

Box 1 | Glossary of terms.

Minimal Genome—a chromosome that contains only the genes that are necessary to sustain life. Pseudogenes and other non-essential DNA are removed from the chromosome.
MAGE—Multiplex Automated Genome Engineering.
CAGE—Conjugative Assembly Genome Engineering.
Orthogonality—(greek: orthos—"straight," and gonia—angle) Modification of one component of a system that does not propagate side effects to other components of the system.
Xeno—greek: xeno—"foreign."

RISK ANALYSIS IN SYNTHETIC BIOLOGY

REPORTING RISK-RELATED DATA

Synthetic biology is at an opportune stage of development where current scientists can make risk-related analysis and data reporting a standard practice. Currently, we have methods to predict and test the environmental impact of an engineered microbe on indigenous microbial populations in soil (Corich et al., 2007; reviewed in Urgun-Demirtas et al., 2006). However, this work is limited to a few representative cases. The field needs an accepted, standard analytical method for determining the safety of a newly designed synthetic organism. We should avoid the temptation to allow representative studies and anecdotal evidence to define risk universally for every newly engineered organism.

There are many questions that must be addressed before determining appropriate risk and safety analyses. Should we expect all types of synthetic organisms to be subjected to the same risk analysis? What should standard methods for determining the safety of newly designed synthetic organism be? Until we can answer these questions, it would be prudent to include risk-related data in the original scientific report for every synthetic organism. A community-wide effort to report risk-related data would produce a wealth of information that could be parsed for meta-analyses and follow-up studies. We propose a method for

reporting risk-related data (**Box 2**), based the Woodrow Wilson group's four focus areas for determining the safety of synthetic organisms: survival of synthetic organisms in receiving environments, gene transfer, interactions between synthetic and natural organisms, and adaptation of synthetic organisms to new ecological niches (Dana et al., 2012).

We can try to predict environmental risk by considering known characteristics of the parent organism species or strain, such as the most likely ecological niches of the organism (e.g., soil, water, within a host cell). Risk that is related to synthetic modifications should also be reported. Scientists can measure synthetic versus wild type organism growth rates to determine any artificially enhanced growth (Londo et al., 2011). It is essential to determine the behavior of the synthetic organism in relevant microcosms (Bej et al., 1988; Knudsen et al., 1995; Ronchel et al., 1995; Ronchel and Ramos, 2001; Steidler et al., 2003; Balan and Schenberg, 2005), especially for engineered cells that are intended for environmental release or human and animal exposure. Reports would also include any characteristics that might aid biological containment (e.g., compromised fitness, kill switches, etc.).

The risk of gene transfer depends upon the ability of the host microbe to undergo conjugation, the viral motility of the engineered DNA, and the likelihood of plant pollination (van Elsas and Bailey, 2002; Brigulla and Wackernagel, 2010; Londo et al., 2011). The release of high-copy plasmids from dead cells might also result in gene transfer. Scientists should report rDNA transfer rates if they have collected such data (see **Table 2** for examples).

In some cases, we can assume interactions between synthetic and natural organisms when the former has certain synthetic modifications. Alterations that enable synthetic cells to adhere with natural cells, invade cell membranes (Agapakis et al., 2011), or kill other cells (Russell et al., 2011) should be explicitly reported. When the synthetic organism is not explicitly designed for cellular interaction, its impact on natural cells is more difficult to predict. In this case, experiments should be done to measure the synthetic system's synergistic or toxic effect on cells it will most likely come into contact with.

The potential of synthetic organisms to adapt to new ecological niches can be reported by highlighting engineered functions that could impart adaptive behavior. For instance, synthetic systems that are engineered to survive in multiple environments could pose a containment risk. A microbe that is engineered to consume pollutants (Contreras et al., 1991) has the potential to thrive on a greater variety of nutrients than its wild-type precursor. Scientists might develop ways to make synthetic organisms more robust, perhaps by making cells less sensitive to normally toxic conditions, or by making cells invisible to the human immune system.

Box 2 | A hypothetical journal article section that reports risk and biosafety information for a seminal engineered genetic toggle switch (Gardner et al., 2000).

RISK ANALYSIS AND BIOSAFETY DATA
Environmental risk:

- **Parent organism species/strain**—*E. coli* JM2.300.
- **Most likely ecological niche(s)**—None. JM2.300 is a derivative of *E. coli* K-12, a debilitated strain that does not normally colonize the human intestine and survives poorly in the environment ("*Escherichia coli* K-12 Derivatives Final Risk Assessment," last accessed October 22, 2012, http://epa.gov/oppt/biotech/pubs/fra/fra004.htm).
- **Growth rate compared to unmodified parent strain**—Not determined.
- **Containment**—A *thi*-mutation renders JM2.300 dependent upon thiamine for growth.

Gene transfer potential—The toggle switch is carried on a low copy number plasmid (pBR322 ColE1 replication origin, 15–20 copies per cell). JM2.300 is an F-strain (Brenner et al., 2007). It is capable of receiving F plasmids through conjugation, and is not capable of transmitting plasmids to other microbes.
Potential interactions—Not determined.
Adaptive behavior—None identified.

Table 2 | Efficacy of gene-flow barriers.

References	Donor	Recipient	Gene-flow barrier	rDNA transfer
Díaz et al., 1994	*E. coli*, *P. putida*	*P. putida*	Colicin E3 RNase	1.60E-8
Ronchel et al., 1995	*P. putida*	*P. putida* (in soil, water)	*Tn5* chromosome insert	Below detection limits
Munthali et al., 1996	*P. putida*	*P. putida*	Colicin E3 RNase, *Tn5* chromosome insert	1.00E-4
Torres et al., 2000	*E. coli*	*A. tumefaciens, E. coli, P. putida, R. eutropha*	EcoRI DNase	1.00E-4
Ronchel and Ramos, 2001	*P. putida*	*P. putida*	*Tn5* chromosome insert	1.00E-8
Torres, 2003	*E. coli*	*E. coli*	Colicin E3 RNase, EcoRI DNase	1.00E-8

Lowest reported frequencies of recombinant DNA (rDNA) transfer in controlled laboratory tests are shown.

These functions could also enhance adaptation. By predicting possible adaptations for environmental survival in advance, scientists can engineer the organisms with safety mechanisms for adequate containment.

USING SYNTHETIC BIOLOGY TO DETECT ENGINEERED ORGANISMS IN THE ENVIRONMENT

An environmental incident is an unsettling possibility that may someday call into question the safety of a synthetic organism. In this case, effective forensic tools would be critical for distinguishing synthetic from natural organisms and determining what role, if any, the synthetic organism played in the incident. Tracking techniques based on ELISA and PCR have successfully identified genetic modification markers along the agricultural pipeline, from farming (Watrud et al., 2004; Dyer et al., 2009) and harvest, through the processing (Auer, 2003). Further tracking potential is evident in a wide range of engineered markers, including detectable DNA sequences, enzymatic activity, cell surface markers could be added to synthetic organisms to aid the tracking process (Urgun-Demirtas et al., 2006). A team of college art students proposed a citizen science driven system that combined balloon cartography with PCR testing of soil samples to track the appearance of BioBrick rDNA across rural areas in India ("Searching for the Ubiquitous Genetically Engineered Machines," last accessed October 22, 2012, http://2011.igem.org/Team:ArtScienceBangalore). This creative vision suggests how adopting genetic tagging as a standard practice today might enable surveillance methods in the future.

INTEGRATING RISK ASSESSMENT INTO THE COMPUTER-AIDED DESIGN PROCESS

Software designers in the synthetic biology community are developing safeguards to help scientists prevent unintentional creation of dangerous organisms. These tools are intended to help scientists design safe synthetic systems before the system is actually built. CLOTHO is a design software tool that helps synthetic biologists construct and simulate engineered genetic devices ("Clotho," last accessed October 22, 2012, http://www.clothocad.org/). A homology search (BLAST) against virulence factors (Chen et al., 2012) assigns risk score to modules, then alerts the user of significant overlap with potentially dangerous sequences. GenoGUARD is another open-source software tool that warns against the use of potential bioterrorism-enabling DNA agents (Adam et al., 2011; "GenoGUARD," last accessed October 22, 2012, http://genoguard.sourceforge.net/). It uses the "best match" screening protocol method recommended by the guidelines of the US Department of Health and Human Services ("Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA," November 19, 2010, available at http://www.phe.gov/preparedness/legal/guidance/syndna/Pages/default.aspx).

How effective are these safeguards? The simple-catch approach utilized by CLOTHO is based on nucleic acid sequence homology, which is insufficient to detect multiple genetic codes that produce the same harmful protein, and does not consider emergent properties that might cause harm at the system level. Furthermore, there is no standard to rate the type and degree of potential harm. These problems can be solved with additional software features. For instance, existing protein homology algorithms could be incorporated into programs such as CLOTHO. Exploratory collaborations between computer scientists and biologists might yield new ways to predict harmfulness in higher-level properties. A more well-defined ontology for "risk" will help developers to create an extremely critical and powerful biosafety tool. This tool will prevent unintended harm at the design stage, before the synthetic organism is ever created.

STAKEHOLDERS' ROLES IN SAFE SYNTHETIC BIOLOGY

In discussing best practices for biosafety, it is critical to consider the stakeholders and their roles in the growth of the synthetic biology industry. Key stakeholders include scientists, industry leaders, regulatory agencies, and the public. Exciting opportunities lie ahead, in which these various stakeholders can cooperate to shape synthetic biology. If successful, synthetic biology will stand apart from other technologies in that it is conducted in an open and ethical way. The synthetic biology industry is in its nascent stages, an ideal time to establish biosafety norms.

Many companies have sprung directly from innovations in research labs, thus scientists from those labs are also industry leaders. These industry leaders should communicate with government officials to help shape policy with effective, research-based, achievable biosafety aims instead of allowing policy to be formed from worst-case hypotheticals. However, the companies cannot objectively do their own risk assessment and Congress does not have the expertise. A recent report showed that a majority of Americans were wary of voluntary guidelines developed jointly by industry and government ("Awareness and Impressions of Synthetic Biology," September 9, 2010, available at http://www.synbioproject.org/library/publications/archive/6456/). Thus, additional voices should also participate in the discourse around safe synthetic biology technologies. To this end, synthetic biologists, environmental microbiologists, public officials, law firm representatives, and public interest group members have recently met to discuss the best ways to address uncertainty when assessing the environmental impacts of synthetic biology ("Beyond Containment—Assessing, Testing and Demonstrating Safety on Release of Synbio Devices and Chassis," last accessed October 22, 2012, http://www.synbioproject.org/events/archive/6635/). Similar activities in Europe, such as those organized by the SYNBIOSAFE consortium ("Synbiosafe," last accessed October 21, 2012, http://www.synbiosafe.eu/), show that the shift toward inclusive discussion is far-reaching.

Regulatory agencies can be leveraged to monitor personal and environmental use of synthetic organisms. The US Department of Health and Human Services has released a set of guidelines to help DNA synthesis companies to only distribute safe, non-pathogenic, non-virulent nucleic acids ("Screening Framework Guidance for Providers of Synthetic Double-Stranded DNA," November 19, 2010, available at http://www.phe.gov/preparedness/legal/guidance/syndna/Pages/default.aspx). However, more information is needed on whether novel synthetic systems might present different risks than current familiar technology. There is a gap between useful technical data in synthetic biology research reports and risk analysis. Our proposed risk-related data report (**Box 2**) might help to fill this gap by providing additional synthetic organism aspects, other than DNA sequences, to inform risk.

The public's power to shape biotechnology practices and industry is largely limited to antagonistic situations, such as class action lawsuits after a clinical treatment has done widespread harm. For the growing field of synthetic biology, we should ensure that the public has a chance to broaden its influence by engaging in cooperative and open dialog to help maximize the benefits of the technology as scientists seek new ways to serve society's needs.

There are many open questions regarding the safety of synthetic organisms and the repercussions of unintended harm. How will we know when an accidental release is cleaned? What will the responsible party (e.g., a for-profit company) owe to a community affected by a spill? Which governmental agency will be responsible for monitoring the company? What guidelines will the company be evaluated on? Diverse stakeholders must develop a strong culture of cooperative discourse to ensure that the technology moves forward while providing minimal harm and maximum benefit to society.

ACKNOWLEDGMENTS

We thank J. Alling for help in finalizing the manuscript. Karmella A. Haynes is supported by the Ira A. Fulton School of Biological and Health Systems Engineering. Rene Davis is supported by SBHSE and the Biological Design Graduate Program.

REFERENCES

Adam, L., Kozar, M., Letort, G., Mirat, O., Srivastava, A., Stewart, T., et al. (2011). Strengths and limitations of the federal guidance on synthetic DNA. *Nat. Biotechnol.* 29, 208–210.

Agapakis, C. M., Niederholtmeyer, H., Noche, R. R., Lieberman, T. D., Megason, S. G., Way, J. C., et al. (2011). Towards a synthetic chloroplast. *PLoS ONE* 6:e18877. doi: 10.1371/journal.pone.0018877

Ahrenholtz, I., Lorenz, M. G., and Wackernagel, W. (1994). A conditional suicide system in *Escherichia coli* based on the intracellular degradation of DNA. *Appl. Environ. Microbiol.* 60, 3746–3751.

Anderson, J. C., Wu, N., Santoro, S. W., Lakshman, V., King, D. S., and Schultz, P. G. (2004). An expanded genetic code with a functional quadruplet codon. *Proc. Natl. Acad. Sci. U.S.A.* 101, 7566–7571.

Auer, C. A. (2003). Tracking genes from seed to supermarket: techniques and trends. *Trends Plant Sci.* 8, 591–597.

Bahey-El-Din, M. (2012). *Lactococcus lactis*-based vaccines from laboratory bench to human use: an overview. *Vaccine* 30, 685–690.

Balan, A., and Schenberg, A. C. G. (2005). A conditional suicide system for *Saccharomyces cerevisiae* relying on the intracellular production of the *Serratia marcescens* nuclease. *Yeast* 22, 203–212.

Bej, A. K., Molin, S., Perlin, M., and Atlas, R. M. (1992). Maintenance and killing efficiency of conditional lethal constructs in *Pseudomonas putida*. *J. Ind. Microbiol.* 10, 79–85.

Bej, A. K., Perlin, M. H., and Atlas, R. M. (1988). Model suicide vector for containment of genetically engineered microorganisms. *Appl. Environ. Microbiol.* 54, 2472–2477.

Betenbaugh, M. J., Beaty, C., and Dhurjati, P. (1989). Effects of plasmid amplification and recombinant gene expression on the growth kinetics of recombinant *E. coli*. *Biotechnol. Bioeng.* 33, 1425–1436.

Betz, K., Malyshev, D. A., Lavergne, T., Welte, W., Diederichs, K., Dwyer, T. J., et al. (2012). KlenTaq polymerase replicates unnatural base pairs by inducing a Watson-Crick geometry. *Nat. Chem. Biol.* 8, 612–614.

Braat, H., Rottiers, P., Hommes, D. W., Huyghebaert, N., Remaut, E., Remon, J. P., et al. (2006). A phase I trial with transgenic bacteria expressing Interleukin-10 in Crohn's disease. *Clin. Gastroenterol. Hepatol.* 4, 754–759.

Brenner, K., Karig, D. K., Weiss, R., and Arnold, F. H. (2007). Engineered bidirectional communication mediates a consensus in a microbial biofilm consortium. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17300–17304.

Brigulla, M., and Wackernagel, W. (2010). Molecular aspects of gene transfer and foreign DNA acquisition in prokaryotes with regard to safety issues. *Appl. Microbiol. Biotechnol.* 86, 1027–1041.

Caluwaerts, S., Vandenbroucke, K., Steidler, L., Neirynck, S., Vanhoenacker, P., Corveleyn, S., et al. (2010). AG013, a mouth rinse formulation of *Lactococcus lactis* secreting human Trefoil Factor 1, provides a safe and efficacious therapeutic tool for treating oral mucositis. *Oral Oncol.* 46, 564–570.

Chen, L., Xiong, Z., Sun, L., Yang, J., and Jin, Q. (2012). VFDB (2012). update: toward the genetic diversity and molecular evolution of bacterial virulence factors. *Nucleic Acids Res.* 40, D641–D645.

Chin, J. W. (2003). An expanded eukaryotic genetic code. *Science* 301, 964–967.

Contreras, A., Molin, S., and Ramos, J. L. (1991). Conditional-suicide containment system for bacteria which mineralize aromatics. *Appl. Environ. Microbiol.* 57, 1504–1508.

Corich, V., Giacomini, A., Vendramin, E., Vian, P., Carlot, M., Concheri, G., et al. (2007). Long term evaluation of field-released genetically modified rhizobia. *Environ. Biosafety Res.* 6, 167–181.

Dana, G. V., Kuiken, T., Rejeski, D., and Snow, A. A. (2012). Synthetic biology: four steps to avoid a synthetic-biology disaster. *Nature* 483, 29.

DeWall, M. T., and Cheng, D. W. (2011). The minimal genome–a metabolic and environmental comparison. *Brief. Funct. Genomics* 10, 312–315.

Díaz, E., Munthali, M., de Lorenzo, V., and Timmis, K. N. (1994). Universal barrier to lateral spread of specific genes among microorganisms. *Mol. Microbiol.* 13, 855–861.

Dyer, G. A., Serratos-Hernández, J. A., Perales, H. R., Gepts, P., Piñeyro-Nelson, A., Chávez, A., et al. (2009). Dispersal of transgenes through maize seed systems in Mexico. *PLoS ONE* 4:e5734. doi: 10.1371/journal.pone.0005734

Eisenstein, M. (2010). Synthetic DNA firms embrace hazardous agents guidance but remain wary of automated 'best-match'. *Nat. Biotechnol.* 28, 1225–1226.

Friedland, A. E., Lu, T. K., Wang, X., Shi, D., Church, G., and Collins, J. J. (2009). Synthetic gene networks that count. *Science* 324, 1199–1202.

Gardner, T. S., Cantor, C. R., and Collins, J. J. (2000). Construction of a genetic toggle switch in *Escherichia coli*. *Nature* 403, 339–342.

Gerdes, K., Bech, F. W., Jørgensen, S. T., Løbner-Olesen, A., Rasmussen, P. B., Atlung, T., et al. (1986). Mechanism of postsegregational killing by the hok gene product of the parB system of plasmid R1 and its homology with the relF gene product of the *E. coli* relB operon. *EMBO J.* 5, 2023–2029.

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., et al. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56.

Heinemann, M., and Panke, S. (2006). Synthetic biology–putting engineering into biology. *Bioinformatics* 22, 2790–2799.

Heuer, H., and Smalla, K. (2007). Horizontal gene transfer between bacteria. *Environ. Biosafety Res.* 6, 3–13.

Hirao, I., Kimoto, M., and Yamashige, R. (2012). Natural versus artificial creation of base pairs in DNA: origin of nucleobases from the perspectives of unnatural base pair studies. *Acc. Chem. Res.* 45, 2055–2065.

Isaacs, F. J., Carr, P. A., Wang, H. H., Lajoie, M. J., Sterling, B., Kraal, L., et al. (2011). Precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement. *Science* 333, 348–353.

Jensen, L. B., Ramos, J. L., Kaneva, Z., and Molin, S. (1993). A substrate-dependent biological containment system for *Pseudomonas putida* based on the *Escherichia coli* gef gene. *Appl. Environ. Microbiol.* 59, 3713–3717.

Knudsen, S., Saadbye, P., Hansen, L. H., Collier, A., Jacobsen, B. L., Schlundt, J., et al. (1995). Development and testing of improved suicide functions for biological containment of bacteria. *Appl. Environ. Microbiol.* 61, 985–991.

Knudsen, S. M., and Karlström, O. H. (1991). Development of efficient suicide mechanisms for biological containment of bacteria. *Appl. Environ. Microbiol.* 57, 85–92.

Kristoffersen, P., Jensen, G. B., Gerdes, K., and Piskur, J. (2000). Bacterial toxin-antitoxin gene system as containment control in yeast cells. *Appl. Environ. Microbiol.* 66, 5524–5526.

Krueger, A. T., Lu, H., Lee, A. H. F., and Kool, E. T. (2007). Synthesis and properties of size-expanded DNAs: toward designed, functional

genetic systems. *Acc. Chem. Res.* 40, 141–150.

Krueger, A. T., Peterson, L. W., Chelliserry, J., Kleinbaum, D. J., and Kool, E. T. (2011). Encoding phenotype in bacteria with an alternative genetic set. *J. Am. Chem. Soc.* 133, 18447–18451.

Lavergne, T., Malyshev, D. A., and Romesberg, F. E. (2011). Major groove substituents and polymerase recognition of a class of predominantly hydrophobic unnatural base pairs. *Chem. Eur. J.* 18, 1231–1239.

Leconte, A. M., Hwang, G. T., Matsuda, S., Capek, P., Hari, Y., and Romesberg, F. E. (2008). Discovery, characterization, and optimization of an unnatural base pair for expansion of the genetic alphabet. *J. Am. Chem. Soc.* 130, 2336–2343.

Li, Q., and Wu, Y.-J. (2009). A fluorescent, genetically engineered microorganism that degrades organophosphates and commits suicide when required. *Appl. Microbiol. Biotechnol.* 82, 749–756.

Link, H.-J. (2012). Playing God and the intrinsic value of life: moral problems for synthetic biology? *Sci. Eng. Ethics*. doi: 10.1007/s11948-012-9353-z. [Epub ahead of print].

Liu, C. C., and Schultz, P. G. (2010). Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* 79, 413–444.

Londo, J. P., Bollman, M. A., Sagers, C. L., Lee, E. H., and Watrud, L. S. (2011). Changes in fitness-associated traits due to the stacking of transgenic glyphosate resistance and insect resistance in *Brassica napus* L. *Heredity* 107, 328–337.

Lu, T. K., Khalil, A. S., and Collins, J. J. (2009). Next-generation synthetic gene networks. *Nat. Biotechnol.* 27, 1139–1150.

Luria, S. E., and Delbrück, M. (1943). Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* 28, 491–511.

Lyon, D. Y., Monier, J.-M., Dupraz, S., Freissinet, C., Simonet, P., and Vogel, T. M. (2010). Integrity and biological activity of DNA after UV exposure. *Astrobiology* 10, 285–292.

Malyshev, D. A., Dhami, K., Quach, H. T., Lavergne, T., Ordoukhanian, P., Torkamani, A., et al. (2012). Efficient and sequence-independent replication of DNA containing a third base pair establishes a functional six-letter genetic alphabet. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12005–12010.

Marlière, P., Patrouix, J., Döring, V., Herdewijn, P., Tricot, S., Cruveiller, S., et al. (2011). Chemical evolution of a bacterium's genome. *Angew. Chem. Int. Ed.* 50, 7109–7114.

Martínez-García, E., Calles, B., Arévalo-Rodríguez, M., and de Lorenzo, V. (2011). pBAM1: an all-synthetic genetic tool for analysis and construction of complex bacterial phenotypes. *BMC Microbiol.* 11:38. doi: 10.1186/1471-2180-11-38

Milo, R., Jorgensen, P., Moran, U., Weber, G., and Springer, M. (2009). BioNumbers–the database of key numbers in molecular and cell biology. *Nucleic Acids Res.* 38, D750–D753.

Molin, S., Klemm, P., Poulsen, L. K., Biehl, H., Gerdes, K., and Andersson, P. (1987). Conditional suicide system for containment of bacteria and plasmids. *Nat. Biotechnol.* 5, 1315–1318.

Munthali, M. T., Timmis, K. N., and Díaz, E. (1996). Use of colicin e3 for biological containment of microorganisms. *Appl. Environ. Microbiol.* 62, 1805–1807.

Neumann, H., Wang, K., Davis, L., Garcia-Alai, M., and Chin, J. W. (2010). Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome. *Nature* 464, 441–444.

Nguyen, L. V., Cox, K. M., Ke, J. S., Peele, C. G., and Dickey, L. F. (2012). Genetic engineering of a Lemna isoleucine auxotroph. *Transgenic Res.* 21, 1071–1083.

Panke, S., Sánchez-Romero, J. M., and de Lorenzo, V. (1998). Engineering of quasi-natural *Pseudomonas putida* strains for toluene metabolism through an ortho-cleavage degradation pathway. *Appl. Environ. Microbiol.* 64, 748–751.

Pinheiro, V. B., and Holliger, P. (2012). The XNA world: progress towards replication and evolution of synthetic genetic polymers. *Curr. Opin. Chem. Biol.* 16, 245–252.

Poulsen, L. K., Larsen, N. W., Molin, S., and Andersson, P. (1989). A family of genes encoding a cell-killing function may be conserved in all gram-negative bacteria. *Mol. Microbiol.* 3, 1463–1472.

Recorbet, G., Robert, C., Givaudan, A., Kudla, B., Normand, P., and Faurie, G. (1993). Conditional suicide system of *Escherichia coli* released into soil that uses the *Bacillus subtilis* sacB gene. *Appl. Environ. Microbiol.* 59, 1361–1366.

Ronchel, M. C., Ramos, C., Jensen, L. B., Molin, S., and Ramos, J. L. (1995). Construction and behavior of biologically contained bacteria for environmental applications in bioremediation. *Appl. Environ. Microbiol.* 61, 2990–2994.

Ronchel, M. C., and Ramos, J. L. (2001). Dual system to reinforce biological containment of recombinant bacteria designed for rhizoremediation. *Appl. Environ. Microbiol.* 67, 2649–2656.

Russell, A. B., Hood, R. D., Bui, N. K., LeRoux, M., Vollmer, W., and Mougous, J. D. (2011). Type VI secretion delivers bacteriolytic effectors to target cells. *Nature* 475, 343–347.

Schmidt, M. (2010). Xenobiology: a new form of life as the ultimate biosafety tool. *Bioessays* 32, 322–331.

Schmidt, M., and de Lorenzo, V. (2012). Synthetic constructs in/for the environment: managing the interplay between natural and engineered biology. *FEBS Lett.* 586, 2199–2206.

Sismour, A. M. (2004). PCR amplification of DNA containing non-standard base pairs by variants of reverse transcriptase from Human Immunodeficiency Virus-1. *Nucleic Acids Res.* 32, 728–735.

Steidler, L., Neirynck, S., Huyghebaert, N., Snoeck, V., Vermeire, A., Goddeeris, B., et al. (2003). Biological containment of genetically modified *Lactococcus lactis* for intestinal delivery of human interleukin 10. *Nat. Biotechnol.* 21, 785–789.

Svab, Z., and Maliga, P. (2007). Exceptional transmission of plastids and mitochondria from the transplastomic pollen parent and its impact on transgene containment. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7003–7008.

Szafranski, P., Mello, C. M., Sano, T., Smith, C. L., Kaplan, D. L., and Cantor, C. R. (1997). A new approach for containment of microorganisms: dual control of streptavidin expression by antisense RNA and the T7 transcription system. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1059–1063.

Torres, B. (2003). A dual lethal system to enhance containment of recombinant micro-organisms. *Microbiology* 149, 3595–3601.

Torres, B., Jaenecke, S., Timmis, K. N., García, J. L., and Díaz, E. (2000). A gene containment strategy based on a restriction-modification system. *Environ. Microbiol.* 2, 555–563.

Urgun-Demirtas, M., Stark, B., and Pagilla, K. (2006). Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. *Crit. Rev. Biotechnol.* 26, 145–164.

van Elsas, J. D., and Bailey, M. J. (2002). The ecology of transfer of mobile genetic elements. *FEMS Microbiol. Ecol.* 42, 187–197.

Wang, L., Brock, A., Herberich, B., and Schultz, P. G. (2001). Expanding the genetic code of *Escherichia coli*. *Science* 292, 498–500.

Watrud, L. S., Lee, E. H., Fairbrother, A., Burdick, C., Reichman, J. R., Bollman, M., et al. (2004). Evidence for landscape-level, pollen-mediated gene flow from genetically modified creeping bentgrass with CP4 EPSPS as a marker. *Proc. Natl. Acad. Sci. U.S.A.* 101, 14533–14538.

Wilson, D. J. (1993). NIH guidelines for research involving recombinant DNA molecules. *Account. Res.* 3, 177–185.

Yamashige, R., Kimoto, M., Takezawa, Y., Sato, A., Mitsui, T., Yokoyama, S., et al. (2012). Highly specific unnatural base pair systems as a third base pair for PCR amplification. *Nucleic Acids Res.* 40, 2793–2806.

Yang, Z., Chen, F., Alvarado, J. B., and Benner, S. A. (2011). Amplification, mutation, and sequencing of a six-letter synthetic genetic system. *J. Am. Chem. Soc.* 133, 15105–15112.

Yang, Z., Hutter, D., Sheng, P., Sismour, A. M., and Benner, S. A. (2006). Artificially expanded genetic information system: a new base pair with an alternative hydrogen bonding pattern. *Nucleic Acids Res.* 34, 6095–6101.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 October 2012; accepted: 04 January 2013; published online: 25 January 2013.

Citation: Moe-Behrens GHG, Davis R and Haynes KA (2013) Preparing synthetic biology for the world. Front. Microbio. ***4****:5. doi: 10.3389/fmicb.2013.00005*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 19 April 2013
doi: 10.3389/fmicb.2013.00092

Design and development of synthetic microbial platform cells for bioenergy

Sang Jun Lee[1], Sang-Jae Lee[2] and Dong-Woo Lee[2]*

[1] *Systems and Synthetic Biology Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea*
[2] *School of Applied Biosciences, Kyungpook National University, Daegu, South Korea*

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Liang Shi, Wright State University, USA
Shawn Chen, Ohio University, USA

****Correspondence:***
Dong-Woo Lee, School of Applied Biosciences, College of Agriculture and Life Sciences, Kyungpook National University, 80 Daehak-ro, Buk-gu, Daegu 702-701, South Korea.
e-mail: leehicam@knu.ac.kr

The finite reservation of fossil fuels accelerates the necessity of development of renewable energy sources. Recent advances in synthetic biology encompassing systems biology and metabolic engineering enable us to engineer and/or create tailor made microorganisms to produce alternative biofuels for the future bio-era. For the efficient transformation of biomass to bioenergy, microbial cells need to be designed and engineered to maximize the performance of cellular metabolisms for the production of biofuels during energy flow. Toward this end, two different conceptual approaches have been applied for the development of platform cell factories: forward minimization and reverse engineering. From the context of naturally minimized genomes,non-essential energy-consuming pathways and/or related gene clusters could be progressively deleted to optimize cellular energy status for bioenergy production. Alternatively, incorporation of non-indigenous parts and/or modules including biomass-degrading enzymes, carbon uptake transporters, photosynthesis, CO_2 fixation, and etc. into chassis microorganisms allows the platform cells to gain novel metabolic functions for bioenergy. This review focuses on the current progress in synthetic biology-aided pathway engineering in microbial cells and discusses its impact on the production of sustainable bioenergy.

Keywords: microbial platform cells, bioenergy, synthetic biology, genome reduction, metabolic engineering

INTRODUCTION

Biological processes using microorganisms have a wide range of superior advantages (e.g., renewability, sustainability, and carbon neutralization) over conventional chemical processes for the production of biofuels. However, besides from compatibility with existing fuel infrastructure, production yields of the advanced biofuels are nevertheless not yet great enough to compete with and replace fossil fuels. The key issues to overcome in biological systems are the cost of substrates and biofuel toxicity/inhibition of fermenting microorganisms, which are directly related to biofuel productivity, titer, and yield (Ezeji et al., 2007; Fischer et al., 2008). To overcome these impediments, development of a robust and high-yielding microbe is required. Recently, a variety of engineered microorganisms, by metabolic engineering integrated with genome engineering and synthetic biology, appear to be quite promising with improved yields of biofuel production (e.g., ethanol, biodiesel, butanol, terpenoids, syngas, and H_2; Bokinsky et al., 2011; Srirangan et al., 2011; Zhang et al., 2011; Lan and Liao, 2012; Li et al., 2012; Westfall et al., 2012).

Synthetic biology provides us with innovative approaches to a wide range of applications (Purnick and Weiss, 2009): sustainable bioenergy production, bioremediation, biorefinery, and biopharmaceuticals. Based on a wealth of genome sequences, systems biology and metabolic engineering integrated with advanced genetic tools have enabled us to make engineered microbes as a blueprint for the near future (Gibson et al., 2008a, 2009; Benders et al., 2010; Jewett and Forster, 2010). Indeed, not only artificial microorganisms based on parasites (Fraser et al., 1995) and small size-genome microorganisms (Gibson et al., 2008b, 2010), but also engineered microorganisms were successfully generated for the production of biofuels, fine chemicals, pharmaceuticals, and biosensors (Martin et al., 2003; Keasling, 2010; Huo et al., 2011; Zhang and Keasling, 2011; Zhang et al., 2011). Thus, construction of efficient biofuel-producing microbial cell factories is now conceivable by design-based engineering of biological systems (Forster and Church, 2006, 2007; Carothers et al., 2009; Nitschke, 2009; Holtz and Keasling, 2010; Nielsen and Keasling, 2011; Tolonen et al., 2011).

To design and engineer microorganisms for the high-yields of biofuel production, we need to better understand how microbial cells can coordinate their metabolic pathways under different environmental conditions, underlying essential and non-essential genes for bacterial life and metabolic networks. This information will help us to modulate the efficiency of production pathway and to optimize the energy balances between bioproduction and biosynthesis in cell factories. To date, there are increasing examples of engineering metabolic pathways tightly linked to the cellular energy balance that is one of the key determining factors of cell factories in the yield and productivity of biofuels (Johnson and Schmidt-Dannert, 2008; Trinh et al., 2008; Portnoy et al., 2010; Lan and Liao, 2011, 2012). Recently, molecular engineering using protein or RNA scaffolds also could be applied for pathway engineering in synthetic cell factories (Conrado et al., 2008; Delebecque et al., 2011; Medema et al., 2011). For example, organizing mevalonate pathway enzymes on scaffolds have been developed for efficient production of isoprenoids (Dueber et al., 2009). In

this review, we will focus on the current strategies for designing and developing cell factories for the maximized production of sustainable bioenergy in the context of "forward engineering" and/or "reverse engineering" of efficiently energy-optimized cells.

"TOP-DOWN" REDUCTION OF MICROBIAL GENOME BY FORWARD ENGINEERING

Recent advances in sequencing techniques have generated enormous amounts of microbial genome databases, which can be invaluable information for the physiology and metabolism of the sequenced microorganisms (Brochier-Armanet et al., 2011; Gonzalez and Knight, 2012). This information also provides insight into the diversity of microorganisms and the molecular basis of their adaptive evolutionary mechanisms in different environments (Forterre et al., 2000). Comparison of genomes can often reveal similar and/or distinctly different metabolic pathways in bacteria, archaea, and eukarya (Siebers and Schonheit, 2005; Brochier-Armanet et al., 2011). In particular, the genome sequences of diverse microorganisms including extremophiles showed genetic traits of adaptation through gene duplication and/or deletion (Riehle et al., 2001; Averhoff, 2009). It suggests that gain and loss of genes is one of the major adaptation mechanisms for their cellular viability under selection pressure in nature. Thus, the genome sequences of diverse microorganisms that have an ancestor in common have diverged in a variety of ways, indicating that specific genes of a microorganism that are not found in others and highly conserved genes among organisms can be feasibly categorized. Accordingly, this leads us to have a fundamental question of which genes are indispensable for cellular lives and are involved in their essential and distinct metabolisms in comparison with other microbes. Moreover, these fundamental informations can provide indirect but very essential knowledge to overcome the endogenous regulation of biofuel-producing pathways to achieve high yields in using native hosts to convert feedstocks into biofuels.

GENOME COMPARISON OF ARCHAEA

Minimal cells comprise only the genes and biomolecular machineries required for basic life. During the past decade, over 100 genomes of archaea that form the third domain of life (Brochier-Armanet et al., 2011) have been sequenced. In fact, their genome data provide insights into the evolution of key central metabolisms, which are directly correlated with the minimal functionality for cell viability and adaptation under extreme environments. Intriguingly, the euryarchaea *Picrophilus torridus*, which thrives optimally at 60°C and pH 0.7, has 1.55 Mb genome (1535 ORFs), the smallest genome among non-parasitic aerobic microorganisms (Futterer et al., 2004). In particular, an exceptionally high ratio (5.6:1) of secondary over ATP-consuming primary transport systems represents a highly relevant strategy for the adaptation of this organism to its extremely acidic environment. Although *Picrophilus torridus* has several distinct gene traits in energy metabolism at low pH, not only all genes required for the Embden–Meyerhof–Parnas (EMP) pathway but also a complete set of genes for the oxidative tricarboxylic acid (TCA) cycle are present. Currently, there is a variety of genome data available for other archaea and bacteria, such as *Thermoplasma acidophilum* (1.56 Mb; Ruepp et al., 2000), *Archaeoglobus profundus* (1.56 Mb; von Jan et al., 2010), *Prochlorococcus marinus* (1.75 Mb; Dewall and Cheng, 2011), and *Methanocaldococcus jannaschii* (1.74 Mb; Bult et al., 1996), of which genome size is less than 2 Mb. Thus, these microorganisms seem to be "closer" to the minimal genome for life, which will also provide the fundamental basis of minimal gene sets for the construction of a genome-minimized platform cell (**Figure 1**). If this is the case, can we design and construct an engineered platform cell to perform our wanted tasks such as biofuel production? If so, then can we selectively choose which genes are friends or foes for the high productivity between cell mass and biofuels? Prior to considering this issue, we need to categorize essential sets of genes for cellular viability even with imperfect accuracy.

GENOME OF PHOTO-AUTOTROPHIC AND -HETEROTROPHIC CYANOBACTERIA

Genes involved in informational processes (not only DNA replication, but also transcription and translation represented by rRNA, tRNA, or structural RNA genes) plus protein folding and processing with strain-dependent metabolisms, are essential. In addition, a complex array of functional systems, including those for membrane transport, energy conversion, the synthesis of vitamins, and nucleic acid precursors, is indispensable for maintaining cellular integrity. Notable examples are the marine cyanobacteria *Prochlorococcus*, which is the smallest known oxygen-evolving photoautotroph (Chisholm et al., 1988). Although the high light-adapted ecotype MED4 strain has a genome of 1.66 Mb that codes for 1717 proteins, its low light-adapted counterpart contains a larger genome of 2.4 Mb (2275 genes; Rocap et al., 2003). Remarkably, the comparative analysis with their genomes revealed that only 1350 genes are common, whereas the remaining genetic loci are quite different, implying that the variable genes appear to be a consequence of a selective process favoring the bacterial adaptation to their environments (Dufresne et al., 2003, 2005; Rocap et al., 2003). Indeed, the photoheterotrophic marine bacterium *Pelagibacter ubique* has the smallest genome (1.3 Mb) of any cell known for a free-living microorganism (Giovannoni et al., 2005). Its genome coding for 1354 ORF shows the nearly complete absence of non-functional or redundant DNA, with very short intergenic regions, and the lack of pseudogenes and phage genes, reflecting an adaptive strategy that resembles the highly successful marine unicellular cyanobacteria in its simple metabolism and small genome size.

NON-ESSENTIAL GENES

Synthesizing minimal and minimized cells will improve understanding of core biology, accelerate development of biotechnology strains of bacteria, and enable evolutionary optimization of natural and unnatural biopolymers (Jewett and Forster, 2010). Genome reduction is of particular importance to identify non-essential genes for understanding of not only how many genes are essential for cellular viability, but also which genes are necessary for cellular beneficial properties. Reduction and engineering of microbial genome is the fundamental basis of design and development of synthetic minimal platform cells for estimation of the minimal

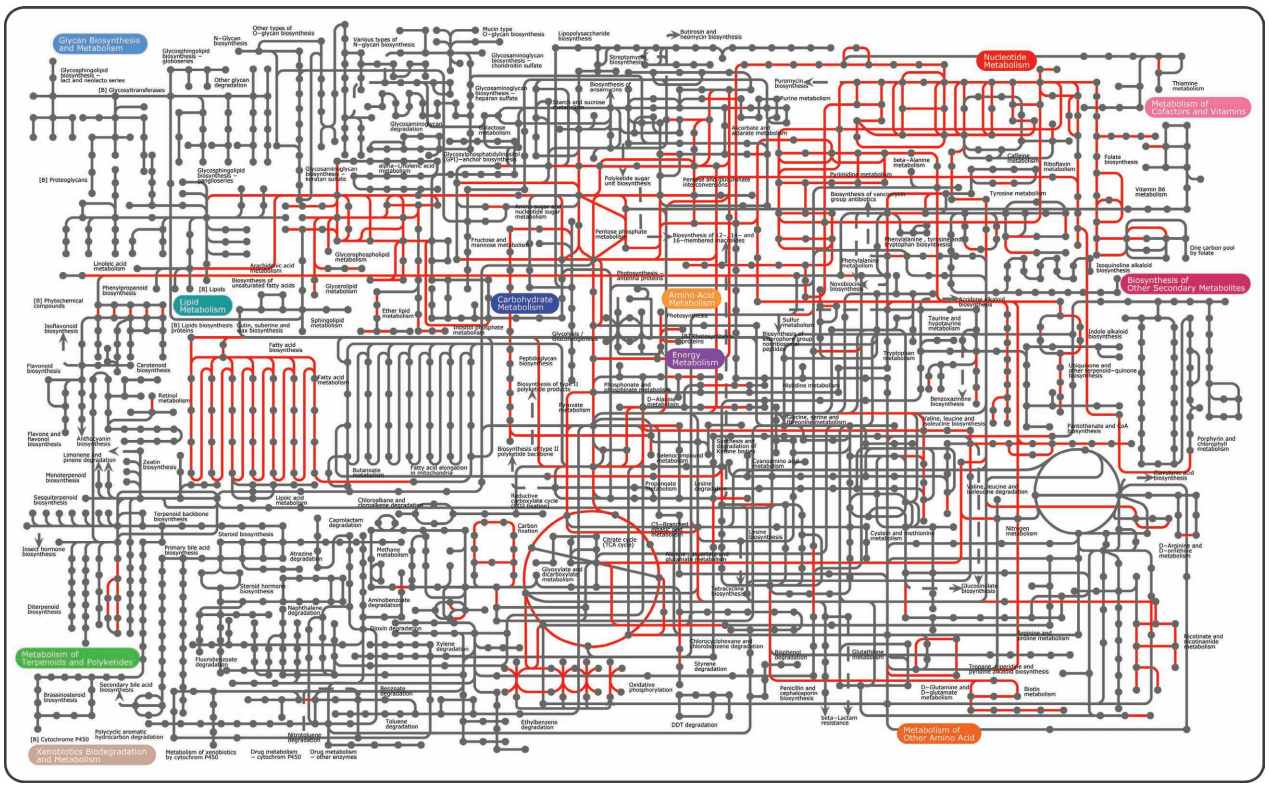


FIGURE 1 | Overview of the microbial pathways on the KEGG pathways using the iPath tool (Letunic et al., 2008). To date, conserved pathways known as essential are shown in red. Hypothetical proteins found as essential are excluded.

gene set required to sustain growth of microorganisms (Fleischmann et al., 1995; Fraser et al., 1995; Mushegian and Koonin, 1996). By use of comparative genomics, non-essential genes have been sought to reconstruct ancestral life forms (Mushegian and Koonin, 1996; Koonin, 2003) to define, by transposon-mediated disruption study, with *Mycoplasma genitalium* (Glass et al., 2006), and to validate and compare the minimal gene sets in *Bacillus subtilis* (Kobayashi et al., 2003; Ara et al., 2007), *E. coli* (Gerdes et al., 2003; Joyce et al., 2006), *Aquifex aeolicus* (Deckert et al., 1998), *Streptococcus sanguinis* (Xu et al., 2011), and Yeast (Kellis et al., 2003, 2004). Although symbiotic archeal organisms apparently have a much smaller genome (Huber et al., 2002; McCutcheon and Moran, 2012), these are out of scope with respect to the minimal gene-set that is necessary and sufficient to support cellular life. Notably, toxin–antitoxin (TA) loci functioning as stress-response elements are not generally essential to basic life (Pandey and Gerdes, 2005). Yet, combined mutations in two or more genes lead to cell death, whereas a single mutation in only one of those genes does not (Tucker and Fields, 2003). Thus, synthetic lethality should be considered when non-essential genes are deleted for genome minimization (Stieber et al., 2008).

MINIMAL GENOME FOR SYNTHETIC CELLS

Intriguingly, Posfai et al. (2006) successfully reduced the *E. coli* K-12 genome (up to 15%) to generate multiple-deletion series of strains without physiological compromise. In addition, genome reduction would provide unexpected benefits, such as high electroporation efficiency and accurate propagation of recombinant plasmids. Several laboratory evolution studies have also been carried out to characterize the genetic traits of adaptation to environmental stresses (e.g., thermal adaptation, salt stress tolerance, utilization of unusual substrates, and susceptibility to antibiotics, etc.; Herring et al., 2006; Dhar et al., 2011; Tran et al., 2011). Remarkably, reduction of the metabolic pathways by selective sorting and deletion of aerobic/anaerobic reactions based on the biomass and biofuel productions enabled cells to have theoretically maximal yields of ethanol even with minimized metabolic functionality under anaerobic conditions (Trinh et al., 2006, 2008). To convert the biomass-derived hexoses and pentoses to ethanol at high yields and productivities, an efficient and robust microorganism has been designed and developed by the removal of seemingly unnecessary pathways for the purpose (Trinh et al., 2006). To construct a minimal *E. coli* cell that is dedicated to producing ethanol, the functional space of the central metabolic network was reduced with eight gene knockout mutations (e.g., *poxB*, *pta* etc.) from over 15,000 pathway possibilities to 6 pathway options that support cell function (Trinh et al., 2008). The remaining pathways consist of four pathways with non-growth-associated conversion of pentoses and hexoses into ethanol at theoretical yields and two pathways with tight coupling of anaerobic cell growth with ethanol formation at high yields. Remarkably, catabolite

repression was completely absent during anaerobic growth by the deletion of acetate-producing pathways, resulting in the simultaneous utilization of pentoses and hexoses for ethanol production in the most efficient way. Thus, this study demonstrated that the ethanol yields of engineered strains with minimized metabolic functionality closely matched the theoretical predictions, implying that reduction of non-essential genes could be quite beneficial with respect to economical production yields in synthetic microorganisms.

"BOTTOM-UP" CREATION OF MICROBIAL CELL FACTORY BY REVERSE ENGINEERING

To avoid and overcome our coming issues with global warming and energy crises, increasing efforts to replace fossil fuels with renewable biofuels are still actively being undertaken in biotechnology fields, resulting in several renewable alternatives such as bioalcohol and plant oil-derived biodiesel from biomass feedstock (Atsumi et al., 2008; Nielsen et al., 2009; Lan and Liao, 2011; Wargacki et al., 2012). Recently, First generation biofuels such as ethanol and biodiesel have provided an avenue to sustainable biofuels in the near future, but they also appear to have their own limitations, such as a lower efficiency of combustion (Gray et al., 2006; Chisti, 2008). Thus, scientists are seeking to develop more sustainable and economically feasible second generation microbial biofuels (i.e., butanol, hydrocarbon, alkanes, H_2), which have great potential to convert renewable sources into energy rich, fuel-like molecules or fuel precursors (Schirmer et al., 2010; Steen et al., 2010; Shen et al., 2011). Many efforts in the fields of metabolic engineering, systems biology, synthetic biology, and genome engineering for biofuel production enable us to modulate indigenous metabolic fluxes, or to insert novel pathways by employing heterologous ones into host microorganisms for (i) increased productivity/titer of biofuels and (ii) energy-efficiency of metabolic pathways. In fact, the former effect is highly dependent on the substrate utilization and tolerance of host cells for product inhibition (Papoutsakis, 2008; Geddes et al., 2011; Peralta-Yahya et al., 2012). On the other hand, the latter seems to be much more important for significantly improving the overall productivity, because it is tightly coordinated with cellular energy transduction through redox homeostasis in cells under specific conditions (Martin et al., 2003; Trinh et al., 2008; Lan and Liao, 2012). Thus, design of microbial platform cells for high yields of biofuel production requires understanding how energy transduction systems including respiratory chains are partitioned and matched stoichiometrically with central metabolisms. Moreover, when foreign energy transduction pathways are employed, they should be compatible with indigenous central and energy metabolisms in host cells. Indeed, this is major challenges in improving the kinetics of metabolic enzymes and generating metabolic driving forces to maximize metabolic flux. In light of this, less genetically tractable hosts that have high biofuel tolerance or the ability to use non-sugar substrates are potentially applicable alternatives. This requires not only whole genome sequences of platform cells but also genome-wide systems analysis. Recent advent of next-generation DNA sequencing expands the width of genomic diversity and allows the exploitation of a variety of novel metabolic enzymes/pathways.

GENOME ENGINEERING TOOLS

To date, a wealth of gene disruption and shuttle vector systems has been developed for *E. coli* as a model system. Simple inactivation of chromosomal genes by the PCR-mediated gene replacement (Datsenko and Wanner, 2000) has greatly facilitated the generation of specific mutants in the functional analysis of the microbial genome. It has been noted that disseminated throughout the genome are mobile DNA elements, which mediate recombination events, such as transposition and horizontal gene transfer, including insertion sequence (IS) elements, transposases, defective phages, integrases, and site-specific recombinases (Frost et al., 2005). To stabilize the genome and streamline metabolism, these elements must be deleted and unwanted functions removed. These unwanted functions include those specific for human hosts or particular environments. Indeed, a Tn5-targeted Cre/IoxP excision system (Yu et al., 2002) and a high-throughput method for the systematic mutagenesis of the genome by Tn5 transposon (Kang et al., 2004) enabled us to create individual *E. coli* deletion and insertion mutants without loss of normal growths. In particular, a multiplex automated genome engineering (MAGE) technique as a powerful high-throughput genome engineering tool has been developed (Wang et al., 2009a). MAGE simultaneously targets many locations on the chromosome for modification in a single cell or across a population of cells using allelic replacement to produce combinatorial genomic diversity through an oligonucleotide-directed recombineering technique. In addition, precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement as well (Isaacs et al., 2011). Further, as described above genome reductions may improve metabolic efficiency and decrease the redundancy among microbial genes and regulatory circuits (Posfai et al., 2006). Therefore, a rational design allows us to attempt to delete genes more extensively while avoiding loss of robustness, which can allow a chassis cell to be incorporated with biofuel-producing synthetic and/or engineered pathways.

OPTIMIZATION OF CELLULAR ENERGY METABOLISM

The attractive strategy described above immediately tempts us to ask questions like "Can we selectively delete the alternative energy transduction pathways as an engineering target?" and "Are minimal sets of genes beneficial in terms of energy efficiency?" This might be true simply because extreme environments already show a variety of extremophiles that can grow optimally with relatively small genome sizes (less than 2 Mb), as discussed above. In fact, their energy metabolisms appear to be designed and adapted to survive under their own specific environments in the most minimal but efficient way. This rationale seems to be supported by several challenging experiments about the effect of the modulation of respiratory chains on fermentation products (Portnoy et al., 2008, 2010). In these studies, a series of aerobic respiratory chains in *E. coli* were progressively knocked out and aerobically adapted to generate an evolved mutant deficient in three terminal oxidases. Initially, this mutant could not grow on M9 minimal medium containing D-glucose. However, 60-day adaptive evolution on the same medium created *E. coli* mutants that exhibited the ability to undergo mixed-acid fermentation during aerobic growth and to produce lactate as a fermentation product from D-glucose.

Moreover, the removal of three terminal cytochrome oxidase genes (*cydAB*, *cyoABCD*, and *cbdAB*) and a quinol monooxygenase gene (*ygiN*) yielded an *E. coli* mutant that exhibits anaerobic physiology even in the presence of oxygen, through the activation of the anoxic redox control transcriptional regulator ArcA (Portnoy et al., 2010). The knockout strain exhibited nearly identical physiological behaviors and produced D-lactate as the sole by-product under oxic and anoxic conditions, suggesting that the mutations resulted in significant metabolic and regulatory perturbations.

Genome-scale transcriptome analysis, ^{13}C tracing experiments, and physiological characterization demonstrated that the deletions resulted in the activation of anaerobic respiration under oxic conditions and a consequential shift in the content of the quinone pool (Portnoy et al., 2010). This result suggests that composition of quinone pool may be tightly coordinated with the activation of ArcB/ArcA regulatory system, concomitantly linked to a major shift in the metabolic flux distribution through the central metabolism in cells (Alexeeva et al., 2000). Therefore, respiratory control and/or modulation could be an efficient engineering strategy for changing the central metabolic flux of cells for the high yields of biofuel production (Fischer et al., 2008; Portnoy et al., 2010). This strategy can be validated by a systems-level characterization of fermentative profiles, using single gene knockouts in *E. coli*, which are related to redox reactions (Lee and Lee, unpublished data). The data demonstrated that single gene deletion mutations in *guaB*, *pyrD*, and *serA* increased D-lactic acid production. Combined knockouts of *guaB*, *pyrD*, *serA*, *fnr*, *arcA*, or *arcB* further enhanced D-lactate production.

Very recently, the hyperthermophilic archaeon *Pyrococcus furiosus* with the small genome size of 1.91 Mb, which grows optimally at 100°C, can be engineered to produce important organic chemicals from CO_2 by use of low potential reducing power from H_2 (Keller et al., 2013). For this, five genes of the carbon fixation cycle of the archaeon *Metallosphaera sedula,* which grows autotrophically at 73°C were heterologously expressed in *Pyrococcus furiosus.* The engineered *Pyrococcus furiosus* strain is able to use H_2 and incorporate CO_2 into 3-hydroxypropionic acid, a chemical building block for the production of acrylic acid, acrylamide, and 1,3-propanediol. Remarkably, this is operated at temperatures that are suboptimal for its growth to minimize the metabolic burden of the engineered microorganisms during chemical production from H_2 and CO_2. Such a strategic operation support only minimal growth but maintain sufficient metabolic activity to sustain the production of 3-hydroxypropionate. The unique temperature-dependent approach (Basen et al., 2012) that confers on a microorganism the capacity to use CO_2, a reaction that it does not occur naturally circumvents the overall low efficiency of photosynthesis and the production of sugar intermediates.

BIOMASS-BASED BIOFUEL PRODUCTION BY ENGINEERED MICROORGANISMS

A wide variety of metabolic engineering and systems biology approaches, including synthetic biology with microorganisms, has been made for exploitation of diverse biomass resources (Walter et al., 2007; Rude and Schirmer, 2009; Oh et al., 2011; Zhang et al., 2011). Although these approaches are promising, there are still limitations in terms of the technical feasibility of cost-effective energy resources and the availability of rapid genetic tools and in-depth physiological knowledge for the effective manipulation of energy transduction systems. Accordingly, a variety of research works have been focused on the development of a cost-effective and energy-efficient engineered microorganisms as platform cells to produce biofuels (Nakamura and Whited, 2003; Johnson and Schmidt-Dannert, 2008; Nielsen et al., 2009; Bokinsky et al., 2011; Yim et al., 2011). Implementation of heterologous pathways and/or metabolisms by the incorporation of single enzyme or a metabolic pathway module into host platform cells is the most frequently used strategy (Johnson et al., 2010; Hopkins et al., 2011; Shen et al., 2011; Lan and Liao, 2012; Westfall et al., 2012). With a macroscopic aspect, cellular metabolisms can be divided into two metabolic pathways: "feed" and "production" pathways (Fischer et al., 2008). Toward the high yields of titer and productivity in an engineered platform cell, tuning the redox balance between central energy and carbon metabolisms via metabolic intermediates is a key factor to improve biofuel productivity, while preventing from the reduction of bioenergetic waste reactions. As shown in **Figure 2**, microbial pathways for production of biofuels were categorized into four subgroups: non-fermentative alcohols, fatty acid-derived hydrocarbons, isoprenoid-derived hydrocarbons, and fermentative alcohols (Rude and Schirmer, 2009).

CELLULOSIC BIOFUELS

In contrast to starches and simple sugars derived from sugar cane and corn, lignocellulosic crops are regarded as sustainable and renewable. However, they also have some technical hurdles due to recalcitrance of cellulosic biomass rich in lignin, resistance to enzymatic hydrolysis and the presence of five carbon sugars (Demain et al., 2005). In this respect, consolidated bioprocessing is a promising strategy to overcome biomass recalcitrance by using cellulolytic microorganisms. One of the most closely studied of the cellulolytic microbes, *Clostridium thermocellum* (Wiegel et al., 1985), is being used for the production of ethanol through the consolidated bioprocessing of plant biomass (Bayer et al., 2008; Lynd et al., 2008). Recently, several genome sequences of thermophilic, plant biomass-degrading members of this genus (Blumer-Schuette et al., 2011) indicate that significant differences in glycoside hydrolase inventories and numbers of carbohydrate transporters exist, which likely relates to variability observed in plant biomass degradation capacity (Dam et al., 2011). In addition, the proteomic analysis of *C. phytofermentans*, which contains 161 carbohydrate-active enzymes, has been performed to identify hydrolases and metabolic enzymes to engineer microorganisms for improved cellulosic bioconversion (Tolonen et al., 2011). Intriguingly, it has been found that increase in tryptophan and nicotinamide synthesis was entailed with cellulosic fermentation for the production of ethanol, providing novel genetic targets for more efficient conversion of biomass to fuels and biomaterials (Tolonen et al., 2011). Alternatively, degradation of cellulosic biomass by extremely thermophilic bacteria *Caldicellulosiruptor* strains could have high potential for the production of biofuels (Blumer-Schuette et al., 2011).

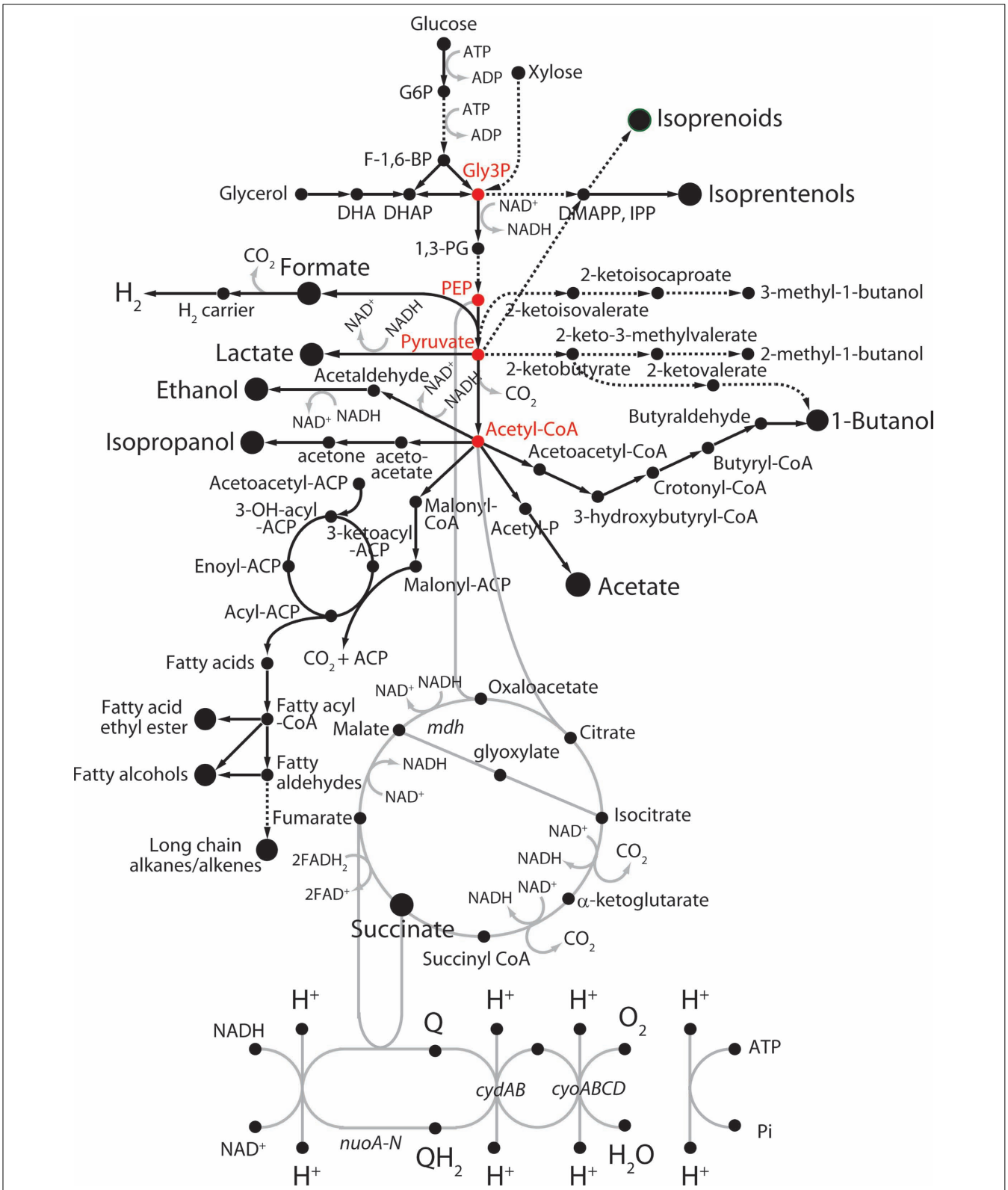


FIGURE 2 | Fermentative and non-fermentative pathways for the production of biofuels in *E. coli*. Dashed lines represent multiple reaction steps. Red circles represent metabolic intermediates. ACP, acyl-carrier protein; DHAP, dihydroxyacetone phosphate; DMAPP, dimethylallyl pyrophosphate; F-1,6-BP, fructose-1,6-bisphosphate; G6P, glucose-6-phosphate; Gly3P, glyceraldehyde 3-phosphate; IPP, isopentenyl pyrophosphate; 1,3-PG, 1,3-diphosphoglycerate; PEP, phosphoenolpyruvate.

Moreover, several attempts to tackle these practical issues to promote biomass waste solutions and biofuel production have been made through metabolic engineering and synthetic biology (Himmel et al., 2007; Vazana et al., 2012). To efficiently degrade crystalline cellulose, artificial enzymatic cellulosome complexes were designed and examined. There are an increasing number of research papers describing the production of designer cellulosomes either *in vitro, ex vivo,* or *in vivo* (Vazana et al., 2012). In designer cellulosomes, each enzyme is equipped with a dockerin module that interacts specifically with one of the cohesin modules of the chimeric scaffoldin. Artificial scaffoldins serve as docking backbones and contain a cellulose-specific carbohydrate-binding module that directs the enzymatic complex to the cellulosic substrate, as well as one or more cohesin modules from different natural cellulosomal species, each exhibiting a different specificity that allows the specific incorporation of the desired matching dockerin-bearing enzymes (Demain et al., 2005).

Another interesting approach in algae is that carbohydrates as a primary store of photosynthates can be exploited for biofuels through conversion to alcohols (Santelia and Zeeman, 2011). Algal polysaccharides can be hydrolyzed and then either fermented to ethanol by yeast or used as a heterotrophic carbon source for the production of a variety of biofuels (Harun et al., 2010). These carbohydrate productions in algae could be advantageous for biofuel production through overexpression of key enzymes in starch biosynthesis and secretion of soluble carbohydrates (Work et al., 2010).

HYDROCARBONS

To overcome the challenges (e.g., limited supply and land yield, inconsistent performance, and challenging economics) for production of biodiesels derived from plant oils, microbial fatty acid esters production could be an alternative way (Reijnders, 2008; Work et al., 2010; Li et al., 2012). In contrast to chemical refining to obtain designed biodiesels, microbial fatty acid esters can be easily altered in their composition (Mayer and Shanklin, 2007) and degree of saturation through manipulation of key regulators of the fatty acid biosynthesis (Peralta-Yahya et al., 2012) and introduction of foreign genes encoding wax synthase/diacylglycerol acyltransferase from other microbes (Stoveken and Steinbuchel, 2008). In addition, when *Arabidopsis* fatty acyl-CoA reductases (Rowland et al., 2006), that catalyze the formation of a fatty alcohol from an acyl-CoA, were expressed in *E. coli,* more favorable short fatty alcohols were synthesized (Doan et al., 2009), indicating that more efficient fatty alcohol-producing enzymes might be paramount for the commercial production of fatty alcohols.

To improve the efficiency of electrical energy in storage, Li et al. (2012) reported a method to store electrical energy as chemical energy in higher alcohols, which used synthetic biology approach for converting electricity and CO_2 to liquid biofuels. They genetically engineered a lithoautotrophic microorganism, *Ralstonia eutropha* H16, to produce isobutanol and 3-methyl-1-butanol in an electro-bioreactor using CO_2 as the sole carbon source and electricity as the sole energy input. This recent breakthrough technology integrated with electrochemical formate production and biological CO_2 fixation and higher alcohol synthesis, opens the possibility of electricity-driven bioconversion of CO_2 to commercial chemicals.

As an ideal replacement for diesel fuel, fatty acid-derived hydrocarbons such as alkanes are produced directly from fatty acid metabolites in numerous organisms. Although two biochemical routes (decarbonylation vs. reduction of fatty alcohols) are available (Dennis and Kolattukudy, 1992; Wackett et al., 2007), their biosynthetic pathways to alkanes are not fully understood. Recently, Schirmer et al. (2010) performed comparative genomics and subtractive genome analysis with more than 50 genome sequences of cyanobacteria to find out two hypothetical enzymes and propose these as acyl-ACP reductase and aldehyde decarbonylase, responsible for alkane biosynthesis. Subsequently, their co-expression in *E. coli* enabled *in vivo* alkane biosynthesis (C13 to C17 mixtures), which is a major step toward the goal of low-cost renewable transportation fuels.

NON-FERMENTATIVE SHORT-CHAIN ALCOHOLS

In contrast to fermentative alcohols produced from carbohydrates and lipids (**Figure 2**) like ethanol and butanol, the non-fermentative short-chain alcohols can be generated with protein sources by introducing exogenous transamination and deamination cycles (Huo et al., 2011). In order to develop a process for conversion of mixtures of peptides and amino acids to biofuels or chemicals, carbon skeletons could be provided from 2-keto acids through deamination of amino acids by 2-keto acid decarboxylases, and then to alcohols by alcohol dehydrogenases as shown in **Figure 2** (Atsumi et al., 2008). Alternatively, other amino acids could be deaminated to TCA cycle intermediates, which can be directed to pyruvate by malic enzymes or phosphoenolpyruvate carboxykinase. Pyruvate can be further extended to longer keto acids by acetohydroxy acid synthase or isopropylmalate synthase chain elongation pathways (Zhang et al., 2008). Such implementation of synthetic pathways together with rewiring metabolisms consequently enabled us to develop a protein-based process for biorefining and fuel production in potential platform cells such as *E. coli*, *B. subtilis*, yeast, and microalgae. Indeed, several *E. coli* variants that were improved for amino acid utilization were screened, and then isobutanol synthesis pathways were introduced into the cells to yield a strain that grew despite the stress generated by increased fuel production (Huo et al., 2011). Further, deletion of ammonium-assimilation genes, *gdhA* and *glnA*, increased the production of alcohols in the presence of the keto acid pathway. Thus, in contrast to previous metabolic engineering of carbon metabolic modules, application of nitrogen metabolic modules can be a significantly considerable alternative strategy in biofuel metabolic engineering.

H_2

Biological hydrogen (H_2) is a potentially favorable, renewable, and ideal fuel for future demand in terms of climate change and sustainability. At present, biological H_2 has been produced through three major processes: biophotolysis, photofermentation, and dark fermentation (Oh et al., 2011). In green algae and cyanobacteria, photosynthesis coupled to H_2 evolution requires only water and sunlight (Prince and Kheshgi, 2005). Several cyanobacteria utilize an indirect pathway wherein storage

carbohydrates generated through photosynthesis are fermentatively used to produce H_2. Significant improvements for H_2 production yield could be achieved by truncation of light-harvesting complexes and reduction of chlorophyll content in green algae and cyanobacteria (Beckmann et al., 2009; Mitra and Melis, 2010; Srirangan et al., 2011). Impaired cyclic electron transport also results in increased electron flow to the hydrogenase (Kruse et al., 2005a). Further findings of alternative anaerobic pathways (Hemschemeier and Happe, 2011), auxiliary electron transport (Peltier et al., 2010), and other distinct anaerobic H_2 metabolisms (Meuser et al., 2009) could be potential targets to improve H_2 production. Most hydrogenases are extremely oxygen-sensitive, which is one of the greatest challenges facing the establishment of an industrial biophotolysis process. For this reason, either control of O_2 levels (Surzycki et al., 2007) or use of protein engineering should be implemented to create enzymes with greater O_2 tolerance (Fritsch et al., 2011; Goris et al., 2011; Liebgott et al., 2011).

Alternatively, strictly anaerobic extremophiles, such as methanogenic archaea and hyperthermophilic bacteria, could be an attractive option for the production of biological H_2 (Kim et al., 2010). H_2 provides an excellent source of low potential reducing power for growth and biosynthesis of archaea (Sun et al., 2010). Recently, the heterologous expression in *E. coli* of a functional [NiFe]-hydrogenase from a hyperthermophilic archaea, *Pyrococcus furiosus*, was performed by employing a novel set of compatible vectors modified with an anaerobically induced *E. coli* hydrogenase promoter (Hopkins et al., 2011). Together with successful engineering of hydrogenase and nitrogenase (O_2 sensitivity and higher molar production; Stripp et al., 2009; Yamamoto et al., 2009; Liebgott et al., 2010, 2011; Fritsch et al., 2011), development of versatile genetic systems and improvements in such oxygen-sensitive and intricate maturation requiring enzymes enable us to design and exploit the use of novel microorganisms and their constituent hydrogenases for biohydrogen production.

PHOTOSYNTHESIS AND CO_2 FIXATION

Solar energy-derived biofuels are one of the most abundant and favorable energy resources with respect to carbon neutralization and sustainability. The photosynthetic process, comprising the reduction of CO_2, utilizing light and water by plants and algae (**Figure 3**), conserves solar energy in the form of reduced carbon compounds at a rate of approximately 120 TW, far exceeding the current annual global energy demand of approximately 14.9 TW (Barber, 2009; Hambourger et al., 2009). Hence, many researchers have focused on utilization of marine algae as a potential source of sustainable energy for biofuels, which can contribute to global energy independence (Robertson et al., 2011).

Photosynthetic microorganisms can serve as feedstocks for biofuels (Dismukes et al., 2008; Radakovits et al., 2010; Wijffels and Barbosa, 2010). Toward this aim, pathway engineering and culture modification have been developed with a result of high yields of biohydrogen, lipids, and carbohydrates. Nevertheless, the photosynthesis-derived biofuels with green algae still have significant challenges in the inefficiency of photosynthesis (Kruse et al., 2005b; Barber, 2009; Blankenship et al., 2011), the productivity of biomass, and the availability of genetic manipulation (Hambourger et al., 2009; Stephenson et al., 2011). In particular, optimization of light capture, energy transfer, and carbon fixation through manipulation of these pathways is essential for improvements in their photosynthetic efficiency, which is the principal determinant of productivity (Work et al., 2012). Several recent

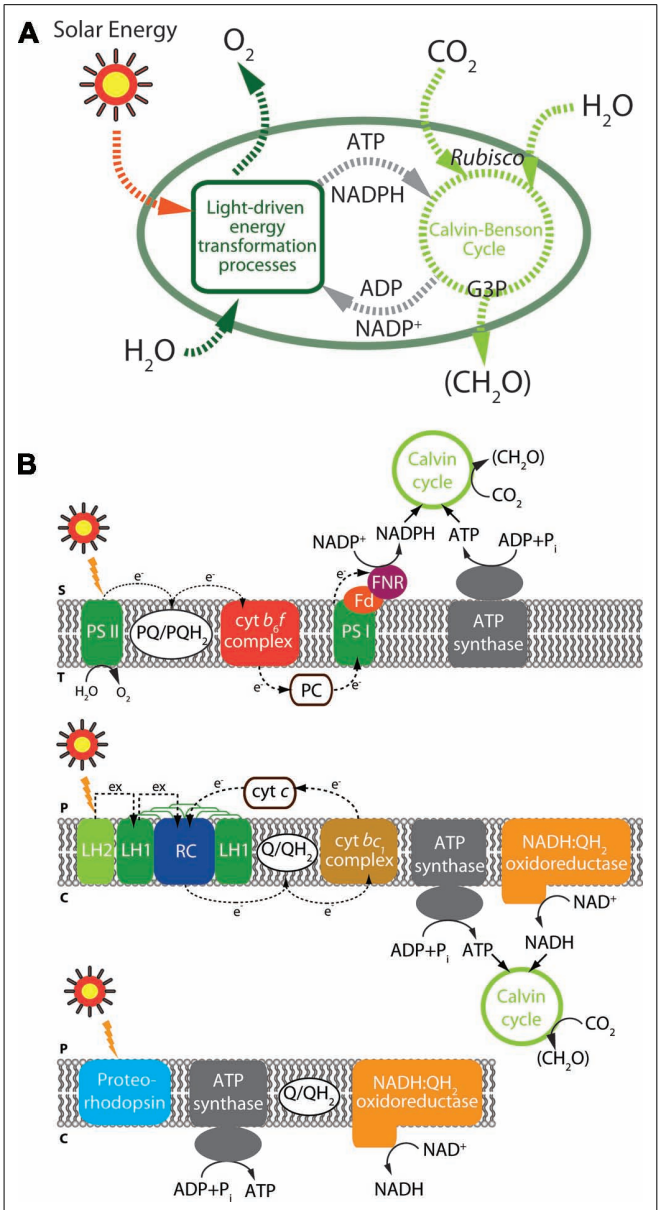


FIGURE 3 | Illustration of photosynthesis and carbon fixation (A) and key light-driven processes in microorganisms (B). Black solid and dashed arrows represent reactions catalyzed by each enzyme and electron flows, respectively. (Top panel) Overview of oxygenic photosynthesis from cyanobacteria. (Middle panel) Overview of anoxygenic photosynthesis from α-proteobacteria. (Bottom panel) Light-driven proton pump. The major components of photosynthesis and carbon fixation including elements are depicted: S, chloroplast stroma; T, thylakoid lumen; P, periplasm; C, cytoplasm; Cyt *c*, cytochrome *c*; Cyt bc_1, cytochrome bc_1; Cyt b_6f, cytochrome b_6f; Fd, ferredoxin; FNR, ferredoxin-NADP$^+$ reductase; LH, light-harvesting complex; PC, plastocyanin; PS, photosystem; PQ, plastoquinone; Q, ubiquinone; QH_2, ubiquinol.

approaches by truncation or deregulation of light-harvesting antenna complexes (Beckmann et al., 2009; Melis, 2009), implementation of C4-type carbon concentrating mechanisms into C3 plants (Yamano et al., 2010; Reinfelder, 2011), optimization of the Calvin cycle by modulating expression levels of related enzymes (Miyagawa et al., 2001; Zhu et al., 2007), and expanding the solar spectrum (Chen et al., 2010; Chen and Blankenship, 2011) seem to be quite successful. For example, engineering and overexpression of ribulose-1,5-bisphosphate carboxylase oxygenase (RUBISCO) is a clear target for raising the efficiency of light energy conversion, resulting in productivity improvements (Lefebvre et al., 2005; Whitney et al., 2011).

In addition, lipids (i.e., triacylglycerols) in microalgae serve as an attractive biofuel feedstock, which can be converted to biodiesel through not only transesterification in biorefining (He et al., 2012) but also genetically interrupting starch biosynthesis (Wang et al., 2009b; Work et al., 2010). Accordingly, several attempts have been made to increase the lipid productivity of algae by screening and/or engineering algae using new enabling technologies, such as whole-genome sequencing, transcriptomics, metagenomics, and flow cytometry (Scott et al., 2010; Work et al., 2010; Stephenson et al., 2011). In addition, cyanobacteria as an excellent system for biodiesel production have been engineered by overexpression of a bacterial diacylglycerol acyltransferase, a phosphatidate phosphatase, and an acetyl-CoA carboxylase for increased lipid production (James et al., 2010). Other important and reasonable strategies for photosynthesis with microalgal biofuels (e.g., truncation of antenna complexes and lipid accumulation) are well described in (Wijffels and Barbosa, 2010; Work et al., 2012).

Alternatively, production of chemicals and fuels directly from CO_2 is an attractive approach to solving energy and environmental problems (Lan and Liao, 2011). Previously, production of 1-butanol as a potential fuel substitute and an important chemical feedstock by the fermentative coenzyme A (CoA)-dependent pathway (Ezeji et al., 2007; Papoutsakis, 2008) using the reversal of β-oxidation has been performed in various organisms (Nielsen et al., 2009; Nicolaou et al., 2010; Yu et al., 2011). In addition, a modified clostridial 1-butanol pathway, including synthetic build-up of NADH and acetyl-CoA, enabled *E. coli* cells to produce a high titer and a high yield of 1-butanol production (Shen et al., 2011). Subsequently, the thermodynamically unfavorable step of the condensation of acetyl-CoA to acetoacetyl-CoA could be driven by artificially engineered ATP consumption through a pathway modification together with substitution of bifunctional aldehyde/alcohol dehydrogenase with separate butyraldehyde (butanal) dehydrogenase and NADPH-dependent alcohol dehydrogenase to improve the direct photosynthetic production of 1-butanol from cyanobacteria *Synechococcus elongatus* PCC 7942 (Lan and Liao, 2012). Therefore, these approaches, based on the importance of ATP and cofactor driving forces, have made a novel avenue to designing an efficient principle to alter metabolic flux by use of non-natural pathways.

FUTURE PERSPECTIVE

A wealth of genome information dramatically expands our understanding of a variety of microbial metabolic pathways available for our purposes. This leads us to attempt to design and engineer microbial cell factories devoted to producing high yields of biofuels by treating metabolic pathways as modules or parts that can be readily moved at will from one organism to another. To date, many successful examples of implementation of non-indigenous metabolic or enzymatic modules in microbial host cells have been made through redesign and rearrangement of pathways, and the creative engineering of metabolic enzymes. Nonetheless, there are still limitations to obtaining the theoretical maximal yields of biofuels to meet our practical demands. Toward these aims, we need to further understand how microbial cells can coordinate their metabolic pathways under different environmental conditions, underlying essential and non-essential genes for bacterial life and metabolic networks. This can provide an effective direction for the design of minimal maintenance energy in cells. Moreover, balancing metabolic fluxes between biosynthesis of cellular mass and production of biofuels through modulation of their metabolic efficiency in cells can be an important key factor to achieving high yields of biofuel productions. Hence, both aspects are directly correlated to the choice of feedstock and biofuel-producing pathways, which is the fundamental basis for the cost-effective production of biofuels in high yield.

Overall, to design and construct the ideally synthetic microorganisms for biofuel productions, most desirable and effective are both (i) the increased efficiency of biosynthesis by the reduction of unnecessary energy-transducing components and their coordinated metabolic pathways, and (ii) high yields of biofuel production by the implementation of non-indigenous pathways with which renewable energy is ultimately transferred to the conversion of usable biomass and/or CO_2 to biofuels.

ACKNOWLEDGMENTS

This work was supported by a grant, 311042-05-1-HD120 (AGC0891111), from the Korea Institute of Planning and Evaluation for Technology (iPET), funded by the Ministry for Food, Agriculture, Forestry and Fisheries and the KRIBB Innovative Research Program. We gratefully acknowledge Prof. Jae-Ho Shin for critical reading and helpful discussions.

REFERENCES

Alexeeva, S., de Kort, B., Sawers, G., Hellingwerf, K. J., and De Mattos, M. J. (2000). Effects of limited aeration and of the ArcAB system on intermediary pyruvate catabolism in *Escherichia coli*. *J. Bacteriol.* 182, 4934–4940.

Ara, K., Ozaki, K., Nakamura, K., Yamane, K., Sekiguchi, J., and Ogasawara, N. (2007). *Bacillus* minimum genome factory: effective utilization of microbial genome information. *Biotechnol. Appl. Biochem.* 46, 169–178.

Atsumi, S., Hanai, T., and Liao, J. C. (2008). Non-fermentative pathways for synthesis of branched-chain higher alcohols as biofuels. *Nature* 451, 86–89.

Averhoff, B. (2009). Shuffling genes around in hot environments: the unique DNA transporter of *Thermus thermophilus*. *FEMS Microbiol. Rev.* 33, 611–626.

Barber, J. (2009). Photosynthetic energy conversion: natural and artificial. *Chem. Soc. Rev.* 38, 185–196.

Basen, M., Sun, J., and Adams, M. W. (2012). Engineering a hyperthermophilic archaeon for temperature-dependent product formation. *mBio* 3, e00053-12.

Bayer, E. A., Lamed, R., White, B. A., and Flint, H. J. (2008). From cellulosomes to cellulosomics. *Chem. Rec.* 8, 364–377.

Beckmann, J., Lehr, F., Finazzi, G., Hankamer, B., Posten, C., Wobbe, L., et al. (2009). Improvement of light to biomass conversion by de-regulation of light-harvesting protein translation in *Chlamydomonas reinhardtii*. *J. Biotechnol.* 142, 70–77.

Benders, G. A., Noskov, V. N., Denisova, E. A., Lartigue, C., Gibson, D. G., Assad-Garcia, N., et al. (2010). Cloning whole bacterial genomes in yeast. *Nucleic Acids Res.* 38, 2558–2569.

Blankenship, R. E., Tiede, D. M., Barber, J., Brudvig, G. W., Fleming, G., Ghirardi, M., et al. (2011). Comparing photosynthetic and photovoltaic efficiencies and recognizing the potential for improvement. *Science* 332, 805–809.

Blumer-Schuette, S. E., Ozdemir, I., Mistry, D., Lucas, S., Lapidus, A., Cheng, J. F., et al. (2011). Complete genome sequences for the anaerobic, extremely thermophilic plant biomass-degrading bacteria *Caldicellulosiruptor hydrothermalis*, *Caldicellulosiruptor kristjanssonii*, *Caldicellulosiruptor kronotskyensis*, *Caldicellulosiruptor owensensis*, and *Caldicellulosiruptor lactoaceticus*. *J. Bacteriol.* 193, 1483–1484.

Bokinsky, G., Peralta-Yahya, P. P., George, A., Holmes, B. M., Steen, E. J., Dietrich, J., et al. (2011). Synthesis of three advanced biofuels from ionic liquid-pretreated switchgrass using engineered *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19949–19954.

Brochier-Armanet, C., Forterre, P., and Gribaldo, S. (2011). Phylogeny and evolution of the Archaea: one hundred genomes later. *Curr. Opin. Microbiol.* 14, 274–281.

Bult, C. J., White, O., Olsen, G. J., Zhou, L., Fleischmann, R. D., Sutton, G. G., et al. (1996). Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* 273, 1058–1073.

Carothers, J. M., Goler, J. A., and Keasling, J. D. (2009). Chemical synthesis using synthetic biology. *Curr. Opin. Biotechnol.* 20, 498–503.

Chen, M., and Blankenship, R. E. (2011). Expanding the solar spectrum used by photosynthesis. *Trends Plant Sci.* 16, 427–431.

Chen, M., Schliep, M., Willows, R. D., Cai, Z. L., Neilan, B. A., and Scheer, H. (2010). A red-shifted chlorophyll. *Science* 329, 1318–1319.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends Biotechnol.* 26, 126–131.

Chisholm, S. W., Olson, R. J., Zettler, E. R., Goericke, R., Waterbury, J. B., and Welschmeyer, N. A. (1988). A novel free-living prochlorophyte abundant in the oceanic euphotic zone. *Nature* 334, 340–343.

Conrado, R. J., Varner, J. D., and Delisa, M. P. (2008). Engineering the spatial organization of metabolic enzymes: mimicking nature's synergy. *Curr. Opin. Biotechnol.* 19, 492–499.

Dam, P., Kataeva, I., Yang, S. J., Zhou, F., Yin, Y., Chou, W., et al. (2011). Insights into plant biomass conversion from the genome of the anaerobic thermophilic bacterium *Caldicellulosiruptor bescii* DSM 6725. *Nucleic Acids Res.* 39, 3240–3254.

Datsenko, K. A., and Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97, 6640–6645.

Deckert, G., Warren, P. V., Gaasterland, T., Young, W. G., Lenox, A. L., Graham, D. E., et al. (1998). The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* 392, 353–358.

Delebecque, C. J., Lindner, A. B., Silver, P. A., and Aldaye, F. A. (2011). Organization of intracellular reactions with rationally designed RNA assemblies. *Science* 333, 470–474.

Demain, A. L., Newcomb, M., and Wu, J. H. (2005). Cellulase, clostridia, and ethanol. *Microbiol. Mol. Biol. Rev.* 69, 124–154.

Dennis, M., and Kolattukudy, P. E. (1992). A cobalt-porphyrin enzyme converts a fatty aldehyde to a hydrocarbon and CO. *Proc. Natl. Acad. Sci. U.S.A.* 89, 5306–5310.

Dewall, M. T., and Cheng, D. W. (2011). The minimal genome: a metabolic and environmental comparison. *Brief. Funct. Genomics* 10, 312–315.

Dhar, R., Sagesser, R., Weikert, C., Yuan, J., and Wagner, A. (2011). Adaptation of *Saccharomyces cerevisiae* to saline stress through laboratory evolution. *J. Evol. Biol.* 24, 1135–1153.

Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., and Posewitz, M. C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr. Opin. Biotechnol.* 19, 235–240.

Doan, T. T., Carlsson, A. S., Hamberg, M., Bulow, L., Stymne, S., and Olsson, P. (2009). Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. *J. Plant Physiol.* 166, 787–796.

Dueber, J. E., Wu, G. C., Malmirchegini, G. R., Moon, T. S., Petzold, C. J., Ullal, A. V., et al. (2009). Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* 27, 753–759.

Dufresne, A., Garczarek, L., and Partensky, F. (2005). Accelerated evolution associated with genome reduction in a free-living prokaryote. *Genome Biol.* 6, R14.

Dufresne, A., Salanoubat, M., Partensky, F., Artiguenave, F., Axmann, I. M., Barbe, V., et al. (2003). Genome sequence of the cyanobacterium *Prochlorococcus marinus* SS120, a nearly minimal oxyphototrophic genome. *Proc. Natl. Acad. Sci. U.S.A.* 100, 10020–10025.

Ezeji, T. C., Qureshi, N., and Blaschek, H. P. (2007). Bioproduction of butanol from biomass: from genes to bioreactors. *Curr. Opin. Biotechnol.* 18, 220–227.

Fischer, C. R., Klein-Marcuschamer, D., and Stephanopoulos, G. (2008). Selection and optimization of microbial hosts for biofuels production. *Metab. Eng.* 10, 295–304.

Fleischmann, R. D., Adams, M. D., White, O., Clayton, R. A., Kirkness, E. F., Kerlavage, A. R., et al. (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* 269, 496–512.

Forster, A. C., and Church, G. M. (2006). Towards synthesis of a minimal cell. *Mol. Syst. Biol.* 2, 45.

Forster, A. C., and Church, G. M. (2007). Synthetic biology projects *in vitro*. *Genome Res.* 17, 1–6.

Forterre, P., Bouthier De La Tour, C., Philippe, H., and Duguet, M. (2000). Reverse gyrase from hyperthermophiles: probable transfer of a thermoadaptation trait from archaea to bacteria. *Trends Genet.* 16, 152–154.

Fraser, C. M., Gocayne, J. D., White, O., Adams, M. D., Clayton, R. A., Fleischmann, R. D., et al. (1995). The minimal gene complement of *Mycoplasma genitalium*. *Science* 270, 397–403.

Fritsch, J., Scheerer, P., Frielingsdorf, S., Kroschinsky, S., Friedrich, B., Lenz, O., et al. (2011). The crystal structure of an oxygen-tolerant hydrogenase uncovers a novel iron-sulphur centre. *Nature* 479, 249–252.

Frost, L. S., Leplae, R., Summers, A. O., and Toussaint, A. (2005). Mobile genetic elements: the agents of open source evolution. *Nat. Rev. Microbiol.* 3, 722–732.

Futterer, O., Angelov, A., Liesegang, H., Gottschalk, G., Schleper, C., Schepers, B., et al. (2004). Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proc. Natl. Acad. Sci. U.S.A.* 101, 9091–9096.

Geddes, C. C., Nieves, I. U., and Ingram, L. O. (2011). Advances in ethanol production. *Curr. Opin. Biotechnol.* 22, 312–319.

Gerdes, S. Y., Scholle, M. D., Campbell, J. W., Balazsi, G., Ravasz, E., Daugherty, M. D., et al. (2003). Experimental determination and system level analysis of essential genes in *Escherichia coli* MG1655. *J. Bacteriol.* 185, 5673–5684.

Gibson, D. G., Benders, G. A., Andrews-Pfannkoch, C., Denisova, E. A., Baden-Tillson, H., Zaveri, J., et al. (2008a). Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 319, 1215–1220.

Gibson, D. G., Benders, G. A., Axelrod, K. C., Zaveri, J., Algire, M. A., Moodie, M., et al. (2008b). One-step assembly in yeast of 25 overlapping DNA fragments to form a complete synthetic *Mycoplasma genitalium* genome. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20404–20409.

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., et al. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56.

Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A. III, and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods* 6, 343–345.

Giovannoni, S. J., Tripp, H. J., Givan, S., Podar, M., Vergin, K. L., Baptista, D., et al. (2005). Genome streamlining in a cosmopolitan oceanic bacterium. *Science* 309, 1242–1245.

Glass, J. I., Assad-Garcia, N., Alperovich, N., Yooseph, S., Lewis, M. R., Maruf, M., et al. (2006). Essential genes of a minimal bacterium. *Proc. Natl. Acad. Sci. U.S.A.* 103, 425–430.

Gonzalez, A., and Knight, R. (2012). Advancing analytical algorithms and pipelines for billions of microbial sequences. *Curr. Opin. Biotechnol.* 23, 64–71.

Goris, T., Wait, A. F., Saggu, M., Fritsch, J., Heidary, N., Stein, M., et al. (2011). A unique iron-sulfur cluster is crucial for oxygen tolerance of a [NiFe]-hydrogenase. *Nat. Chem. Biol.* 7, 310–318.

Gray, K. A., Zhao, L., and Emptage, M. (2006). Bioethanol. *Curr. Opin. Chem. Biol.* 10, 141–146.

Hambourger, M., Moore, G. F., Kramer, D. M., Gust, D., Moore, A. L., and Moore, T. A. (2009). Biology and technology for photochemical fuel production. *Chem. Soc. Rev.* 38, 25–35.

Harun, R., Singh, M., Forde, G. M., and Danquah, M. K. (2010). Bioprocess engineering of microalgae to

produce a variety of consumer products. *Renew. Sust. Energ. Rev.* 14, 1037–1047.

He, Y., Bagley, D. M., Leung, K. T., Liss, S. N., and Liao, B. Q. (2012). Recent advances in membrane technologies for biorefining and bioenergy production. *Biotechnol. Adv.* 30, 817–858.

Hemschemeier, A., and Happe, T. (2011). Alternative photosynthetic electron transport pathways during anaerobiosis in the green alga *Chlamydomonas reinhardtii*. *Biochim. Biophys. Acta* 1807, 919–926.

Herring, C. D., Raghunathan, A., Honisch, C., Patel, T., Applebee, M. K., Joyce, A. R., et al. (2006). Comparative genome sequencing of *Escherichia coli* allows observation of bacterial evolution on a laboratory timescale. *Nat. Genet.* 38, 1406–1412.

Himmel, M. E., Ding, S. Y., Johnson, D. K., Adney, W. S., Nimlos, M. R., Brady, J. W., et al. (2007). Biomass recalcitrance: engineering plants and enzymes for biofuels production. *Science* 315, 804–807.

Holtz, W. J., and Keasling, J. D. (2010). Engineering static and dynamic control of synthetic pathways. *Cell* 140, 19–23.

Hopkins, R. C., Sun, J., Jenney, F. E. Jr., Chandrayan, S. K., Mcternan, P. M., and Adams, M. V. V. (2011). Homologous expression of a subcomplex of *Pyrococcus furiosus* hydrogenase that interacts with pyruvate ferredoxin oxidoreductase. *PLoS ONE* 6:e26569. doi: 10.1371/journal.pone.0026569

Huber, H., Hohn, M. J., Rachel, R., Fuchs, T., Wimmer, V. C., and Stetter, K. O. (2002). A new phylum of Archaea represented by a nanosized hyperthermophilic symbiont. *Nature* 417, 63–67.

Huo, Y. X., Cho, K. M., Rivera, J. G., Monte, E., Shen, C. R., Yan, Y., et al. (2011). Conversion of proteins into biofuels by engineering nitrogen flux. *Nat. Biotechnol.* 29, 346–351.

Isaacs, F. J., Carr, P. A., Wang, H. H., Lajoie, M. J., Sterling, B., Kraal, L., et al. (2011). Precise manipulation of chromosomes *in vivo* enables genome-wide codon replacement. *Science* 333, 348–353.

James, R., Fred, C., Paul, W., Javier, M. E., Henry, L. M., and Khadidja, R. (2010). Modified photosynthetic microorganisms for producing triglycerides. US patent US 20100255551 A1.

Jewett, M. C., and Forster, A. C. (2010). Update on designing and building minimal cells. *Curr. Opin. Biotechnol.* 21, 697–703.

Johnson, E. T., Baron, D. B., Naranjo, B., Bond, D. R., Schmidt-Dannert, C., and Gralnick, J. A. (2010). Enhancement of survival and electricity production in an engineered bacterium by light-driven proton pumping. *Appl. Environ. Microbiol.* 76, 4123–4129.

Johnson, E. T., and Schmidt-Dannert, C. (2008). Light-energy conversion in engineered microorganisms. *Trends Biotechnol.* 26, 682–689.

Joyce, A. R., Reed, J. L., White, A., Edwards, R., Osterman, A., Baba, T., et al. (2006). Experimental and computational assessment of conditionally essential genes in *Escherichia coli*. *J. Bacteriol.* 188, 8259–8271.

Kang, Y., Durfee, T., Glasner, J. D., Qiu, Y., Frisch, D., Winterberg, K. M., et al. (2004). Systematic mutagenesis of the *Escherichia coli* genome. *J. Bacteriol.* 186, 4921–4930.

Keasling, J. D. (2010). Manufacturing molecules through metabolic engineering. *Science* 330, 1355–1358.

Keller, M. W., Schut, G. J., Lipscomb, G. L., Menon, A. L., Iwuchukwu, I. J., Leuko, T. T., et al. (2013). Exploiting microbial hyperthermophilicity to produce an industrial chemical, using hydrogen and carbon dioxide. *Proc. Natl. Acad. Sci. U.S.A.* doi: 10.1073/pnas.1222607110 [Epub ahead of print].

Kellis, M., Birren, B. W., and Lander, E. S. (2004). Proof and evolutionary analysis of ancient genome duplication in the yeast *Saccharomyces cerevisiae*. *Nature* 428, 617–624.

Kellis, M., Patterson, N., Endrizzi, M., Birren, B., and Lander, E. S. (2003). Sequencing and comparison of yeast species to identify genes and regulatory elements. *Nature* 423, 241–254.

Kim, Y. J., Lee, H. S., Kim, E. S., Bae, S. S., Lim, J. K., Matsumi, R., et al. (2010). Formate-driven growth coupled with H2 production. *Nature* 467, 352–355.

Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., et al. (2003). Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4678–4683.

Koonin, E. V. (2003). Comparative genomics, minimal gene-sets and the last universal common ancestor. *Nat. Rev. Microbiol.* 1, 127–136.

Kruse, O., Rupprecht, J., Bader, K. P., Thomas-Hall, S., Schenk, P. M., Finazzi, G., et al. (2005a). Improved photobiological H2 production in engineered green algal cells. *J. Biol. Chem.* 280, 34170–34177.

Kruse, O., Rupprecht, J., Mussgnug, J. H., Dismukes, G. C., and Hankamer, B. (2005b). Photosynthesis: a blueprint for solar energy capture and biohydrogen production technologies. *Photochem. Photobiol. Sci.* 4, 957–970.

Lan, E. I., and Liao, J. C. (2011). Metabolic engineering of cyanobacteria for 1-butanol production from carbon dioxide. *Metab. Eng.* 13, 353–363.

Lan, E. I., and Liao, J. C. (2012). ATP drives direct photosynthetic production of 1-butanol in cyanobacteria. *Proc. Natl. Acad. Sci. U.S.A.* 109, 6018–6023.

Lefebvre, S., Lawson, T., Zakhleniuk, O. V., Lloyd, J. C., Raines, C. A., and Fryer, M. (2005). Increased sedoheptulose-1,7-bisphosphatase activity in transgenic tobacco plants stimulates photosynthesis and growth from an early stage in development. *Plant Physiol.* 138, 451–460.

Letunic, I., Yamada, T., Kanehisa, M., and Bork, P. (2008). iPath: interactive exploration of biochemical pathways and networks. *Trends Biochem. Sci.* 33, 101–103.

Li, H., Opgenorth, P. H., Wernick, D. G., Rogers, S., Wu, T. Y., Higashide, W., et al. (2012). Integrated electromicrobial conversion of CO2 to higher alcohols. *Science* 335, 1596.

Liebgott, P. P., de Lacey, A. L., Burlat, B., Cournac, L., Richaud, P., Brugna, M., et al. (2011). Original design of an oxygen-tolerant [NiFe] hydrogenase: major effect of a valine-to-cysteine mutation near the active site. *J. Am. Chem. Soc.* 133, 986–997.

Liebgott, P. P., Leroux, F., Burlat, B., Dementin, S., Baffert, C., Lautier, T., et al. (2010). Relating diffusion along the substrate tunnel and oxygen sensitivity in hydrogenase. *Nat. Chem. Biol.* 6, 63–70.

Lynd, L. R., Laser, M. S., Bransby, D., Dale, B. E., Davison, B., Hamilton, R., et al. (2008). How biotech can transform biofuels. *Nat. Biotechnol.* 26, 169–172.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21, 796–802.

Mayer, K. M., and Shanklin, J. (2007). Identification of amino acid residues involved in substrate specificity of plant acyl-ACP thioesterases using a bioinformatics-guided approach. *BMC Plant Biol.* 7:1. doi: 10.1186/1471-2229-7-1

McCutcheon, J. P., and Moran, N. A. (2012). Extreme genome reduction in symbiotic bacteria. *Nat. Rev. Microbiol.* 10, 13–26.

Medema, M. H., Breitling, R., Bovenberg, R., and Takano, E. (2011). Exploiting plug-and-play synthetic biology for drug discovery and production in microorganisms. *Nat. Rev. Microbiol.* 9, 131–137.

Melis, A. (2009). Solar energy conversion efficiencies in photosynthesis: minimizing the chlorophyll antennae to maximize efficiency. *Plant Sci.* 177, 272–280.

Meuser, J. E., Ananyev, G., Wittig, L. E., Kosourov, S., Ghirardi, M. L., Seibert, M., et al. (2009). Phenotypic diversity of hydrogen production in chlorophycean algae reflects distinct anaerobic metabolisms. *J. Biotechnol.* 142, 21–30.

Mitra, M., and Melis, A. (2010). Genetic and biochemical analysis of the TLA1 gene in *Chlamydomonas reinhardtii*. *Planta* 231, 729–740.

Miyagawa, Y., Tamoi, M., and Shigeoka, S. (2001). Overexpression of a cyanobacterial fructose-1,6-/sedoheptulose-1,7-bisphosphatase in tobacco enhances photosynthesis and growth. *Nat. Biotechnol.* 19, 965–969.

Mushegian, A. R., and Koonin, E. V. (1996). A minimal gene set for cellular life derived by comparison of complete bacterial genomes. *Proc. Natl. Acad. Sci. U.S.A.* 93, 10268–10273.

Nakamura, C. E., and Whited, G. M. (2003). Metabolic engineering for the microbial production of 1,3-propanediol. *Curr. Opin. Biotechnol.* 14, 454–459.

Nicolaou, S. A., Gaida, S. M., and Papoutsakis, E. T. (2010). A comparative view of metabolite and substrate stress and tolerance in microbial bioprocessing: from biofuels and chemicals, to biocatalysis and bioremediation. *Metab. Eng.* 12, 307–331.

Nielsen, D. R., Leonard, E., Yoon, S. H., Tseng, H. C., Yuan, C., and Prather, K. L. (2009). Engineering alternative butanol production platforms in heterologous bacteria. *Metab. Eng.* 11, 262–273.

Nielsen, J., and Keasling, J. D. (2011). Synergies between synthetic biology and metabolic engineering. *Nat. Biotechnol.* 29, 693–695.

Nitschke, J. R. (2009). Systems chemistry: molecular networks come of age. *Nature* 462, 736–738.

Oh, Y. K., Raj, S. M., Jung, G. Y., and Park, S. (2011). Current status of the metabolic engineering of microorganisms for biohydrogen production. *Bioresour. Technol.* 102, 8357–8367.

Pandey, D. P., and Gerdes, K. (2005). Toxin–antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Res.* 33, 966–976.

Papoutsakis, E. T. (2008). Engineering solventogenic clostridia. *Curr. Opin. Biotechnol* 19, 420–429.

Peltier, G., Tolleter, D., Billon, E., and Cournac, L. (2010). Auxiliary electron transport pathways in chloroplasts of microalgae. *Photosynth. Res.* 106, 19–31.

Peralta-Yahya, P. P., Zhang, F., Del Cardayre, S. B., and Keasling, J. D. (2012). Microbial engineering for the production of advanced biofuels. *Nature* 488, 320–328.

Portnoy, V. A., Herrgard, M. J., and Palsson, B. O. (2008). Aerobic fermentation of D-glucose by an evolved cytochrome oxidase-deficient *Escherichia coli* strain. *Appl. Environ. Microbiol.* 74, 7561–7569.

Portnoy, V. A., Scott, D. A., Lewis, N. E., Tarasova, Y., Osterman, A. L., and Palsson, B. O. (2010). Deletion of genes encoding cytochrome oxidases and quinol monooxygenase blocks the aerobic-anaerobic shift in *Escherichia coli* K-12 MG1655. *Appl. Environ. Microbiol.* 76, 6529–6540.

Posfai, G., Plunkett, G. III, Feher, T., Frisch, D., Keil, G. M., Umenhoffer, K., et al. (2006). Emergent properties of reduced-genome *Escherichia coli*. *Science* 312, 1044–1046.

Prince, R. C., and Kheshgi, H. S. (2005). The photobiological production of hydrogen: potential efficiency and effectiveness as a renewable fuel. *Crit. Rev. Microbiol.* 31, 19–31.

Purnick, P. E., and Weiss, R. (2009). The second wave of synthetic biology: from modules to systems. *Nat. Rev. Mol. Cell Biol.* 10, 410–422.

Radakovits, R., Jinkerson, R. E., Darzins, A., and Posewitz, M. C. (2010). Genetic engineering of algae for enhanced biofuel production. *Eukaryot. Cell* 9, 486–501.

Reijnders, L. (2008). Do biofuels from microalgae beat biofuels from terrestrial plants? *Trends. Biotechnol.* 26, 349–350

Reinfelder, J. R. (2011). Carbon concentrating mechanisms in eukaryotic marine phytoplankton. *Ann. Rev. Mar. Sci.* 3, 291–315.

Riehle, M. M., Bennett, A. F., and Long, A. D. (2001). Genetic architecture of thermal adaptation in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 98, 525–530.

Robertson, D. E., Jacobson, S. A., Morgan, F., Berry, D., Church, G. M., and Afeyan, N. B. (2011). A new dawn for industrial photosynthesis. *Photosynth. Res.* 107, 269–277.

Rocap, G., Larimer, F. W., Lamerdin, J., Malfatti, S., Chain, P., Ahlgren, N. A., et al. (2003). Genome divergence in two *Prochlorococcus* ecotypes reflects oceanic niche differentiation. *Nature* 424, 1042–1047.

Rowland, O., Zheng, H., Hepworth, S. R., Lam, P., Jetter, R., and Kunst, L. (2006). CER4 encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in *Arabidopsis*. *Plant Physiol.* 142, 866–877.

Rude, M. A., and Schirmer, A. (2009). New microbial fuels: a biotech perspective. *Curr. Opin. Microbiol.* 12, 274–281.

Ruepp, A., Graml, W., Santos-Martinez, M. L., Koretke, K. K., Volker, C., Mewes, H. W., et al. (2000). The genome sequence of the thermoacidophilic scavenger *Thermoplasma acidophilum*. *Nature* 407, 508–513.

Santelia, D., and Zeeman, S. C. (2011). Progress in *Arabidopsis* starch research and potential biotechnological applications. *Curr. Opin. Biotechnol.* 22, 271–280.

Schirmer, A., Rude, M. A., Li, X., Popova, E., and Del Cardayre, S. B. (2010). Microbial biosynthesis of alkanes. *Science* 329, 559–562.

Scott, S. A., Davey, M. P., Dennis, J. S., Horst, I., Howe, C. J., Lea-Smith, D. J., et al. (2010). Biodiesel from algae: challenges and prospects. *Curr. Opin. Biotechnol.* 21, 277–286.

Shen, C. R., Lan, E. I., Dekishima, Y., Baez, A., Cho, K. M., and Liao, J. C. (2011). Driving forces enable high-titer anaerobic 1-butanol synthesis in *Escherichia coli*. *Appl. Environ. Microbiol.* 77, 2905–2915.

Siebers, B., and Schonheit, P. (2005). Unusual pathways and enzymes of central carbohydrate metabolism in Archaea. *Curr. Opin. Microbiol.* 8, 695–705.

Srirangan, K., Pyne, M. E., and Perry Chou, C. (2011). Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. *Bioresour. Technol.* 102, 8589–8604.

Steen, E. J., Kang, Y., Bokinsky, G., Hu, Z., Schirmer, A., Mcclure, A., et al. (2010). Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature* 463, 559–562.

Stephenson, P. G., Moore, C. M., Terry, M. J., Zubkov, M. V., and Bibby, T. S. (2011). Improving photosynthesis for algal biofuels: toward a green revolution. *Trends Biotechnol.* 29, 615–623.

Stieber, D., Gabant, P., and Szpirer, C. (2008). The art of selective killing: plasmid toxin/antitoxin systems and their technological applications. *Biotechniques* 45, 344–346.

Stoveken, T., and Steinbuchel, A. (2008). Bacterial acyltransferases as an alternative for lipase-catalyzed acylation for the production of oleochemicals and fuels. *Angew. Chem. Int. Ed. Engl.* 47, 3688–3694.

Stripp, S. T., Goldet, G., Brandmayr, C., Sanganas, O., Vincent, K. A., Haumann, M., et al. (2009). How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. *Proc. Natl. Acad. Sci. U.S.A.* 106, 17331–17336.

Sun, J., Hopkins, R. C., Jenney, F. E., Mcternan, P. M., and Adams, M. W. (2010). Heterologous expression and maturation of an NADP-dependent [NiFe]-hydrogenase: a key enzyme in biofuel production. *PLoS ONE* 5:e10526. doi: 10.1371/journal.pone.0010526

Surzycki, R., Cournac, L., Peltier, G., and Rochaix, J. D. (2007). Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *Proc. Natl. Acad. Sci. U.S.A.* 104, 17548–17553.

Tolonen, A. C., Haas, W., Chilaka, A. C., Aach, J., Gygi, S. P., and Church, G. M. (2011). Proteome-wide systems analysis of a cellulosic biofuel-producing microbe. *Mol. Syst. Biol.* 7, 461.

Tran, L., Van Baarsel, J. A., Washburn, R. S., Gottesman, M. E., and Miller, J. H. (2011). Single-gene deletion mutants of *Escherichia coli* with altered sensitivity to bicyclomycin, an inhibitor of transcription termination factor Rho. *J. Bacteriol.* 193, 2229–2235.

Trinh, C. T., Carlson, R., Wlaschin, A., and Srienc, F. (2006). Design, construction and performance of the most efficient biomass producing *E. coli* bacterium. *Metab. Eng.* 8, 628–638.

Trinh, C. T., Unrean, P., and Srienc, F. (2008). Minimal *Escherichia coli* cell for the most efficient production of ethanol from hexoses and pentoses. *Appl. Environ. Microbiol.* 74, 3634–3643.

Tucker, C. L., and Fields, S. (2003). Lethal combinations. *Nat. Genet.* 35, 204–205.

Vazana, Y., Morais, S., Barak, Y., Lamed, R., and Bayer, E. A. (2012). Designer cellulosomes for enhanced hydrolysis of cellulosic substrates. *Methods Enzymol.* 510, 429–452.

von Jan, M., Lapidus, A., Del Rio, T. G., Copeland, A., Tice, H., Cheng, J. F., et al. (2010). Complete genome sequence of *Archaeoglobus profundus* type strain (AV18). *Stand. Genomic Sci.* 2, 327–346.

Wackett, L. P., Frias, J. A., Seffernick, J. L., Sukovich, D. J., and Cameron, S. M. (2007). Genomic and biochemical studies demonstrating the absence of an alkane-producing phenotype in *Vibrio furnissii* M1. *Appl. Environ. Microbiol.* 73, 7192–7198.

Walter, J. M., Greenfield, D., Bustamante, C., and Liphardt, J. (2007). Light-powering *Escherichia coli* with proteorhodopsin. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2408–2412.

Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R., et al. (2009a). Programming cells by multiplex genome engineering and accelerated evolution. *Nature* 460, 894–898.

Wang, Z. T., Ullrich, N., Joo, S., Waffenschmidt, S., and Goodenough, U. (2009b). Algal lipid bodies: stress induction, purification, and biochemical characterization in wild-type and starchless *Chlamydomonas reinhardtii*. *Eukaryot. Cell* 8, 1856–1868.

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N., Kim, P. B., et al. (2012). An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335, 308–313.

Westfall, P. J., Pitera, D. J., Lenihan, J. R., Eng, D., Woolard, F. X., Regentin, R., et al. (2012). Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* 109, E111–E118.

Whitney, S. M., Houtz, R. L., and Alonso, H. (2011). Advancing our understanding and capacity to engineer nature's CO2-sequestering enzyme, Rubisco. *Plant Physiol.* 155, 27–35.

Wiegel, J., Mothershed, C. P., and Puls, J. (1985). Differences in xylan degradation by various noncellulolytic thermophilic anaerobes and *Clostridium thermocellum*. *Appl. Environ. Microbiol.* 49, 656–659.

Wijffels, R. H., and Barbosa, M. J. (2010). An outlook on microalgal biofuels. *Science* 329, 796–799.

Work, V. H., D'adamo, S., Radakovits, R., Jinkerson, R. E., and Posewitz, M. C. (2012). Improving photosynthesis and metabolic networks for the competitive production of

phototroph-derived biofuels. *Curr. Opin. Biotechnol.* 23, 290–297.

Work, V. H., Radakovits, R., Jinkerson, R. E., Meuser, J. E., Elliott, L. G., Vinyard, D. J., et al. (2010). Increased lipid accumulation in the *Chlamydomonas reinhardtii* sta7-10 starchless isoamylase mutant and increased carbohydrate synthesis in complemented strains. *Eukaryot. Cell* 9, 1251–1261.

Xu, P., Ge, X., Chen, L., Wang, X., Dou, Y., Xu, J. Z., et al. (2011). Genome-wide essential gene identification in *Streptococcus sanguinis*. *Sci. Rep.* 1, 125.

Yamamoto, H., Kurumiya, S., Ohashi, R., and Fujita, Y. (2009). Oxygen sensitivity of a nitrogenase-like protochlorophyllide reductase from the cyanobacterium *Leptolyngbya boryana*. *Plant Cell Physiol.* 50, 1663–1673.

Yamano, T., Tsujikawa, T., Hatano, K., Ozawa, S., Takahashi, Y., and Fukuzawa, H. (2010). Light and low-CO2-dependent LCIB-LCIC complex localization in the chloroplast supports the carbon-concentrating mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* 51, 1453–1468.

Yim, H., Haselbeck, R., Niu, W., Pujol-Baxley, C., Burgard, A., Boldt, J., et al. (2011). Metabolic engineering of *Escherichia coli* for direct production of 1,4-butanediol. *Nat. Chem. Biol.* 7, 445–452.

Yu, B. J., Sung, B. H., Koob, M. D., Lee, C. H., Lee, J. H., Lee, W. S., et al. (2002). Minimization of the *Escherichia coli* genome using a Tn5-targeted Cre/loxP excision system. *Nat. Biotechnol.* 20, 1018–1023.

Yu, M., Zhang, Y., Tang, I. C., and Yang, S. T. (2011). Metabolic engineering of *Clostridium tyrobutyricum* for n-butanol production. *Metab. Eng.* 13, 373–382.

Zhang, F., and Keasling, J. (2011). Biosensors and their applications in microbial metabolic engineering. *Trends Microbiol.* 19, 323–329.

Zhang, F., Rodriguez, S., and Keasling, J. D. (2011). Metabolic engineering of microbial pathways for advanced biofuels production. *Curr. Opin. Biotechnol.* 22, 775–783.

Zhang, K., Sawaya, M. R., Eisenberg, D. S., and Liao, J. C. (2008). Expanding metabolism for biosynthesis of nonnatural alcohols. *Proc. Natl. Acad. Sci. U.S.A.* 105, 20653–20658.

Zhu, X. G., De Sturler, E., and Long, S. P. (2007). Optimizing the distribution of resources between enzymes of carbon metabolism can dramatically increase photosynthetic rate: a numerical simulation using an evolutionary algorithm. *Plant Physiol.* 145, 513–526.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 January 2013; accepted: 03 April 2013; published online: 19 April 2013.

Citation: Lee SJ, Lee S-J and Lee D-W (2013) Design and development of synthetic microbial platform cells for bioenergy. Front. Microbiol. ***4****:92. doi: 10.3389/fmicb.2013.00092*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

MINI REVIEW ARTICLE
published: 04 April 2013
doi: 10.3389/fmicb.2013.00075

Microbial production of isoprenoids enabled by synthetic biology

Cheryl M. Immethun, Allison G. Hoynes-O'Connor, Andrea Balassy and Tae Seok Moon*

Department of Energy, Environmental & Chemical Engineering, Washington University in St. Louis, Saint Louis, MO, USA

Edited by:
David Nielsen, Arizona State University, USA

Reviewed by:
Ian Wheeldon, University of California Riverside, USA
Danielle Tullman-Ercek, University of California Berkeley, USA

****Correspondence:***
Tae Seok Moon, Department of Energy, Environmental & Chemical Engineering, Washington University in St. Louis, Box 1180, 1 Brookings Drive, Saint Louis, MO 63130, USA.
e-mail: tsmoon@seas.wustl.edu

Microorganisms transform inexpensive carbon sources into highly functionalized compounds without toxic by-product generation or significant energy consumption. By redesigning the natural biosynthetic pathways in an industrially suited host, microbial cell factories can produce complex compounds for a variety of industries. Isoprenoids include many medically important compounds such as antioxidants and anticancer and antimalarial drugs, all of which have been produced microbially. While a biosynthetic pathway could be simply transferred to the production host, the titers would become economically feasible when it is rationally designed, built, and optimized through synthetic biology tools. These tools have been implemented by a number of research groups, with new tools pledging further improvements in yields and expansion to new medically relevant compounds. This review focuses on the microbial production of isoprenoids for the health industry and the advancements though synthetic biology.

Keywords: microbial biosynthesis, synthetic biology, metabolic engineering, isoprenoids, health industry

INTRODUCTION

Microbial biosynthesis of natural products provides advantages over biomass extraction and chemical synthesis. The native hosts of the natural products, usually plants, grow slowly with differences in the plant's climate and geography, leading to detrimental variations in the product concentration and composition (Chang and Keasling, 2006; Asadollahi et al., 2008; Engels et al., 2008). In addition, biomass extraction often requires substantial energy and resource consumption for miniscule product recovery (Martin et al., 2003; Shiba et al., 2007; Ajikumar et al., 2008). Chemical synthesis struggles to create the natural product's integral complexity (Chang and Keasling, 2006; Engels et al., 2008; Nakagawa et al., 2011). It also requires significant amounts of energy while producing toxic by-products (Chemler et al., 2006; Winter and Tang, 2012; Wu et al., 2013). Microbial biosynthesis of natural products can help avert these problems. The biosynthetic pathway from the native host is redesigned in a tractable platform organism, often *Escherichia coli* or *Saccharomyces cerevisiae*, which serves as a microbial cell factory (Shiba et al., 2007; Ajikumar et al., 2008; Albertsen et al., 2011; Du et al., 2011; Misawa, 2011). The microbes can utilize inexpensive carbon sources with short doubling times to produce highly functionalized and value-added products with no toxic by-products (Chemler et al., 2006; Ajikumar et al., 2008; Tang and Zhao, 2009; Hong and Nielsen, 2012). Microbial biosynthesis is well suited for the production of many natural products, including isoprenoids.

Isoprenoids are important secondary metabolites for the health industry. Built from five carbon isoprene units that are cyclized, rearranged, and adorned in a multitude of ways, isoprenoids, sometimes called terpenoids, include more than 40,000 structurally unique compounds (Chang and Keasling, 2006; Ajikumar et al., 2008; Farhi et al., 2011). Terpenoids are classified based on their number of isoprene units. Monoterpenes consist of two isoprene units, with sesquiterpenes, diterpenes, triterpenes, and carotenoids (or tetraterpenes) built from three, four, six, and eight isoprene units, respectively (Withers and Keasling, 2007; Asadollahi et al., 2008; Misawa, 2011; Walter and Strack, 2011). Important compounds for the health industry can be found in many of the terpenoids classes, including the carotenoid lycopene, the sesquiterpene artemisinin, the diterpene paclitaxel, and triterpene herbal medicines (Das et al., 2007; Ajikumar et al., 2008; Asadollahi et al., 2008; Misawa, 2011).

Synthetic biology tools can help boost the delivery of isoprenoids to market. While the natural biosynthetic pathway could be simply transferred to an industrially suited host, such as *E. coli* or *S. cerevisiae*, the final titers of the desired product would become economically feasible when the pathway is rationally designed, built, and optimized (Klein-Marcuschamer et al., 2007; Maury et al., 2008; Anthony et al., 2009). While reasonable titers for the commercial-scale production are currently unknown, a review indicates that 0.5 g/l is an adequate starting point for high value compounds (Ajikumar et al., 2008). Innovations in genomics and systems biology have facilitated the ability to engineer biology for commercial applications through a set of clear steps (Ajikumar et al., 2008; Keasling, 2012). First, the metabolic pathways needed to produce the desired products are selected. Second, a host suitable for industrial production and genetic manipulation is chosen. Third, what must be redesigned for the pathway and host to operate together is determined, followed by optimization so that production can become commercially relevant (Jarboe et al., 2010). These steps have been implemented by a number of research groups for the creation of biofuels, commodity chemicals, and products for the health industry. This review focuses on the microbial production of isoprenoids for the health industry and the advancements through synthetic biology. Four of the 10 isoprenoids discussed (**Table 1**)

Table 1 | Isoprenoid production.

Isoprenoid	Approach[1]	Microbial production (fold improvement)	Microbe	Natural source and extraction	Chemical synthesis
Amorphadiene[2]	Express heterologous pathway in two operons and codon-optimize amorphadiene synthase	24 mg caryophyllene equivalent/l (300-fold) (Martin et al., 2003)	*E. coli*	*Artemesia annua* 0.01–1.0% of dry leaf weight (Liu et al., 2006)	29–42% Overall yield (Zhu and Cook, 2012)
	Redesign the mevalonate pathway to increase FPP and express *Artemisia annua*'s amorphadiene synthase and cytochrome P450	153 mg/l (500-fold) (Ro et al., 2006)	*S. cerevisiae*		
	Identify the limiting reaction enzymes and balance gene expression through plasmid copy number and promoter strength	293 mg/l (7-fold) (Anthony et al., 2009)	*E. coli*		
	Overexpress every enzyme in the mevalonate pathway as well as modify fermentation conditions	40 g/l (250-fold) (Westfall et al., 2012)	*S. cerevisiae*		
	Express heterologous pathway in a strain of *Streptomyces avermitilis* with minimized genome	30 mg/l (from 0 mg/l) (Komatsu et al., 2010)	*S. avermitilis*		
	Truncate and deregulate HMG1 and co-localize heterologous FDP synthase and amorphadiene synthase to the mitochondria	20 mg/l (20-fold) (Farhi et al., 2011)	*S. cerevisiae*		
Astaxanthin	Overexpress native *idi* and *gps* from *Archaeoglobus fulgidus* and express the gene cluster *crtBIYZW* from *Agrobacterium aurantiacum*	1.4 mg/g dcw (50-fold) (Wang et al., 1999)	*E. coli*	*Haematococcus* microalgae 1.5–3.0% by dry weight (Lorenz and Cysewski, 2000)	Mixture of isomers, not approved for human consumption (Li et al., 2011)
	Overexpress *idi* and *dxs* and balance expression of *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Pantoea ananatis* and *crtW148* (NpF4798) from *Nostoc punctiforme*, which were inserted into the chromosome	1.4 mg/g dcw (20-fold) (Lemuth et al., 2011)	*E. coli*		
Levopimaradiene	Combinatorially mutate the GGPPS–LPS pathway	700 mg/l (2,600-fold) (Leonard et al., 2010)	*E. coli*	Young *Ginkgo biloba* trees 1–7 mg/g dry weight (Matsuda and Schepmann, 2008)	<3% Overall yield (Matsuda and Schepmann, 2008)
Lycopene[2]	Express *Erwinia* carotenoid biosynthesis gene cluster and *idi* from *Haematococcus pluvialis*	1.03 mg/g dcw (4.5-fold) (Kajiwara et al., 1997)	*E. coli*	Tomatoes 0.15–0.25 mg/g (Rath, 2009)	0.13 mg/g and 70% trans configurations (Olempska-Beer, 2006)
	Redesign the global regulatory system, the Ntr regulon	160 mg/l (from 0 mg/l) (Farmer and Liao, 2000)	*E. coli*	94–96% trans configurations (Olempska-Beer, 2006)	
	Overexpress the catalytic domain of HMG and disrupt *ERG9*	7.8 mg/g dcw (7-fold) (Shimada et al., 1998)	*C. utilis*		

(Continued)

Table 1 | Continued

Isoprenoid	Approach[1]	Microbial production (fold improvement)	Microbe	Natural source and extraction	Chemical synthesis
	Overexpress genes identified by the FSEOF strategy combined with gene knockouts	12.32 mg/g dcw (4-fold) (Choi et al., 2010)	*E. coli*		
	Overexpress and knockout genes selected from a metabolic landscape	16 mg/g dcw (4-fold) (Jin and Stephanopoulos, 2007)	*E. coli*		
	Use "global transcription machinery engineering" to improve phenotypes	7.7 mg/l (1.8-fold) (Alper and Stephanopoulos, 2007)	*E. coli*		
	Optimize DXP pathway with "multiplex automated genome engineering"	9 mg/g dcw (5-fold) (Wang et al., 2009)	*E. coli*		
Miltiradiene	Fuse SmCPS and SmKSL as well as BTS1 and ERG20	365 mg/l (340-fold) (Zhou et al., 2012)	*S. cerevisiae*	*Salvia miltiorrhiza* <40 mg/g dry weight (Li et al., 2012)	4 mg/ml of the precursor salvianolic acid B (Gu et al., 2008)
Patchoulol	Replace the native *ERG9* promoter with the methionine repressible *MET3* promoter	16.9 mg/l (1.5-fold) (Asadollahi et al., 2008)	*S. cerevisiae*	*Pogostemon cablin* 30–40% total mass (Hybertson, 2007)	6% Overall yield of the precursor norpatchoulenol (Kolek et al., 2009)
	Fuse the native farnesyl diphosphate synthase and the heterologous patchoulol synthase and repress *ERG9*	40.9 mg/l (2-fold) (Albertsen et al., 2011)	*S. cerevisiae*		
Taxadiene	Express genes for GGPPS, taxadiene synthase, three cytochrome P450 hydroxylases, and three acyl/aroyl CoA dependent transferases and build a five step taxoid pathway	1 mg/l (100-fold) (Dejong et al., 2006)	*S. cerevisiae*	*Taxus brevifolia* 0.01–0.1% dry bark weight (Hezari et al., 1995)	18–20% Overall yield (Mendoza et al., 2012)
	Express genes for geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius* and a codon-optimized taxadiene synthase from *Taxus chinensis*	8.7 mg/l (40-fold) (Engels et al., 2008)	*S. cerevisiae*		
	Vary small pathway modules simultaneously to determine the optimally balanced complete pathway ("multivariate modular pathway engineering")	1 g/l (15,000-fold) (Ajikumar et al., 2010)	*E. coli*		
Zeaxanthin[2]	Overexpress different combinations of *idi* from *Xanthophyllomyces dendrorhous*, *dxr* from *Sulfolobus acidocaldarius*, and native *dxs*	1.6 mg/g dcw (3.5-fold) (Albrecht et al., 1999)	*E. coli*	*Tagetes erecta's* red flowers 23% dry weight (Stankovic, 2004)	12% Overall yield of racemic mix (Khachik and Chang, 2009)
	Use the "ordered gene assembly in *Bacillus subtilis* (OGAB) method" to determine optimal gene order	820 μg/g dcw (4.4-fold) (Nishizaki et al., 2007)	*E. coli*		

(Continued)

Table 1 | Continued

Isoprenoid	Approach[1]	Microbial production (fold improvement)	Microbe	Natural source and extraction	Chemical synthesis
α-Santalene	Replace the native *ERG9* promoter with the glucose-responsive *HXT1* promoter, delete the genes for lipid phosphate phosphatase and pyrophosphate phosphatase, and overexpress a truncated 3-hydroxyl-3-methyl-glutaryl-CoA reductase	0.21 mg/g dcw (3.4-fold) (Scalcinati et al., 2012)	*S. cerevisiae*	*Santalum album* 1–2% by weight of oil (Jones et al., 2011)	8% Overall yield (Bastiaansen et al., 1996)
β-Carotene[2]	Overexpress different combinations of *idi* from *Xanthophyllomyces dendrorhous*, *dxr* from *Sulfolobus acidocaldarius*, and native *dxs*	1.5 mg/g dcw (3.5-fold) (Albrecht et al., 1999)	*E. coli*	Mostly *Dunaliella salina* 300 mg/m^2/day (Hosseini Tafreshi and Shariati, 2009)	85% Yield using triphenyl-phosphine oxide, which is harmful to aquatic organisms (USDA, 2011)
	Replace the native promoters for the chromosomal genes *dxs*, *ispDispF*, *idi*, and *ispB* with strong T5 bacteriophage promoters	6 mg/g dcw (24.5-fold) Yuan et al., 2006)	*E. coli*		

Dcw, dry cell weight.

[1]Acronyms are defined in the main text.

[2]Currently produced or produced in the near term by microbial biosynthesis.

are currently manufactured or will be manufactured in the near future.

ISOPRENOID PATHWAY

Although isoprenoids include a wide range of compounds, they are synthesized through a common metabolic pathway. The isoprenoid pathway (**Figure 1**) begins with the conversion of acetyl-CoA to isopentenyl diphosphate (IPP). IPP is then isomerized to dimethylallyl diphosphate (DMAPP), which forms geranyl diphosphate (GPP), then farnesyl diphosphate (FPP), followed by geranylgeranyl diphosphate (GGPP). At this point different isoprenoids begin to branch off into individualized pathways (Kajiwara et al., 1997; Schmidt-Dannert, 2000; Walter and Strack, 2011). Two distinct pathways exist for the production of the precursor compounds IPP and DMAPP, the mevalonate pathway, and the methylerythritol phosphate (MEP) pathway. Thus, researchers enjoy multiple options when selecting the metabolic pathway for production of the chosen isoprenoid (Chang and Keasling, 2006). Furthermore, the isoprenoid pathway has been expressed in a variety of hosts and assembled using genes from a diversity of sources (Misawa and Shimada, 1997; Schmidt-Dannert, 2000; Das et al., 2007; Nishizaki et al., 2007; Maury et al., 2008).

The IPP precursor supply has been engineered through several techniques to improve the commercial viability of isoprenoid biosynthesis. Martin et al. (2003) began their work on the synthesis of the sesquiterpene artemisinin by assembling *S. cerevisiae*'s mevalonate pathway into two operons that were co-expressed in *E. coli*. Post-transcriptional processes made balanced expression of genes within operons difficult. To overcome this problem, libraries of "tunable intergenic regions" (TIGRs) and recombined control elements (RBS sequestering sequences, mRNA secondary structures, and RNase cleavage sites) were screened to select the *E. coli* strain that produced sevenfold more mevalonate (Pfleger et al., 2006). Shiba et al. amplified the precursor flux to the mevalonate pathway in *S. cerevisiae* by overexpressing acetaldehyde dehydrogenase and incorporating *Salmonella enterica's* acetyl-CoA synthetase. This kept more carbon flow in the cytosol, as opposed to the mitochondria, resulting in more mevalonate (Shiba et al., 2007). To increase lycopene production by boosting the precursor supply, a synthetic mevalonate pathway was assembled in *E. coli*, which included yeast mevalonate kinase (yMVK), human 5-phosphomevalonate kinase (hPMK), yeast 5-diphosphomevalonate decarboxylase (yPMD), and *E. coli* IPP/DMAPP isomerase (Rodriguez-Villalon et al., 2008). Dueber et al. (2009) created synthetic protein scaffolds to co-localize mevalonate pathway enzymes, leading to a 77-fold improvement in product titers. This approach demonstrated that high production of mevalonate can be achieved with low enzyme expression and reduced metabolic burden.

CAROTENOIDS

Carotenoids are among the first natural products whose titers were improved through synthetic biology tools. Early work on the microbial production focused on increasing the supply of intermediates in the first steps of the isoprenoid pathway. In 1997, the production of carotenoids in *E. coli* was improved by a factor of 2.7, for a total of 1.3 mg/g dry cell weight (dcw), by introducing heterologous genes for IPP isomerase (Kajiwara et al., 1997). Albrecht et al. (1999) increased the nutrients β-carotene and zeaxanthin 3.5-fold, to reach 1.5 and 1.6 mg/g dcw respectively, by overexpressing

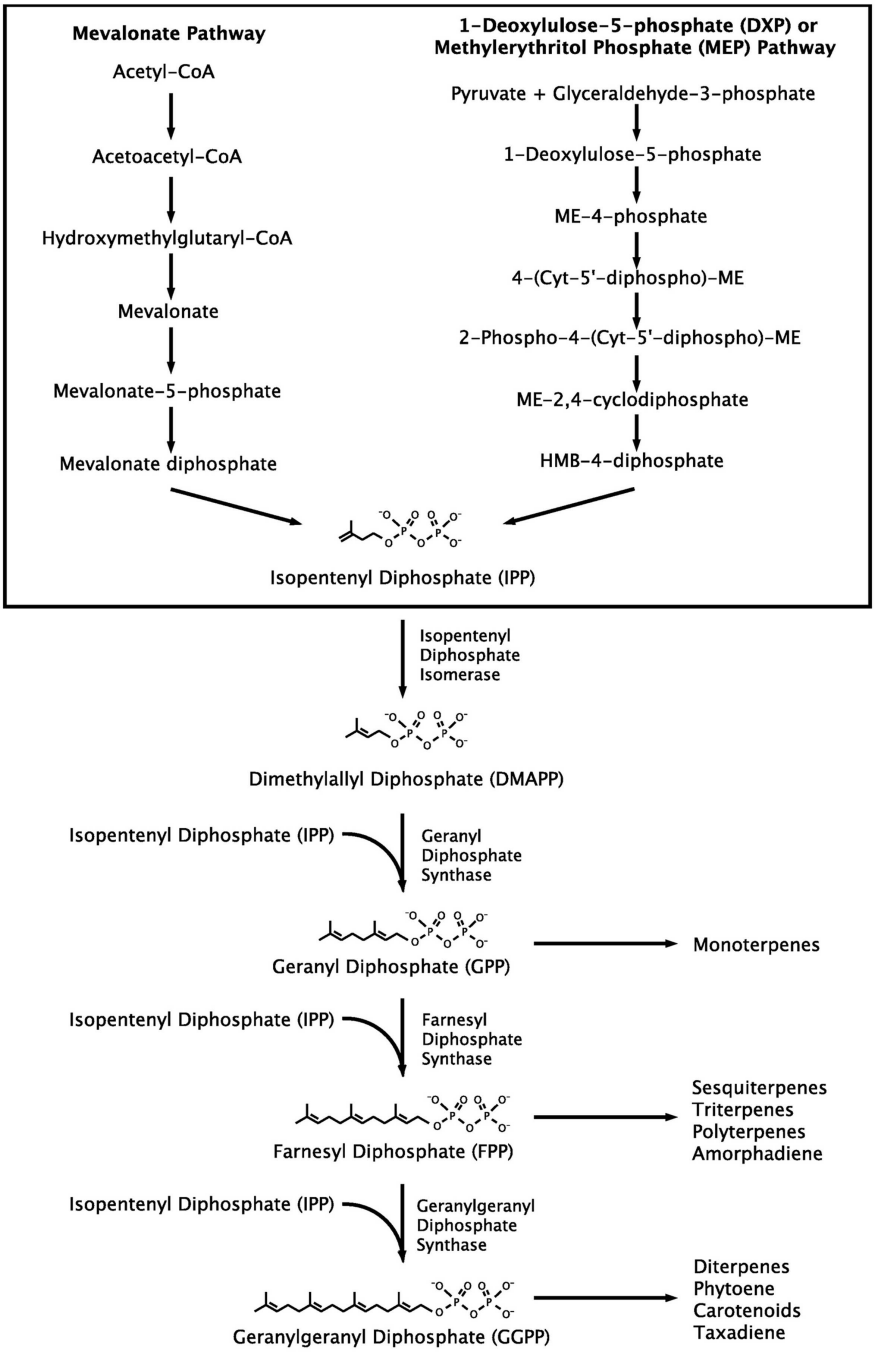


FIGURE 1 | Isoprenoid pathway. Two distinct pathways for IPP production are shown together, but they exist in different organisms.

different combinations of the genes for IPP isomerase from *Xanthophyllomyces dendrorhous,* GGPP synthase from *Sulfolobus acidocaldarius,* and native DXP synthase. More than a 50-fold improvement was made in the production of astaxanthin, used to treat several degenerative nerve diseases, by boosting IPP and GGPP formation as well as expressing the gene cluster *crtBIYZW* from *Agrobacterium aurantiacum* in *E. coli* for a total of 1.4 mg/g dcw (Wang et al., 1999). However, unhindered metabolite production can lead to metabolic imbalance. Farmer and Liao (2000) redesigned a global regulatory system in *E. coli* to allow lycopene production only in the presence of sufficient glucose, as indicated by acetyl phosphate availability. This control loop decreased the metabolic imbalance, thus increasing the final yield of the nutritional supplement. Shimada et al. (1998) also improved lycopene

production by focusing on the later steps of the isoprenoid pathway. In *Candida utilis* that expressed exogenous *crtE*, *crtB*, and *crtI*, the gene *ERG9*, which diverts FPP to the ergosterol pathway, was disrupted and the catalytic domain of HMG was overexpressed, resulting in 7.8 mg lycopene/g dcw. Novel carotenoids were created in *E. coli* through the expression of mutagenic libraries of phytoene desaturase and lycopene cyclase, enzymes that regulate branchpoints in the later stages of carotenoid biosynthesis. A wide variety of metabolites, including 3,4,3′,4′-tetradehydrolycopene, a fully conjugated carotenoid, and torulene, a new cyclic carotenoid, were observed (Schmidt-Dannert et al., 2000).

More recently, powerful new techniques have been employed to further improve carotenoid production. Jin and Stephanopoulos (2007) constructed a lycopene production metabolic landscape using *E. coli* strains that incorporated different combinations of overexpression and knockout targets. From this landscape, the best combination of genes increased lycopene production to 16 mg/g dcw. β-carotene production reached 6 mg/g dcw by replacing the native *E. coli* promoters for the chromosomal genes *dxs*, *ispDispF*, *idi*, and *ispB* with strong T5 bacteriophage promoters. Modifying chromosomal genes, instead of introducing high-copy vectors to overexpress the target genes, decreased the metabolic burden (Yuan et al., 2006). Lemuth et al. also used similar techniques by balancing expression of *crtE*, *crtB*, *crtI*, *crtY*, and *crtZ* from *Pantoea ananatis* and *crtW148* (NpF4798) from *Nostoc punctiforme*, which were inserted into the chromosome of *E. coli*. This plasmid-free strain created astaxanthin as its only carotenoid at 1.4 mg/g dcw (Lemuth et al., 2011). Using the "ordered gene assembly in *Bacillus subtilis* (OGAB) method" to put together multiple genes in a single step, Nishizaki et al. (2007) determined the optimum gene order which subsequently produced 820 μg zeaxanthin/g dcw. The "flux scanning based on enforced objective flux" (FSEOF) strategy identified targets for gene amplification that were not intuitive. When combined with gene knockouts, 12.32 mg lycopene/g dcw was achieved (Choi et al., 2010). Alper and Stephanopoulos (2007) randomly mutated the *E. coli* sigma factor σ^{70} to look for desired complex phenotypes. This "global transcription machinery engineering" (gTME) improved lycopene production. "Multiplex automated genome engineering" (MAGE) was proposed by Wang et al. They modified 24 genetic components at once from a degenerate pool of synthetic DNA, achieving a fivefold increase in lycopene production in just 3 days (Wang et al., 2009).

ARTEMISININ

The microbial production of the potent anti-malaria drug artemisinin has utilized a number of advances in the synthetic biology field. Balancing metabolic flux with the codon-optimized amorphadiene synthase gene improved the titer of amorphadiene, an artemisinin precursor, beyond what had been accomplished by increasing IPP precursor supply (Martin et al., 2003). Ro et al. (2006) redesigned the mevalonate pathway in *S. cerevisiae* to increase production of FPP and introduced *Artemisia annua's* amorphadiene synthase and cytochrome P450 for the final oxidation steps. The modifications resulted in 100 mg/l of artemisinic acid (Ro et al., 2006). Anthony et al. (2009) achieved the amorphadiene titer of 293 mg/l by identifying the limiting reaction enzymes and balancing gene expression through plasmid copy number and promoter strength. Building upon all of the previous works in the Keasling lab, production of >40 g/l amorphadiene was achieved by overexpressing every enzyme in the mevalonate pathway and modifying fermentation conditions. Subsequently, the amorphadiene was chemically converted to dihydroartemisinic acid, the precursor of the antimalarial agent artemisinin (Westfall et al., 2012). *A. annua's* amorphadiene synthase, codon-optimized and placed under the control of the *rpsJ* promoter, and the native FPP synthase were expressed in a genome-minimized strain of *Streptomyces avermitilis*. This approach led to heterologous biosynthesis of 30 mg/l of amorphadiene while not producing any of the major endogenous secondary metabolites (Komatsu et al., 2010). Farhi et al. (2011) co-localized heterologous FDP synthase and amorphadiene synthase to the mitochondria to improve the amorphadiene titer by 20-fold, for a total of 20 mg/l.

DITERPENES AND OTHER SESQUITERPENES

Biosynthetic pathways for various diterpenes and sesquiterpenes have also been engineered for improved production through synthetic biology. To maximize production of several sesquiterpenes, Asadollahi et al. replaced the native *ERG9* promoter, which is responsible for diverting the terpenoid precursor FPP to a competing pathway, with the methionine repressible *MET3* promoter. After optimizing methionine levels, 16.9 mg/l of patchoulol, the starting compound in the chemical synthesis of the chemotherapeutic drug paclitaxel (Taxol), was achieved (Asadollahi et al., 2008). Scalcinati et al. chose to control *ERG9* expression by coupling it with the glucose-responsive *HXT1* promoter. In addition to using this promoter, the genes encoding lipid phosphate phosphatase and pyrophosphate phosphatase were deleted, and a truncated 3-hydroxyl-3-methyl-glutaryl-CoA reductase (HMGR) was overexpressed to produce α-santalene, a skin cancer chemopreventative, at 0.21 mg/g dcw (Scalcinati et al., 2012). The native FPP synthase and the heterologous patchoulol synthase were fused to reduce metabolic diffusion distance between enzymes, increasing patchoulol production twofold, to a total of 40.9 mg/l, in *S. cerevisiae* (Albertsen et al., 2011). Miltiradiene, related to the Chinese medicinal herb *Salvia miltiorrhiza*, was produced up to 365 mg/l in a 15l bioreactor, by fusing labdadienyl/copalyl diphosphate synthase (SmCPS) and kaurene synthase-like (SmKSL) as well as GGPP synthase (BTS1) and FPP synthase (ERG20) in *S. cerevisiae* (Zhou et al., 2012). The capacity of downstream pathways can also limit titers. The geranylgeranyl diphosphate synthase – levopimaradiene synthase (GGPPS – LPS) pathway was combinatorially mutated to accommodate the engineered upsurge in precursors. This approach led to a 2,600-fold increase, for a total of 700 mg/l, of the diterpene levopimaradiene, used to produce the ancient medicinal ginkgolides (Leonard et al., 2010).

PACLITAXEL

Application of synthetic biology tools to microbial production of the cancer chemotherapy drug paclitaxel will decrease its cost and increase its availability. Paclitaxel, known as Taxol, is a potent chemotherapy drug, which is very difficult to chemically synthesize (Chandran et al., 2011) and is extracted at very low efficiency from the bark of the rare Pacific yew (Ajikumar et al., 2008). Dejong et al. (2006) were the first to express genes for a portion of the Taxol pathway in *S. cerevisiae*, but production levels of the

Taxol intermediate, taxadiene, were low. Several changes to taxadiene synthesis in yeast were introduced, including an alternate geranylgeranyl diphosphate synthase from *S. acidocaldarius* and a codon-optimized taxadiene synthase from *Taxus chinensis*, ultimately resulting in a 40-fold titer increase to 8.7 mg/l (Engels et al., 2008). Using *E. coli* as a host, Ajikumar et al. (2010) divided the metabolic pathway into smaller modules and varied the expression levels simultaneously to determine the optimally balanced pathway without requiring high throughput screening. This "multivariate modular pathway engineering" resulted in the taxadiene titer of 1 g/l. Although challenges remain for the biosynthesis of Taxol and other compounds, the range of advancements in isoprenoid production by microbial biosynthesis shows promise for increasing their availability at reduced cost.

CONCLUSION

The past decade has witnessed the potential of synthetic biology to make the microbial isoprenoid production become industrially relevant. However, further improvements in yield and expansion to new medically important compounds can be attained through the development of additional tools. An incomplete understanding of the complexity of biosynthetic pathways limits the ability to fully forward engineer microbial production (Nielsen and Keasling, 2011; Stephanopoulos, 2012). Continued innovations in systems biology to elucidate the complex regulatory and metabolic networks will advance the predictive potential of mathematical models, and therefore the ability to generate optimized microbial cell factories (Jarboe et al., 2010; Nielsen and Keasling, 2011; Keasling, 2012). Genome mining, scanning genome sequences for natural functions, will accelerate the rate of new compound discoveries. Improved enzyme engineering will also support the *de novo* design of biosynthetic pathways (Ajikumar et al., 2008; Jarboe et al., 2010). Moreover, biological devices built from well characterized and standardized genetic parts can be used to control metabolic pathways. Incorporation of these strategies would lead to engineered microbes for industrial-scale production of medically important compounds.

REFERENCES

Ajikumar, P. K., Tyo, K., Carlsen, S., Mucha, O., Phon, T. H., and Stephanopoulos, G. (2008). Terpenoids: opportunities for biosynthesis of natural product drugs using engineered microorganisms. *Mol. Pharm.* 5, 167–190.

Ajikumar, P. K., Xiao, W. H., Tyo, K. E., Wang, Y., Simeon, F., Leonard, E., et al. (2010). Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli*. *Science* 330, 70–74.

Albertsen, L., Chen, Y., Bach, L. S., Rattleff, S., Maury, J., Brix, S., et al. (2011). Diversion of flux toward sesquiterpene production in *Saccharomyces cerevisiae* by fusion of host and heterologous enzymes. *Appl. Environ. Microbiol.* 77, 1033–1040.

Albrecht, M., Misawa, N., and Sandmann, G. (1999). Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechnol. Lett.* 21, 791–795.

Alper, H., and Stephanopoulos, G. (2007). Global transcription machinery engineering: a new approach for improving cellular phenotype. *Metab. Eng.* 9, 258–267.

Anthony, J. R., Anthony, L. C., Nowroozi, F., Kwon, G., Newman, J. D., and Keasling, J. D. (2009). Optimization of the mevalonate-based isoprenoid biosynthetic pathway in *Escherichia coli* for production of the anti-malarial drug precursor amorpha-4,11-diene. *Metab. Eng.* 11, 13–19.

Asadollahi, M. A., Maury, J., Moller, K., Nielsen, K. F., Schalk, M., Clark, A., et al. (2008). Production of plant sesquiterpenes in *Saccharomyces cerevisiae*: effect of ERG9 repression on sesquiterpene biosynthesis. *Biotechnol. Bioeng.* 99, 666–677.

Bastiaansen, P. M. F. M., Wijnberg, J. B. P. A., and De Groot, A. (1996). A new enantiospecific synthesis of α-santalanes via homofragmentation of 1,4-diol monosulfonate esters. *J. Org. Chem.* 61, 4955–4958.

Chandran, S. S., Kealey, J. T., and Reeves, C. D. (2011). Microbial production of isoprenoids. *Process Biochem.* 46, 1703–1710.

Chang, M. C., and Keasling, J. D. (2006). Production of isoprenoid pharmaceuticals by engineered microbes. *Nat. Chem. Biol.* 2, 674–681.

Chemler, J. A., Yan, Y., and Koffas, M. A. (2006). Biosynthesis of isoprenoids, polyunsaturated fatty acids and flavonoids in *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 5, 20.

Choi, H. S., Lee, S. Y., Kim, T. Y., and Woo, H. M. (2010). In silico identification of gene amplification targets for improvement of lycopene production. *Appl. Environ. Microbiol.* 76, 3097–3105.

Das, A., Yoon, S. H., Lee, S. H., Kim, J. Y., Oh, D. K., and Kim, S. W. (2007). An update on microbial carotenoid production: application of recent metabolic engineering tools. *Appl. Microbiol. Biotechnol.* 77, 505–512.

Dejong, J. M., Liu, Y., Bollon, A. P., Long, R. M., Jennewein, S., Williams, D., et al. (2006). Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 93, 212–224.

Du, J., Shao, Z., and Zhao, H. (2011). Engineering microbial factories for synthesis of value-added products. *J. Ind. Microbiol. Biotechnol.* 38, 873–890.

Dueber, J. E., Wu, G. C., Malmirchegini, G. R., Moon, T. S., Petzold, C. J., Ullal, A. V., et al. (2009). Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* 27, 753–759.

Engels, B., Dahm, P., and Jennewein, S. (2008). Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards taxol (paclitaxel) production. *Metab. Eng.* 10, 201–206.

Farhi, M., Marhevka, E., Masci, T., Marcos, E., Eyal, Y., Ovadis, M., et al. (2011). Harnessing yeast subcellular compartments for the production of plant terpenoids. *Metab. Eng.* 13, 474–481.

Farmer, W. R., and Liao, J. C. (2000). Improving lycopene production in *Escherichia coli* by engineering metabolic control. *Nat. Biotechnol.* 18, 533–537.

Gu, M., Su, Z. G., and Janson, J. C. (2008). One-step purification of 3,4-dihydroxyphenyllactic acid, salvianolic acid B, and protocatechualdehyde from *Salvia miltiorrhiza* Bunge by isocratic stepwise hydrogen bond adsorption chromatography on cross-linked 12% agarose. *J. Chromatogr. Sci.* 46, 165–168.

Hezari, M., Lewis, N. G., and Croteau, R. (1995). Purification and characterization of taxa-4(5),11(12)-diene synthase from Pacific yew (*Taxus brevifolia*) that catalyzes the first committed step of taxol biosynthesis. *Arch. Biochem. Biophys.* 322, 437–444.

Hong, K. K., and Nielsen, J. (2012). Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. *Cell. Mol. Life Sci.* 69, 2671–2690.

Hosseini Tafreshi, A., and Shariati, M. (2009). Dunaliella biotechnology: methods and applications. *J. Appl. Microbiol.* 107, 14–35.

Hybertson, B. M. (2007). Solubility of the sesquiterpene alcohol patchoulol in supercritical carbon dioxide. *J. Chem. Eng. Data.* 52, 235–238.

Jarboe, L. R., Zhang, X., Wang, X., Moore, J. C., Shanmugam, K. T., and Ingram, L. O. (2010). Metabolic engineering for production of biorenewable fuels and chemicals: contributions of synthetic biology. *J. Biomed. Biotechnol.* 2010, 761042.

Jin, Y. S., and Stephanopoulos, G. (2007). Multi-dimensional gene target search for improving lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* 9, 337–347.

Jones, C. G., Moniodis, J., Zulak, K. G., Scaffidi, A., Plummer, J. A., Ghisalberti, E. L., et al. (2011). Sandalwood fragrance biosynthesis involves sesquiterpene synthases of both the terpene synthase (TPS)-a and TPS-b subfamilies, including santalene synthases. *J. Biol. Chem.* 286, 17445–17454.

Kajiwara, S., Fraser, P. D., Kondo, K., and Misawa, N. (1997). Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*. *Biochem. J.* 324, 421–426.

Keasling, J. D. (2012). Synthetic biology and the development of tools for metabolic engineering. *Metab. Eng.* 14, 189–195.

Khachik, F., and Chang, A. N. (2009). Total synthesis of (3R,3′R,6′R)-lutein and its stereoisomers. *J. Org. Chem.* 74, 3875–3885.

Klein-Marcuschamer, D., Ajikumar, P. K., and Stephanopoulos, G. (2007). Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. *Trends Biotechnol.* 25, 417–424.

Kolek, T., Szpineter, A., and Swizdor, A. (2009). The microbiological hydroxylation of patchoulol in *Absidia coerulea* and *Mucor hiemalis*. *Biocatal. Biotransformation* 27, 102–106.

Komatsu, M., Uchiyama, T., Omura, S., Cane, D. E., and Ikeda, H. (2010). Genome-minimized *Streptomyces* host for the heterologous expression of secondary metabolism. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2646–2651.

Lemuth, K., Steuer, K., and Albermann, C. (2011). Engineering of a plasmid-free *Escherichia coli* strain for improved in vivo biosynthesis of astaxanthin. *Microb. Cell Fact.* 10, 29.

Leonard, E., Ajikumar, P. K., Thayer, K., Xiao, W. H., Mo, J. D., Tidor, B., et al. (2010). Combining metabolic and protein engineering of a terpenoid biosynthetic pathway for overproduction and selectivity control. *Proc. Natl. Acad. Sci. U.S.A.* 107, 13654–13659.

Li, J., Zhu, D., Niu, J., Shen, S., and Wang, G. (2011). An economic assessment of astaxanthin production by large scale cultivation of *Haematococcus pluvialis*. *Biotechnol. Adv.* 29, 568–574.

Li, X. B., Wang, W., Zhou, G. J., Li, Y., Xie, X. M., and Zhou, T. S. (2012). Production of salvianolic acid B in roots of *Salvia miltiorrhiza* (Danshen) during the post-harvest drying process. *Molecules* 17, 2388–2407.

Liu, C., Zhao, Y., and Wang, Y. (2006). Artemisinin: current state and perspectives for biotechnological production of an antimalarial drug. *Appl. Microbiol. Biotechnol.* 72, 11–20.

Lorenz, R. T., and Cysewski, G. R. (2000). Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin. *Trends Biotechnol.* 18, 160–167.

Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D., and Keasling, J. D. (2003). Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat. Biotechnol.* 21, 796–802.

Matsuda, S. P. T., and Schepmann, H. G. (2008). Ginkgo biloba levopimaradiene synthase. United States Patent Application 11/166,996 [accessed May 20, 2008].

Maury, J., Asadollahi, M. A., Moller, K., Schalk, M., Clark, A., Formenti, L. R., et al. (2008). Reconstruction of a bacterial isoprenoid biosynthetic pathway in *Saccharomyces cerevisiae*. *FEBS Lett.* 582, 4032–4038.

Mendoza, A., Ishihara, Y., and Baran, P. S. (2012). Scalable enantioselective total synthesis of taxanes. *Nat. Chem.* 4, 21–25.

Misawa, N. (2011). Pathway engineering for functional isoprenoids. *Curr. Opin. Biotechnol.* 22, 627–633.

Misawa, N., and Shimada, H. (1997). Metabolic engineering for the production of carotenoids in non-carotenogenic bacteria and yeasts. *J. Biotechnol.* 59, 169–181.

Nakagawa, A., Minami, H., Kim, J. S., Koyanagi, T., Katayama, T., Sato, F., et al. (2011). A bacterial platform for fermentative production of plant alkaloids. *Nat. Commun.* 2, 326.

Nielsen, J., and Keasling, J. D. (2011). Synergies between synthetic biology and metabolic engineering. *Nat. Biotechnol.* 29, 693–695.

Nishizaki, T., Tsuge, K., Itaya, M., Doi, N., and Yanagawa, H. (2007). Metabolic engineering of carotenoid biosynthesis in *Escherichia coli* by ordered gene assembly in *Bacillus subtilis*. *Appl. Environ. Microbiol.* 73, 1355–1361.

Olempska-Beer, Z. (2006). *Lycopene (Synthetic)*. Rome: 67th Joint FAO/WHO Expert Committee on Food Additives, 1–6.

Pfleger, B. F., Pitera, D. J., Smolke, C. D., and Keasling, J. D. (2006). Combinatorial engineering of intergenic regions in operons tunes expression of multiple genes. *Nat. Biotechnol.* 24, 1027–1032.

Rath, S. (2009). *Lycopene Extract from Tomato*. Rome: 71st Joint FAO/WHO Expert Committee on Food Additives, 1–9.

Ro, D. K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., et al. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943.

Rodriguez-Villalon, A., Perez-Gil, J., and Rodriguez-Concepcion, M. (2008). Carotenoid accumulation in bacteria with enhanced supply of isoprenoid precursors by upregulation of exogenous or endogenous pathways. *J. Biotechnol.* 135, 78–84.

Scalcinati, G., Knuf, C., Partow, S., Chen, Y., Maury, J., Schalk, M., et al. (2012). Dynamic control of gene expression in *Saccharomyces cerevisiae* engineered for the production of plant sesquitepene alpha-santalene in a fed-batch mode. *Metab. Eng.* 14, 91–103.

Schmidt-Dannert, C. (2000). Engineering novel carotenoids in microorganisms. *Curr. Opin. Biotechnol.* 11, 255–261.

Schmidt-Dannert, C., Umeno, D., and Arnold, F. H. (2000). Molecular breeding of carotenoid biosynthetic pathways. *Nat. Biotechnol.* 18, 750–753.

Shiba, Y., Paradise, E. M., Kirby, J., Ro, D. K., and Keasling, J. D. (2007). Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids. *Metab. Eng.* 9, 160–168.

Shimada, H., Kondo, K., Fraser, P. D., Miura, Y., Saito, T., and Misawa, N. (1998). Increased carotenoid production by the food yeast *Candida utilis* through metabolic engineering of the isoprenoid pathway. *Appl. Environ. Microbiol.* 64, 2676–2680.

Stankovic, I. (2004). *Zeaxanthin*. Geneva: 63rd Joint FAO/WHO Expert Committee on Food Additives, 1–7.

Stephanopoulos, G. (2012). Synthetic biology and metabolic engineering. *ACS Synth. Biol.* 1, 514–525.

Tang, W. L., and Zhao, H. (2009). Industrial biotechnology: tools and applications. *Biotechnol. J.* 4, 1725–1739.

USDA. (2011). *β-Carotene Handling and Processing*. Washington: Technical Services Branch for the USDA National Organic Program, 1–20.

Walter, M. H., and Strack, D. (2011). Carotenoids and their cleavage products: biosynthesis and functions. *Nat. Prod. Rep.* 28, 663–692.

Wang, C. W., Oh, M. K., and Liao, J. C. (1999). Engineered isoprenoid pathway enhances astaxanthin production in Escherichia coli. *Biotechnol. Bioeng.* 62, 235–241.

Wang, H. H., Isaacs, F. J., Carr, P. A., Sun, Z. Z., Xu, G., Forest, C. R., et al. (2009). Programming cells by multiplex genome engineering and accelerated evolution. *Nature* 460, 894–898.

Westfall, P. J., Pitera, D. J., Lenihan, J. R., Eng, D., Woolard, F. X., Regentin, R., et al. (2012). Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* 109, E111–118.

Winter, J. M., and Tang, Y. (2012). Synthetic biological approaches to natural product biosynthesis. *Curr. Opin. Biotechnol.* 23, 736–743.

Withers, S. T., and Keasling, J. D. (2007). Biosynthesis and engineering of isoprenoid small molecules. *Appl. Microbiol. Biotechnol.* 73, 980–990.

Wu, J., Du, G., Zhou, J., and Chen, J. (2013). Metabolic engineering of Escherichia coli for (2S)-pinocembrin production from glucose by a modular metabolic strategy. *Metab. Eng.* 16, 48–55.

Yuan, L. Z., Rouviere, P. E., Larossa, R. A., and Suh, W. (2006). Chromosomal promoter replacement of the isoprenoid pathway for enhancing carotenoid production in *E. coli*. *Metab. Eng.* 8, 79–90.

Zhou, Y. J., Gao, W., Rong, Q., Jin, G., Chu, H., Liu, W., et al. (2012). Modular pathway engineering of diterpenoid synthases and the mevalonic acid pathway for miltiradiene production. *J. Am. Chem. Soc.* 134, 3234–3241.

Zhu, C., and Cook, S. P. (2012). A concise synthesis of (+)-artemisinin. *J. Am. Chem. Soc.* 134, 13577–13579.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 January 2013; accepted: 14 March 2013; published online: 04 April 2013.

Citation: Immethun CM, Hoynes-O'Connor AG, Balassy A and Moon TS (2013) Microbial production of isoprenoids enabled by synthetic biology. Front. Microbiol. ***4****:75. doi: 10.3389/fmicb.2013.00075*

This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 26 July 2013
doi: 10.3389/fmicb.2013.00200

Metabolic analyses elucidate non-trivial gene targets for amplifying dihydroartemisinic acid production in yeast

Ashish Misra[1†], Matthew F. Conway[1†], Joseph Johnnie[2], Tabish M. Qureshi[1], Bao Lige[3], Anne M. Derrick[3], Eddy C. Agbo[3] and Ganesh Sriram[1]*

[1] Department of Chemical and Biomolecular Engineering, University of Maryland, College Park, MD, USA
[2] Institute for Systems Engineering, University of Maryland, College Park, MD, USA
[3] Fyodor Biotechnologies, Baltimore, MD, USA

Edited by:
David Nielsen, Arizona State University, USA

Reviewed by:
Stefan Junne, Technische Universität Berlin, Germany
Yinjie Tang, Washington University, USA

***Correspondence:**
Ganesh Sriram, Department of Chemical and Biomolecular Engineering, University of Maryland, 1208D, Chemical and Nuclear Engineering Building 090, College Park, MD 20742, USA
e-mail: gsriram@umd.edu

†Present address:
Ashish Misra, DBT-ICT Centre for Energy Biosciences, Institute of Chemical Technology, Mumbai, India; Matthew F. Conway, Department of Chemical Engineering, Columbia University, New York, NY, USA.

Synthetic biology enables metabolic engineering of industrial microbes to synthesize value-added molecules. In this, a major challenge is the efficient redirection of carbon to the desired metabolic pathways. Pinpointing strategies toward this goal requires an in-depth investigation of the metabolic landscape of the organism, particularly primary metabolism, to identify precursor and cofactor availability for the target compound. The potent antimalarial therapeutic artemisinin and its precursors are promising candidate molecules for production in microbial hosts. Recent advances have demonstrated the production of artemisinin precursors in engineered yeast strains as an alternative to extraction from plants. We report the application of *in silico* and *in vivo* metabolic pathway analyses to identify metabolic engineering targets to improve the yield of the direct artemisinin precursor dihydroartemisinic acid (DHA) in yeast. First, *in silico* extreme pathway (ExPa) analysis identified NADPH-malic enzyme and the oxidative pentose phosphate pathway (PPP) as mechanisms to meet NADPH demand for DHA synthesis. Next, we compared key DHA-synthesizing ExPas to the metabolic flux distributions obtained from *in vivo* ^{13}C metabolic flux analysis of a DHA-synthesizing strain. This comparison revealed that knocking out ethanol synthesis and overexpressing glucose-6-phosphate dehydrogenase in the oxidative PPP (gene *YNL241C*) or the NADPH-malic enzyme ME2 (*YKL029C*) are vital steps toward overproducing DHA. Finally, we employed *in silico* flux balance analysis and minimization of metabolic adjustment on a yeast genome-scale model to identify gene knockouts for improving DHA yields. The best strategy involved knockout of an oxaloacetate transporter (*YKL120W*) and an aspartate aminotransferase (*YKL106W*), and was predicted to improve DHA yields by 70-fold. Collectively, our work elucidates multiple non-trivial metabolic engineering strategies for improving DHA yield in yeast.

Keywords: artemisinin, metabolic engineering, metabolic pathway, extreme pathway, isotope labeling, metabolic flux analysis, flux balance analysis, minimization of metabolic adjustment

INTRODUCTION

Artemisinin-based combination therapy (ACT) is currently the most commonly used treatment for malaria (Weathers et al., 2006; Eastman and Fidock, 2009; Price and Douglas, 2009), an infectious disease that is widespread particularly in regions of Africa, Asia, and South America (Feachem et al., 2010; O'Meara et al., 2010). Artemisinin, the primary component of ACT, is a naturally produced sesquiterpene lactone endoperoxide. Artemisinin and its derivatives have also been found to exhibit anti-cancer properties (Ferreira et al., 2010) such as inducing apoptosis in lung cancer cells (Gao et al., 2013) and preventing cell proliferation in breast cancer cells (Ba et al., 2012; Zhang et al., 2013). In nature, artemisinin is synthesized by the plant *Artemisia annua* through conversion of the intermediate sesquiterpene farnesyl pyrophosphate (FPP; Bertea et al., 2005; Liu et al., 2011). The precursor FPP is synthesized from the primary metabolite acetyl-coenzyme A (CoA) via the mevalonate (MVA) pathway, or from glyceraldehyde-3-phosphate (GAP) and pyruvate via the methylerythritol phosphate (MEP) pathway (**Figure 1**). *A. annua* has been the primary source for meeting almost all the worldwide demand for artemisinin, despite producing artemisinin up to less than 1.0–1.5% of its dry weight (Kindermans et al., 2007; Covello, 2008). Not surprisingly, the price of artemisinin has varied substantially from a lower bound of US$150–170 kg^{-1} to an upper bound of US$1,100–1,500 kg^{-1} (World Health Organization, 2010), partially due to variability in the cultivation of *A. annua*. Therefore, it is necessary to explore avenues for reliable production of artemisinin that offer this drug at the minimal possible cost to developing countries (Covello, 2008). To achieve this goal, researchers have demonstrated the synthesis of artemisinin precursors in microbes (Ro et al., 2006; Zhang et al., 2008; Westfall et al., 2012; Paddon et al., 2013) or plants (e.g., Zhang et al., 2011) engineered to express genes from the *A. annua* artemisinin pathway, thereby enabling conversion of endogenously produced FPP to artemisinin precursors. Orthogonally, chemical syntheses for artemisinin from starting materials ranging from natural

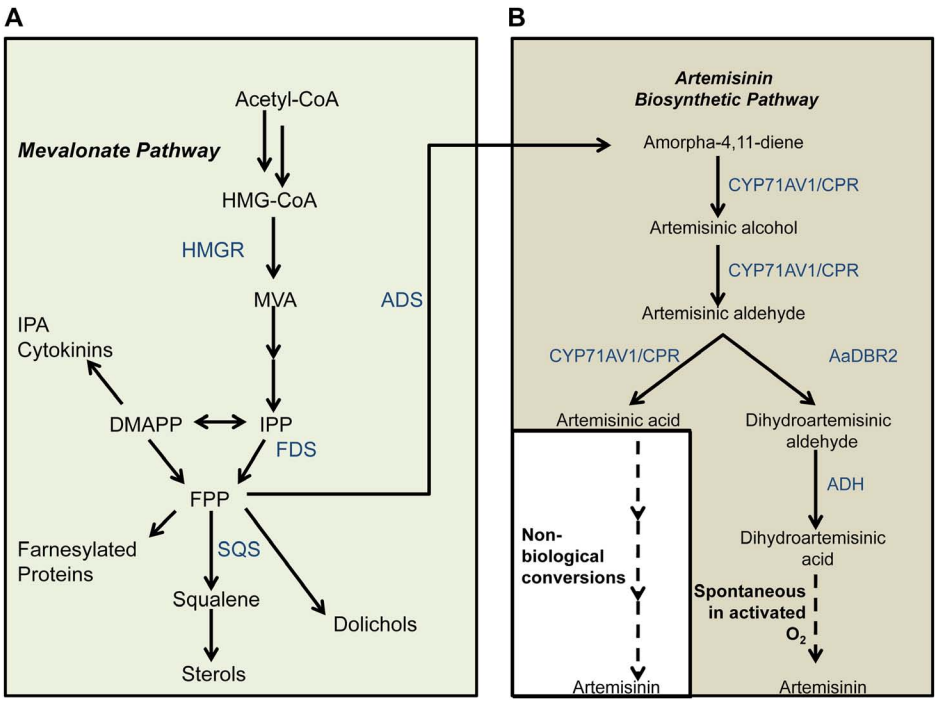


FIGURE 1 | Artemisinin precursor synthesis pathways in yeast. In the native isoprenoid biosynthesis pathway in yeast **(A)**, IPP synthesized via the MVA pathway is converted to FPP. HMGR is a key enzyme in the isoprenoid biosynthetic pathway that feeds the artemisinin precursor synthesis pathway **(B)**. Steps that are not known to be catalyzed by enzymes are depicted with dashed lines. Enzyme and metabolite name abbreviations: AaDBR2, *A. annua* double bond reductase; ADH, (dihydroartemisinic) aldehyde dehydrogenase; ADS, amorpha-4,11-diene synthase; CYP71AV1, cytochrome P450 monooxygenase; CPR, *A. annua* cytochrome P450 reductase; CoA, coenzyme A; DMAPP, dimethylallyl diphosphate; FDS, farnesyl diphosphate synthase; FPP, farnesyl pyrophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HMGR, HMG-CoA reductase; IPA, isopentenyladenine; IPP, isopentenyl diphosphate; MVA, mevalonate; SQS, squalene synthase. Adapted from Zhang et al. (2008) and Teoh et al. (2006).

terpenoids (reviewed in Covello, 2008) to cyclohexenone (e.g., Zhu and Cook, 2012) have been reported.

The first applications of synthetic biology to artemisinin production were those of Keasling and colleagues (Ro et al., 2006) and of Covello and colleagues (Teoh et al., 2006). Both groups reported the engineering of *Saccharomyces cerevisiae* to produce artemisinic acid (AA), which can be converted to artemisinin through a sequence of chemical steps. The breakthrough work by Keasling and colleagues was followed by several reports of significantly improved AA titers from the same group (Westfall et al., 2012; Paddon et al., 2013; see detailed description below). Independently, Covello and colleagues demonstrated the production of dihydroartemisinic acid (DHA) in *S. cerevisiae* (Zhang et al., 2008). As an artemisinin precursor, DHA is preferable to AA for multiple reasons. Firstly, DHA can be oxidized to artemisinin spontaneously without the involvement of enzymes (Sy and Brown, 2002; Brown and Sy, 2004). *In planta* artemisinin biosynthesis is hypothesized to proceed through DHA via this mechanism (Bertea et al., 2005), circumstantial evidence for which comes from the observation that DHA-rich chemotypes of *A. annua* exhibit significantly higher artemisinin production than AA-rich chemotypes (Wallaart et al., 2000; Rydén et al., 2010). Secondly, semi-synthetic routes to artemisinin production have relied upon DHA as the starting material (e.g., Lévesque and Seeberger, 2012; Westfall et al., 2012). Thus, the possibility of engineering DHA biosynthesis in microbes opens up an alternative route for artemisinin synthesis in yeast, which has the potential to be executed completely *in vivo* (Zhang et al., 2008).

The AA route for artemisinin production has recently seen impressive scientific success and commercialization (Hale et al., 2007; Chandran et al., 2008; Lenihan et al., 2008; Westfall et al., 2012; Paddon et al., 2013). The titers and yields of artemisinin or its precursors have been improved substantially by optimization of downstream metabolic pathways as well as process development. The work of Keasling and coworkers improved FPP production, and thereby AA titers, in the initial AA-synthesizing strains (Ro et al., 2006) by ingeniously combining several approaches. These included (i) overexpression of *upc2-1*, a transcription factor involved in the regulation of sterol production, to increase flux through the MVA pathway; (ii) downregulation of squalene synthase (*ERG9*), which diverts FPP away from DHA production to sterol production; and (iii) overexpression of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (*HMGR*) to enhance conversion of HMG-CoA to MVA. By employing these strategies in concert, Keasling and colleagues were able to improve the titer of amorpha-4,11-diene, a precursor of the artemisinin pathway (**Figure 1**), by more than 15-fold over the original AA-synthesizing strain (Ro et al., 2006). Further work (Lenihan et al., 2008) improved AA titers of these strains through process optimization of fermentation parameters. Recently, Keasling and

colleagues employed the strategy of overexpressing all enzymes of the MVA pathway together with repression of *ERG9* to achieve very high titers and yields of amorpha-4,11-diene and AA (Westfall et al., 2012). While all previous attempts employed galactose or glucose as the sole carbon source, Westfall et al. (2012) fed ethanol as a second carbon source and obtained yields of amorpha-4,11-diene higher than 18 C mol % (**Figure 2**). More recently, Paddon et al. (2013) improved AA titers by more than 15-fold to 25 g L^{-1}. Coupled with their development of a scalable chemical process for conversion of AA to artemisinin, this work is a major breakthrough in the commercial production of artemisinin.

The approaches described above have improved AA yields almost exclusively by manipulating flux through downstream pathways, specifically the MVA and sterol biosynthesis pathways. However, another powerful approach to enhance the production of artemisinin precursors would be to engineer upstream (primary and central carbon) metabolism. Several computational and experimental tools such as extreme pathway (ExPa) analysis (Wiback and Palsson, 2002; Llaneras and Picó, 2010), flux balance analysis (FBA; Orth et al., 2010), minimization of metabolic adjustment (MOMA) analysis (Segrè et al., 2002) as well as ^{13}C metabolic flux analysis (^{13}C MFA; e.g., Feng et al., 2010; Papini et al., 2012; Ahn and Antoniewicz, 2013; Nargund and Sriram, 2013) are now available to dissect metabolism, derive insight and propose metabolic engineering strategies. Because primary metabolic pathways supply substantial amounts of carbon and reductant to downstream pathways such as MVA and sesquiterpene synthesis, such strategies may identify non-trivial metabolic engineering strategies and thus play a very significant role in improving flux to AA or DHA. There have been relatively few reports of computationally inferred genetic engineering strategies in upstream metabolism for improving microbial yields of intermediary or secondary metabolites. Studies that have successfully accomplished this include those focusing on the production of L-valine (Park et al., 2007), lycopene (Alper et al., 2005), and C_{14}–C_{16} fatty acids (Ranganathan et al., 2012) by *Escherichia coli*. In one study directed at secondary metabolite production by yeast, Asadollahi et al. (2009) identified genetic interventions toward increased production of sesquiterpenes synthesized via the MVA pathway, by *in silico* analyses of a genome-scale model of yeast metabolism. They predicted that knocking out glutamate dehydrogenase (*GDH1*), whose product assimilates nitrogen at the expense of NADPH, will result in a 10-fold increase in the production of the sesquiterpene cubebol. Mechanistically, this knockout was predicted to shunt carbon via an alternative reaction that consumes NADH instead of NADPH, thus improving NADPH availability to the MVA pathway and sesquiterpene synthesis. Indeed, on *in vivo* implementation, the *GDH1* knockout strategy led to a significant (∼twofold) increase in the titer of cubebol.

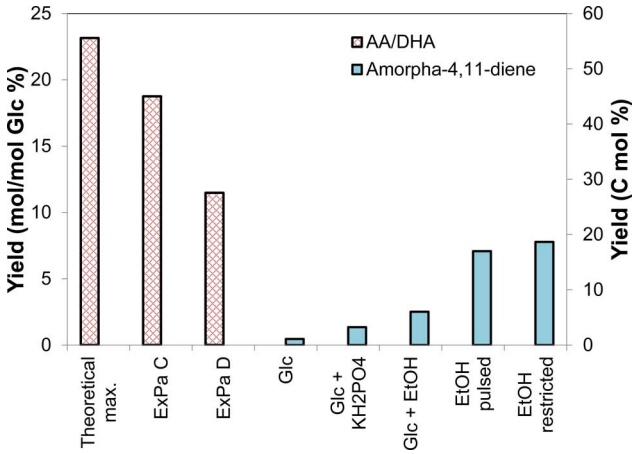


FIGURE 2 | Calculated (red bars) and previously reported (blue bars) yields of artemisinin precursors. The theoretical maximum yield of the artemisinin precursors amorpha-4,11-diene, AA, or DHA is 22 mol/100 mol glucose (Glc) (left axis) or 55 C mol % (right axis). One ExPa (ExPa C, with ME2 active), yields 18 mol DHA/100 mol glucose), with concomitant biomass production (3 mol/100 mol glucose). Here, ME2 supplies reductant to meet the NADPH requirement. Another ExPa (ExPa D, with an active oxidative PPP), yields 11 mol DHA/100 mol glucose, with concomitant biomass production (6 mol/100 mol glucose). Here, the oxidative PPP supplies reductant to meet the NADPH requirement. **Figure 3** depicts flux distributions through these ExPas. The yields of artemisinin precursors in the pioneering microbial production studies (Ro et al., 2006; Zhang et al., 2011) were relatively low; however, subsequent work obtained further improvement in amorpha-4,11-diene and AA yield by increasing flux through the MVA pathway and varying feed composition. Recently, Westfall et al. (2012) achieved drastic improvement in amorpha-4,11-diene yield of close to 20 C mol % by using an optimized yeast strain with a mixed feed consisting of ethanol (EtOH) and Glc. They achieved yields of 1.1 C mol % with glucose as carbon source, which increased to 3.2 C mol % by using KH_2PO_4 with the glucose feed, to 6.0 C mol % by using glucose and EtOH mixed feed. The highest yields obtained were 17.0 and 18.7 C mol % with a restricted EtOH feed.

Because modeling approaches are immensely useful in synthetic biology (Zheng and Sriram, 2010), this article investigates strategies for improving DHA yields in engineered yeast by employing a variety of computation-assisted methodologies. These include ExPa analysis, ^{13}C MFA, FBA, and MOMA analysis. ExPa and elementary flux mode analysis enable detailed investigation of the flux distribution space (phenotypic space) of a metabolic network (Schilling et al., 2000) to obtain insights on the network's capabilities and limitations. Such analyses can suggest genetic intervention strategies to effect a desired outcome from the network Becker et al. (2011). ^{13}C MFA is a powerful methodology currently used for estimating intracellular fluxes. In this methodology, isotope labeling signatures obtained from feeding a mixture of ^{13}C and ^{12}C carbon sources to an organism, in conjunction with extracellular flux measurements, are used to evaluate intracellular flux distributions (e.g., Wiechert, 2001; Sriram et al., 2004, 2008; Murphy et al., 2013). FBA, a complementary approach to ^{13}C MFA, optimizes a metabolic objective to estimate or predict flux distribution seven at the genome-scale (Grafahrend-Belau et al., 2009; Orth et al., 2010). In genome-scale FBA, an organism's inventory of metabolic reactions is used to assemble a stoichiometric matrix. Thermodynamic irreversibility constraints as well as measurements of a few extracellular fluxes such as substrate uptake and product secretion are used to constrain the null space of this matrix, thus generating a phenotypic space of feasible flux distributions. An objective function such as maximization of biomass production is then employed to isolate a particular solution within

the feasible solution space. Several extensions of the FBA approach enable prediction of changes in flux distributions due to genetic interventions, including OptKnock (Burgard et al., 2003), OptStrain (Pharkya et al., 2004), OptForce (Ranganathan et al., 2010), and MOMA (Segrè et al., 2002). MOMA predicts flux alterations resulting from gene knockouts, and thereby enables identification of advantageous knockouts. It is based on the premise that gene knockouts restrict the wild type phenotypic space of an organism, and that organisms respond by minimally adjusting their wild type flux distribution to make it lie within the restricted phenotypic space (Segrè et al., 2002). This contrasts with FBA, which assumes that organisms respond by finding a new optimum of their objective function within the restricted phenotypic space.

To our knowledge, this is the first study utilizing a variety of computational and experimental pathway analysis techniques to identify metabolic engineering targets for enhanced production of artemisinin precursors in yeast. Together, our analyses elucidate upstream bottlenecks in DHA synthesis and suggest non-trivial genetic intervention strategies for pushing carbon toward the DHA pathway and thereby improving DHA yield.

MATERIALS AND METHODS

ExPa ANALYSIS

For ExPa analysis, we constructed a metabolic model of yeast engineered to synthesize DHA. This model consisted of central carbon metabolic pathways native to yeast including glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, MVA pathway, pathways for synthesis of biomass components and a consolidated reaction encompassing the pathway from MVA to DHA. Altogether, this model contained 63 reactions and 51 metabolites. To determine ExPas, we analyzed this model with the *expa* program from Bell and Palsson (2004). The resulting ExPas are tabulated in Data Sheet 1 in Supplementary Material.

CELL CULTURE, STRAIN CHARACTERIZATION, AND METABOLIC MEASUREMENTS

Yeast cell culture in microplates

We used three *S. cerevisiae* strains in this study (Fy_{AA}, Fy_{DHA}, and Fy_0; Zhang et al., 2008), all of which were designer yeast strains carrying lysine and methionine auxotrophies. Strain Fy_{AA} was engineered with genes for the synthesis of AA, specifically *A. annua* farnesyl diphosphate synthase (*FDS*), amorpha-4,11-diene synthase (*ADS*), cytochrome P450 monooxygenase (*CYP71AV1*), and *A. annua* cytochrome P450 reductase (*CPR*). Strain Fy_{DHA} was engineered with genes for the synthesis of DHA, specifically *FDS*, *ADS*, *CYP71AV1*, *CPR*, and *A. annua* double bond reductase (*AaDBR2*). **Figure 1** depicts the reactions catalyzed by the products of these genes. Strain Fy_0 was an empty vector control. Further genetic engineering details are provided in the supplementary material of Zhang et al. (2008). We grew the strains in synthetic defined base yeast minimal medium (Clontech Laboratories, Mountain View, CA, USA) supplemented with trace amounts of lysine and methionine due to the auxotrophies in the strains. This medium was supplemented with 2% (w/v) glucose or galactose as carbon source. All growth and ^{13}C MFA experiments were performed on 2 mL batch cultures in deep-well microplates in a Biotek Synergy HT microplate reader (Biotek Instruments, Winooski, VT, USA) at 30°C with continuous shaking. To induce DHA or AA production, cultures grew for 45–55 h on this medium supplemented with 2% (w/v) galactose as the carbon source. For isotope labeling studies, we used either 20% U-^{13}C galactose or 100% 1-^{13}C galactose in parallel labeling experiments. To harvest cells we centrifuged the cell suspensions, separated the supernatant and immediately froze the pellets in liquid nitrogen to arrest metabolism. The pellets and supernatants were lyophilized and stored at −80°C until further analysis.

Strain characterization

Expression of recombinant genes in the engineered strains was confirmed by RT-PCR, using primers from Zhang et al. (2008). *A. annua actin1* was used as a negative control, whereas the yeast *ACT1* and *TAF10* were used as positive controls (Teste et al., 2009). Primers (sequences are listed in Data Sheet 2 in Supplementary Material) were obtained from Integrated DNA Technologies (Coralville, IA, USA).

Cell growth rates were determined via online optical density measurements on the Biotek microplate reader. Measurements for glucose, ethanol, and glycerol were performed by analyzing the supernatant with a YSI 2700 Select metabolite analyzer (YSI Life Sciences, Yellow Springs, OH, USA), using a measurement kit appropriate for each metabolite. These measurements are listed in Data Sheet 2 in Supplementary Material. AA and DHA were extracted by using protocols adapted from Zhang et al. (2008) and quantified by gas-chromatography-mass spectrometry (GC-MS) of their TMS derivatives, using methyl stearate as an internal standard.

Extraction and analysis of proteinogenic amino acids for ^{13}C isotopomer measurements

Proteinogenic amino acids in cell pellets were obtained by vacuum-hydrolyzing the pellets with 6N hydrochloric acid (Thermo Scientific, Rockford, IL, USA). The hydrolysate was mixed with a known amount of norleucine as an internal standard and derivatized by adding 100 μL *N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide (MTBSTFA; Thermo Scientific) in 100 μL dimethylformamide (Thermo Scientific) and heating at 70°C for 1 h. GC-MS analysis of 1 μL of this derivatized mixture was performed using previously published instrument methods (Sriram et al., 2008) on a Varian 300MS quadrupole GC-MS unit (Bruker, Billerica, MA, USA) equipped with a VF5-ms column of dimensions 0.25 mm × 30 m × 0.25 μm and capable of detecting ions by electron ionization. Quantification of amino acids in the biomass was performed by comparing the chromatographic peak of each amino acid with that of the norleucine internal standard. Isotopomer abundances obtained from the mass spectrum of each amino acid were processed to filter out natural abundances of elements other than metabolic carbon, using a previously developed in-house MATLAB program, whose accuracy has been verified by using amino acid isotopomer mixtures of known isotopomeric composition (Sriram et al., 2008). Isotopomer abundances for the three strains are reported in Data Sheet 2 in Supplementary Material.

Reproducibility and statistics

All experiments were performed with two or three biological replicates, and statistical significance was determined by calculating *p*-values through a Student's *t*-test.

Evaluation of metabolic fluxes from ^{13}C isotopomer data

We evaluated fluxes from the ^{13}C mass isotopomer data and extracellular flux measurements by using our flux evaluation program NMR2Flux+ (Sriram et al., 2004, 2008) to fit this data to a steady state flux-isotopomer model. This model consisted of yeast central carbon metabolic pathways, including glycolysis, PPP, TCA cycle, and anaplerotic pathways. For the retrobiosynthetic reconstruction of metabolic precursor mass isotopomer distributions (MIDs) from the amino acid MIDs (e.g., pyruvate from alanine or oxaloacetate from aspartic acid), we used standard pathways from the precursors to the amino acids (Szyperski, 1995, 1998). These pathways are universally conserved across life (Romano and Conway, 1996). We used standard models for the biosynthesis of amino acids from the intermediates of these pathways (e.g., Szyperski, 1995). We found that labeling of the proteinogenic amino acids except methionine and lysine was very close to 20% for cells grown on 20% U-^{13}C galactose, which indicates attainment of isotopic steady state (data not shown). However, the metabolism is still at pseudo-steady state due to the batch culture. Statistical analysis was performed by the NMR2Flux+ program, using a bootstrap Monte Carlo simulation to execute 100 runs of the flux evaluation by perturbing measurements by their known standard error, as previously described (Sriram et al., 2004, 2008). Evaluated fluxes for the three strains are reported in Data Sheet 2 in Supplementary Material.

FBA AND MOMA ANALYSIS

We modified the iMM904 genome-scale model for *S. cerevisiae* (Mo et al., 2009) by appending it with the reactions necessary to synthesize DHA from FPP. We constrained this model with the measured extracellular uptake fluxes (glucose or galactose) as well as intracellular fluxes estimated from the ^{13}C isotopomer data. The open-source COBRA toolbox (Schellenberger et al., 2011) was used to perform FBA and MOMA analysis on the model. For FBA, we used maximization of biomass growth as the objective function. We performed MOMA to assess the effects of all possible single-gene knockouts on DHA production. For this, we used an in-house MATLAB script (available on request) to automate certain repetitive steps such as recurring calls to COBRA. The non-lethal single-gene knockouts that significantly increased DHA yields were used in a second iteration of the MOMA analysis to identify double-gene knockouts that enhanced DHA production. The results of this analysis are available in Data Sheet 3 in Supplementary Material.

RESULTS AND DISCUSSION

The currently available synthesis routes for DHA (or AA) use galactose or glucose as carbon sources (Ro et al., 2006; Teoh et al., 2006; Zhang et al., 2008; Paddon et al., 2013). Strategizing how to produce DHA from these substrates at high yield is a complex problem, solving which requires answering the following questions. (i) What is the theoretical maximal yield of DHA on glucose or galactose? (ii) Is this yield limited by the availability of reductant cofactors? (iii) Which configuration of pathways and fluxes favors a high yield? (iv) Do current DHA-synthesizing strains operate near or distantly from such a configuration? (v) Which minimal sets of genetic interventions can drastically improve the yield of a low DHA-producing strain? Below, we describe our computational analyses (ExPa analysis and MOMA analysis) and experimental investigations (^{13}C MFA) toward answering these questions.

THEORETICAL MAXIMAL YIELD OF DHA OR AA ON GLUCOSE

The value of the maximal yield of DHA on glucose (or a similar sugar such as galactose) depends on whether the reductant cofactors NADH and NADPH are considered in the analysis, and whether a distinction is made between the cofactors NADH and NADPH. A cofactor-free theoretical yield calculation reveals that yeast can produce a maximum yield of 22 mol DHA/100 mol glucose, or 1 mol DHA per 4.5 mol glucose (**Figure 2**). Metabolically, this would proceed as follows. Glycolysis would convert 4.5 mol glucose to 9 mol acetyl-CoA, which the MVA pathway would condense to form 3 mol dimethylallyl diphosphate (DMAPP). DMAPP would further condense to form FPP in a molar proportion of 3 DMAPP:1 FPP. FPP would then be converted to amorphadiene and ultimately to DHA in a 1:1 molar proportion (Bertea et al., 2005). This DHA yield is equivalent to 55 C mol %, wherein the 45 C mol % carbon loss is due to the release of CO_2 in multiple pathway steps. This calculation and yield value also apply to AA production from glucose or galactose.

The fact that artemisinin, DHA and AA are significantly reduced compounds necessitates the consideration of reductant (cofactor) demand and supply in this yield calculation. In the upstream section of the pathway described above, the conversion of 4.5 mol glucose to 9 mol acetyl-CoA supplies 18 mol NADH. In the downstream section, 6 mol NADPH is required for the conversion of 9 mol acetyl-CoA to 3 mol DMAPP. Therefore, if no distinction is made between the NADH and NADPH, this pathway is self-sufficient in reductant because the demand of 6 mol NADPH is met by a supply of 18 mol NADH.

The interchangeability of NADH and NADPH invoked in the above analysis is contingent upon the availability of a transhydrogenase enzyme that converts NADH to NADPH (Stephanopoulos et al., 1998). However, there is no evidence for a transhydrogenase in yeast (Bruinenberg et al., 1983; Nissen et al., 2001). Therefore, other NADPH-supplying pathways will have to be recruited to make DHA production feasible. This complicates the theoretical yield calculation for three reasons. Firstly, multiple pathways including the oxidative PPP and anaplerotic cycles can supply NADPH either by themselves or in combination. Secondly some of these pathways, such as the oxidative PPP, supply NADPH at the expense of carbon, so that recruiting them for NADPH provision can compromise the maximal yield of DHA. Thirdly, the practical necessity of concurrently producing biomass with DHA will obviously reduce the maximal yield of DHA. To analyze this complex set of possibilities and delineate all possible pathway configurations for DHA synthesis with or without cofactor requirement, we performed ExPa analysis of the underlying metabolic network.

ExPa ANALYSIS OF DHA SYNTHESIS NETWORK

Colloquially, an ExPa represents one way of "walking through a metabolic network." Mathematically, ExPas are the edges of the polyhedral cones that represent the hyperdimensional phenotypic space that a metabolic network can occupy (Bell and Palsson, 2004; Palsson, 2006). ExPa analysis has previously been applied to a variety of systems on different scales from small scale networks of red blood cells to genome-scale pathway analysis of the influenza virus to elucidate important metabolic properties of the systems (Schilling and Palsson, 2000; Wiback and Palsson, 2002). Amongst the three types of ExPas (types I, II, and III) classified by Palsson (2006), we are interested in types I and III. Type I ExPas effect the conversion of a substrate or substrates to a product or products along with the transport of the substrates and products across the cell membrane. Type III ExPas involve intracellular cycles with no transport across the cell membrane. To perform ExPa analysis, we constructed a simplified (less than genome-scale) metabolic model of yeast capable of producing both DHA and biomass (Data Sheet 1 in Supplementary Material). This model includes 63 reactions including exchange reactions and 51 metabolites including cofactors. We then performed ExPa analysis on the model by (i) neglecting and (ii) considering cofactor requirements to gain insights about the capabilities of the network. **Table 1** presents a summary of ExPa families for both cases, and **Figure 3** depicts flux distributions through four selected ExPas. Of these, ExPa A was obtained from a cofactor-free model, and ExPas B, C, and D were obtained by considering cofactor requirements.

COFACTOR-FREE ExPa ANALYSIS REVEALS IMPORTANT CLASSES OF PATHWAYS TOWARD DHA SYNTHESIS

A cofactor-free ExPa analysis of a network, although an incomplete depiction of the network, permits the delineation of major classes of metabolic routes through it. Such an analysis of the metabolic model in Data Sheet 1 in Supplementary Material revealed 50 type I, 6 type II, and 18 type III ExPas (**Table 1** and Data Sheet 1 in Supplementary Material). Of these, only five ExPas synthesized DHA as their sole product, whereas the rest synthesized various secreted metabolites (e.g., glycerol, ethanol, and acetate), biomass or a combination of biomass and secreted metabolites. Interestingly, none of the biomass-producing ExPas concurrently synthesized DHA (**Table 1**). The DHA yield in the DHA-synthesizing ExPa A was equal to the previously calculated theoretical maximum of 22 mol DHA/100 mol glucose (**Figure 3A**).

INCLUSION OF COFACTOR REQUIREMENT RECRUITS NADPH-MALIC ENZYME (ME2) OR THE OXIDATIVE PPP FOR NADPH PROVISION TO DHA SYNTHESIS PATHWAY

We repeated the foregoing ExPa analysis for a more realistic scenario that considered cofactor requirements and did not include a

Table 1 | Summary of ExPa families for a yeast strain synthesizing DHA from glucose, in the absence and presence of cofactor requirement.

	Cofactor-free			With cofactor requirement		
ExPa family[a]	**# of ExPas**	**Maximal yields**		**# of ExPas**	**Maximal yields**	
DHA production[b]	5	DHA:	22	238	DHA:	22
	(#1 to #5)			(#1 to #238)		
DHA and biomass production[c]	0	N/A		94	DHA:	18
				(#239 to #332)	Biomass:	10
Biomass production	11	Biomass:	16	383	Biomass:	11
	(#6 to #16)			(#333 to #715)		
All carbon lost to CO_2	10	CO_2:	600	108	CO_2:	600
	(#17 to #26)			(#716 to #823)		
Metabolite production via glycolysis	10	Glycerol:	200	83	Glycerol:	164
	(#27 to #36)	Ethanol:	200	(#824 to #906)	Ethanol:	200
Metabolite production via PPP	14	Glycerol:	167	161	Glycerol:	137
	(#37 to #50)	Ethanol:	167	(#907 to #1067)	Ethanol:	167
Type II ExPas	6	N/A		0	N/A	
	(#51 to #56)					
Type III ExPas	18	N/A		19	N/A	
	(#57 to #74)			(#1068 to #1086)		

ExPa analysis revealed maximal DHA yield of 22 mol/100 mol glucose in both cases. Simultaneous DHA and biomass production was possible only when cofactor requirements were considered. The numerals following the hashes "#" in the "# of ExPas" columns denote pathway numbers in Data Sheet 1 in Supplementary Materials. All yields are in mol/100 mol glucose.

[a] ExPas are type I unless otherwise specified.See Palsson (2006) and text for definitions of ExPa types.

[b] NADPH corresponding to maximal DHA yield is supplied by the ME2 reaction; supply of NADPH by the PPP results in lower yields.

[c] This is the most important ExPa family, as it provides insights into strategies for improving overall DHA yield.

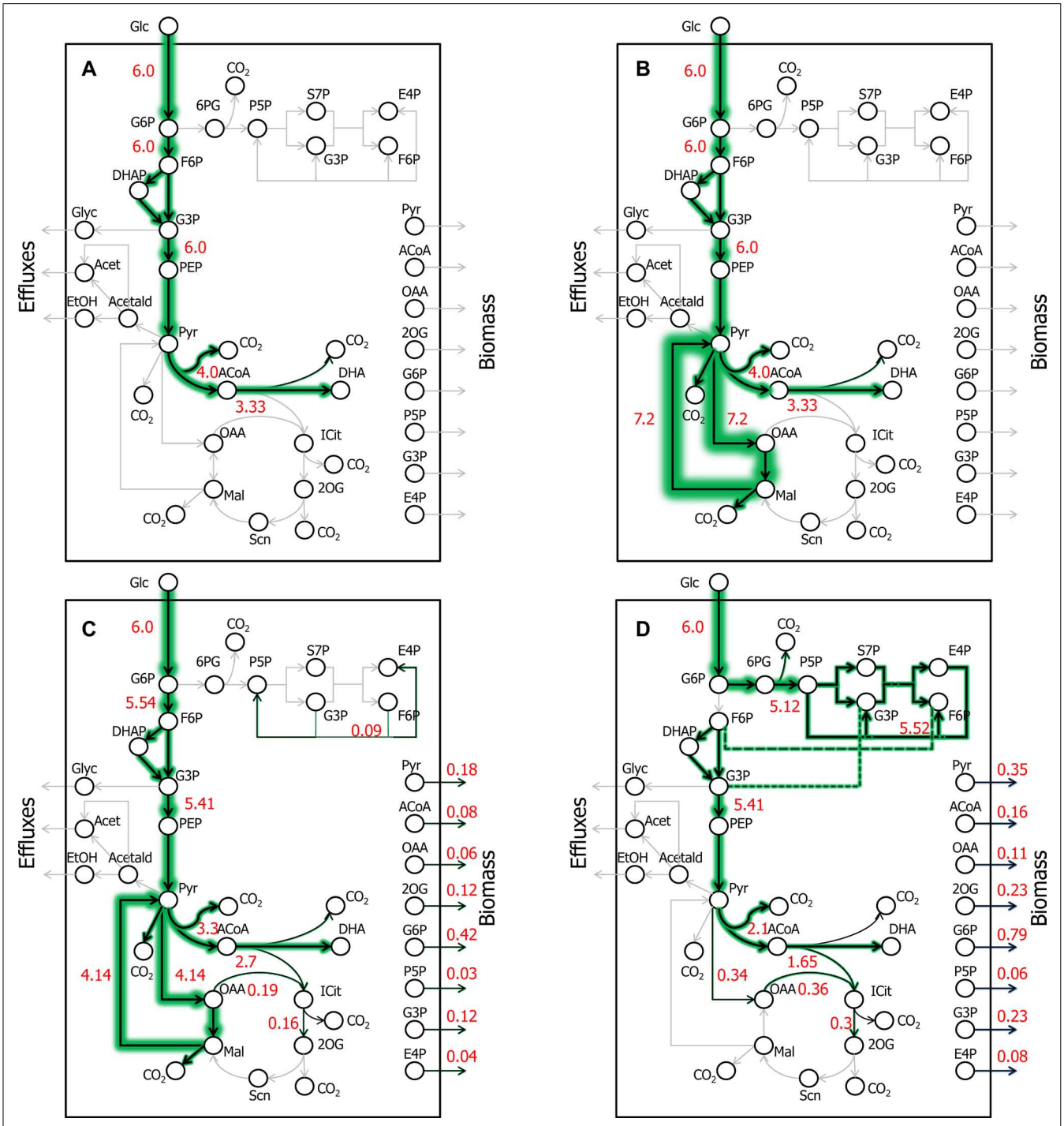


FIGURE 3 | Key DHA- or AA-synthesizing ExPas in yeast. Four ExPas that synthesize DHA (or AA) and/or biomass are shown. Each ExPa is depicted on a network diagram by highlighting the reactions active in it. The intensities of green glows around arrows are proportional to the fluxes of the corresponding reactions; numerals alongside the arrows indicate flux values. **(A)** ExPa A, producing the theoretical maximum DHA yield of 22 mol/100 mol glucose, without considering cofactor requirement or availability. There is no biomass production and all possible carbon is diverted toward DHA synthesis. **(B)** ExPa B, producing the theoretical maximum DHA yield of 22 mol/100 mol glucose, by accounting for cofactor requirement and availability. There is no biomass production. Here, the NADPH requirement is met by a pyruvate ≫ oxaloacetate ≫ malate ≫ pyruvate shuttle via ME2. **(C)** ExPa C, producing a high DHA yield of 18 mol/100 mol glucose, with concomitant biomass production of 3 mol/100 mol glucose. ME2 supplies reductant to meet the NADPH requirement. **(D)** ExPa D, producing substantial DHA yield of 11 mol/100 mol glucose, with concomitant biomass production of 6 mol/100 mol glucose. Here, the oxidative PPP supplies reductant to meet the NADPH requirement.

transhydrogenase to interconvert NADH and NADPH. This analysis resulted in 1086 ExPas with 1067 type I, 0 type II, and 19 type III ExPas (**Table 1** and Data Sheet 1 in Supplementary Material). Interestingly, the maximal DHA yield obtained from this analysis was identical to the theoretical maximal yield, indicating that some reactions in the network are able to supply the desired cofactors without shunting any carbon away from DHA production. For instance, one ExPa (ExPa B) gave a DHA yield of 22 mol/100 mol glucose by providing NADPH via ME2 with no concomitant biomass production (**Figure 3B**). Here, a pyruvate ≫ oxaloacetate ≫ malate ≫ pyruvate shuttle, with the malate ≫ pyruvate conversion catalyzed by ME2, meets the NADPH requirement of DHA synthesis. In all the 238 ExPas that exclusively synthesized DHA, the NADPH requirements were met either by a coupling of the pyruvate carboxylase and ME2 reactions or by operation of the oxidative PPP. In fact, the ME2 reaction for converting malate to pyruvate and the oxidative branch of the PPP are the only two means of producing NADPH in the network. ExPas that recruited the oxidative PPP to meet the NADPH requirement were associated with a DHA yield lower than the theoretical maximum, because the oxidative PPP loses significant carbon as CO_2. Consequently, ME2 is preferable to the oxidative PPP as a source of NADPH for DHA synthesis. This makes ME2 an attractive metabolic engineering target.

Consideration of cofactor requirements resulted in an important set of 94 ExPas that simultaneously produced DHA and biomass (Data Sheet 1 in Supplementary Material and **Table 1**; see examples in **Figures 3C,D**). These pathways are important to our objective because an overall improvement in DHA yield requires the production of both DHA and biomass at high yields. However, concomitant synthesis of DHA and biomass involves many NADPH-consuming reactions with conflicting NADPH demands. The NADPH demands of DHA biosynthesis are explained earlier in this section; biomass synthesis requires NADPH for the conversion of central carbon metabolic precursors to proteinogenic amino acids and lipogenic fatty acids. Thus, an increase in DHA yield is accompanied by a decrease in biomass yield and vice versa. Two important ExPas that coupled DHA and biomass production are depicted in **Figures 3C,D**. ExPa C (**Figure 3C**) produced a high DHA yield of 18 mol/100 mol glucose, with concomitant biomass production of 3 mol/100 mol glucose. ME2 supplied reductant to meet the NADPH requirement. ExPa D (**Figure 3D**) produced substantial DHA yield of 11 mol/100 mol glucose, with concomitant biomass production of 6 mol/100 mol glucose. Here, the oxidative PPP supplied reductant to meet the NADPH requirement. Thus, the ExPa analysis provided insights on the capabilities of the DHA biosynthesis metabolic network, and identified ME2 and the oxidative PPP as two critical metabolic engineering targets.

However, ExPa analysis only identifies the modes of the network, as each ExPa is the edge of the realizable flux cone. Because a real flux distribution would be a linear combination of a number of ExPas, it will contain pathways that produce unneeded metabolites. Furthermore, ExPa analysis does not take in to account the carbon requirement for the *de novo* synthesis of cofactors and energy carriers such as ATP. In the absence of these processes that consume carbon, it overestimates production of biomass and other metabolites compared to experimental results. As a result, the actual improvements in yields would be lower than predicted by the ExPas analysis. To obtain more realistic predictions, we performed flux determination of the DHA-synthesizing strain experimentally using ^{13}C MFA.

^{13}C MFA AND EXTRACELLULAR MEASUREMENTS OF DHA-SYNTHESIZING STRAIN REVEAL NEGLIGIBLE PPP AND LOW TCA CYCLE FLUXES AS WELL AS SUBSTANTIAL FERMENTATIVE FLUXES

We performed comparative ^{13}C MFA of the three yeast strains Fy_0, Fy_{AA}, and Fy_{DHA}. First, we used RT-PCR to verify that these strains correctly express the recombinant genes engineered into them (**Figure 4A**). We also verified the strains produce the secondary metabolites expected of them: Fy_{AA} produced AA but not DHA, Fy_{DHA} produced both DHA and AA, whereas Fy_0 produced neither compound (data not shown). **Figure 4B** depicts transient DHA production by Fy_{DHA}. Extracellular flux measurements on these strains (**Figure 4C**) showed that ∼50% of the consumed galactose was directed toward ethanol, 1% toward glycerol and less than 10% toward biomass. Thus, despite aerobic conditions, the strains exhibited substantial fermentative metabolism. Therefore, an initial strategy for improving DHA yields should be the deletion of the ethanol fermentation pathway, which can be expected to double the carbon available for DHA and biomass production.

For a more profound understanding of the metabolic behavior of these strains, we performed ^{13}C MFA on them. **Figure 5** depicts key isotopomer trends that emerged from the ^{13}C labeling experiments. Surprisingly, we did not observe any major significant differences in the amino acid isotopomer abundances between the three strains (Data Sheet 2 in Supplementary Material). This was confirmed by a principal component analysis of the isotopomer abundance data, in which all three strains clustered quite close together, although the Fy_{AA} and Fy_{DHA} and strains were slightly closer to each other than each was to the control strain Fy_0 (data not shown). **Figure 6** presents the flux maps obtained from ^{13}C MFA, using the metabolic model listed in Data Sheet 2 in Supplementary Material. This model did not consider cofactors. NADPH and other cofactors are often consumed in processes such as scavenging reductive oxygen species, making it difficult to estimate fluxes in real cellular scenarios. Because ^{13}C MFA obtains significant flux information from isotopomer abundance data, it is a powerful method of estimating fluxes independent of cofactors.

^{13}C MFA revealed that almost all the galactose entering the cell was metabolized via glycolysis with ∼10% of it passing through the oxidative PPP (**Figure 6**). This is clear from the MIDs of the fragment of alanine in the 100% 1-^{13}C galactose labeling experiment (**Figures 5A,B** and Data Sheet 2 in Supplementary Material). The [123] fragment of pyruvate (and of alanine, which is derived from it) is synthesized from the [321] and [456] fragments of galactose. The oxidative PPP results in the loss of the C-1 carbon of galactose. Thus, alanine synthesized from carbon that passed through the oxidative PPP should exhibit a [123] fragment with the MID {m+0: 100%; m+1: 0%; m+2: 0%; m+3: 0%}. Conversely, alanine synthesized from carbon that passed through glycolysis should exhibit a [123] fragment with the MID {m+0: 50%; m+1: 50%; m+2: 0%; m+3: 0%}. In our 100% 1-^{13}C galactose labeling experiment, we observed an MID of {m+0: 52%; m+1: 46%;

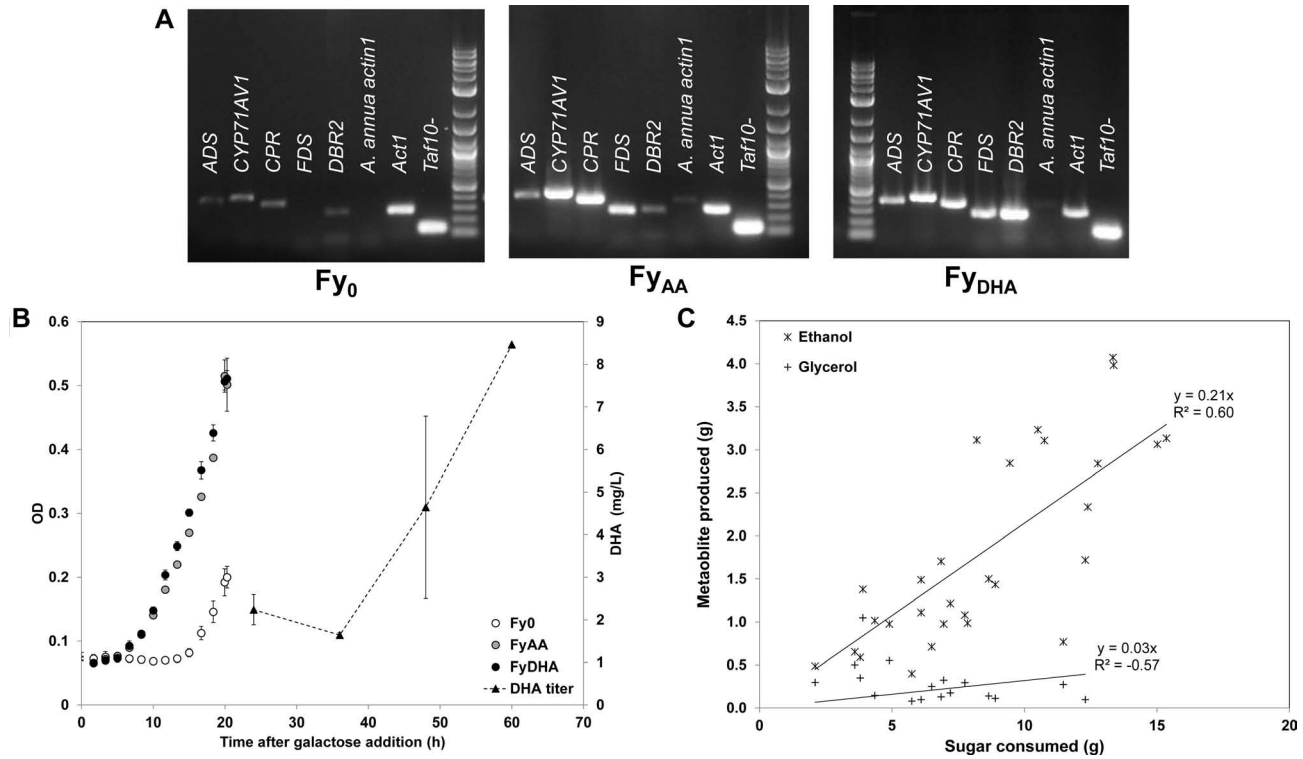


FIGURE 4 | Characterization of engineered yeast strains Fy_0, Fy_{AA}, and Fy_{DHA} used in this study. (A) Gene expression assay of *ADS*, *CYP71AV1*, *CPR*, *FDS*, *DBR2*, a negative control (*A. annua actin1*) and two positive controls (*ACT1* and *TAF10*). This assay was performed in duplicate at multiple time points; only one replicate is shown. This assay showed that as expected, strain Fy_{AA} expresses *ADS*, *CYP71AV1*, *CPR*, and *FDS*, whereas strain Fy_{DHA} expresses these genes and *DBR2*. **(B)** Cell growth curves (left axis) and DHA titer for Fy_{DHA} strain (right axis): white circles, Fy_0; gray circles, Fy_{AA}; black circles, Fy_{DHA}; black triangles, DHA titer of Fy_{DHA}. **(C)** Yield calculations of ethanol (asterisks) and glycerol (plus signs) on glucose and galactose for all three strains.

m+2: 1%; *m*+3: 0%} for the alanine [123] fragment (**Figure 5B**), indicating that the oxidative PPP was nearly inactive. Data from other amino acid fragments in this experiment as well as the 20% U-^{13}C galactose labeling experiment also concur with this result. Because the oxidative PPP generates two NADPH molecules for every molecule of glucose-6-phosphate metabolized through it and is featured in many DHA-synthesizing ExPas identified above, amplifying flux through it is a key metabolic engineering strategy toward improving DHA yield. A possible candidate gene for overexpression is glucose-6-phosphate dehydrogenase *G6PDH2*, which is known to be the rate-limiting enzyme in this pathway in *S. cerevisiae* (Kwon et al., 2006).

Furthermore, the isotopomer data revealed that the TCA cycle carried lower flux than expected under aerobic conditions (**Figures 5C,D**). This is evident from the MIDs of the aspartic acid fragment [1234] {*m*+0: 66%; *m*+1: 15%; *m*+2: 3%; *m*+3: 14%; *m*+4: 2%} and alanine fragment [123] {*m*+0: 78%; *m*+1: 3%; *m*+2: 1%; *m*+3: 18%}from the 20% U-^{13}C galactose labeling experiment (**Figure 5D** and Data Sheet 2 in Supplementary Material). Assuming that the ^{13}C enrichment of intracellular CO_2 is approximately the same as that of the supplied galactose, synthesis of the fragment [1234] of the TCA cycle intermediate oxaloacetate, and thereby of aspartic acid, purely through the anaplerotic reaction pyruvate + $CO_2 \gg$ oxaloacetate should result in the MID {*m*+0: 64%; *m*+1: 16%; *m*+2: 0%; *m*+3: 16%; *m*+4: 4%} for the aspartic acid [1234] fragment. Synthesis of oxaloacetate through the TCA cycle itself should result in a very different MID because of the carbon atom rearrangements in that pathway (**Figure 5D**). The closeness of the aspartic acid [1234] fragment MID to that predicted through pure anaplerotic synthesis indicates that the TCA cycle has very little flux relative to the anaplerotic reactions or glycolysis (**Figure 6**).

COMPARISON OF ^{13}C MFA AND ExPa FLUX DISTRIBUTIONS IDENTIFIES FURTHER METABOLIC ENGINEERING TARGETS

The selection of a preferred DHA-producing ExPa from amongst the 1000+ ExPas elucidated by us would depend on the ease with which the Fy_{DHA} strain can be engineered to emulate the ExPa. Therefore, as a step to identify gene targets for improved DHA production, we compared the flux distribution of strain Fy_{DHA} and the desired DHA- and biomass-synthesizing ExPas C and D from **Figures 3C,D** (**Table 2**). Because these ExPas are optimal DHA- and biomass-producing modes, emulating them should result in an improvement of overall DHA yield. Gene overexpressions necessary for Fy_{DHA} to emulate ExPa C include pyruvate dehydrogenase, ME2 and pyruvate carboxylase. Genetic interventions necessary to emulate ExPa D include overexpression of pyruvate dehydrogenase, glucose-6-phosphate

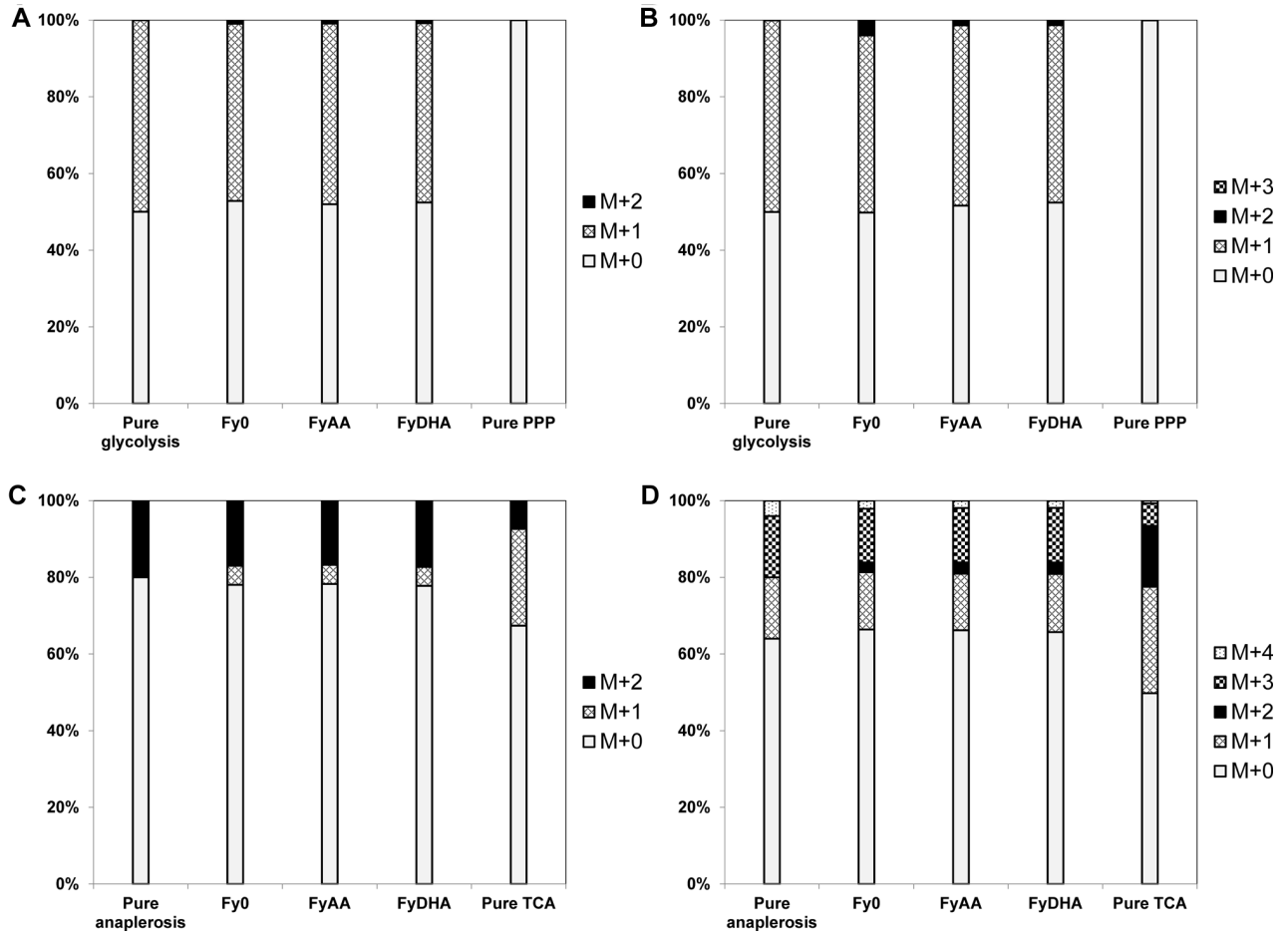


FIGURE 5 | ^{13}C labeling patterns of proteinogenic amino acids evince negligible PPP flux and low TCA cycle flux in all three yeast strains. **(A)** Ala[23] fragment, **(B)** Ala[123] fragment, **(C)** Asp[12] fragment **(D)** Asp[1234] fragment. The observed MIDs of the Ala fragments of all three strains closely resembled MIDs simulated by assuming that all carbon is processed by glycolysis ("pure glycolysis" bar), and were substantially different from MIDs simulated by assuming that all carbon passed through the PPP. This evidences negligible PPP flux in all three strains. The observed MIDs of both the Asp fragments more closely resembled simulated MIDs corresponding to the synthesis of oxaloacetate through anaplerosis than those corresponding to oxaloacetate synthesis through the TCA cycle. This suggests low TCA cycle flux in all three strains.

dehydrogenase, transaldolase, and transketolase as well as knockdown of glucose-6-phosphate isomerase (**Table 2**). Common to both ExPas is pyruvate dehydrogenase, which catalyzes the pyruvate ≫ acetyl-CoA reaction. The rationale for overexpressing it is that the DHA-synthesizing strain has a low flux from pyruvate to acetyl-CoA, which would limit the carbon available for DHA production, as acetyl-CoA is the precursor for the MVA pathway. A strong alternative to pyruvate dehydrogenase would be acetyl-CoA synthetase, which plays a role in the pyruvate ≫ acetaldehyde ≫ acetate ≫ acetyl-CoA route that is reported to be a major source of acetyl-CoA in *S. cerevisiae* (Hynes and Murray, 2010). Another alternative may be ATP citrate lyase, which produces acetyl-CoA from citrate. However, this pathway may not be a major source of cytosolic acetyl-CoA in *S. cerevisiae* (Hynes and Murray, 2010).

Other gene targets in **Table 2** belong to two categories: one involves improving flux through the PPP (overexpression of glucose-6-phosphate dehydrogenase, transketolase, transaldolase) and reducing the glycolytic flux (knockdown of glucose-6-phosphate isomerase), whereas the other involves increasing flux through the malate ≫ pyruvate reaction catalyzed by ME2. Both of these are complementary approaches that look to improve NADPH availability for DHA and biomass production. It should be noted that there is a likelihood of some ME2 flux in the yeast strains of this study. In the model we used for ^{13}C MFA, all anaplerotic flux was consolidated into a single bidirectional reaction maef, because our isotopomer measurements could not resolve the different possibilities for this flux: phosphoenolpyruvate carboxykinase, phosphoenolpyruvate carboxylase, and pyruvate carboxylase (phosphoenolpyruvate or pyruvate + CO_2 ≫ oxaloacetate) as well as NADH-dependent and NADPH-malic enzyme (pyruvate + CO_2 ≪≫ malate). The flux distributions returned by ^{13}C MFA included a low net flux (0.02) but substantial reversibility (0.97–0.98) for this consolidated anaplerotic flux, translating to forward (0.78) and reverse (0.76) fluxes that were significant, yet an order of magnitude smaller than

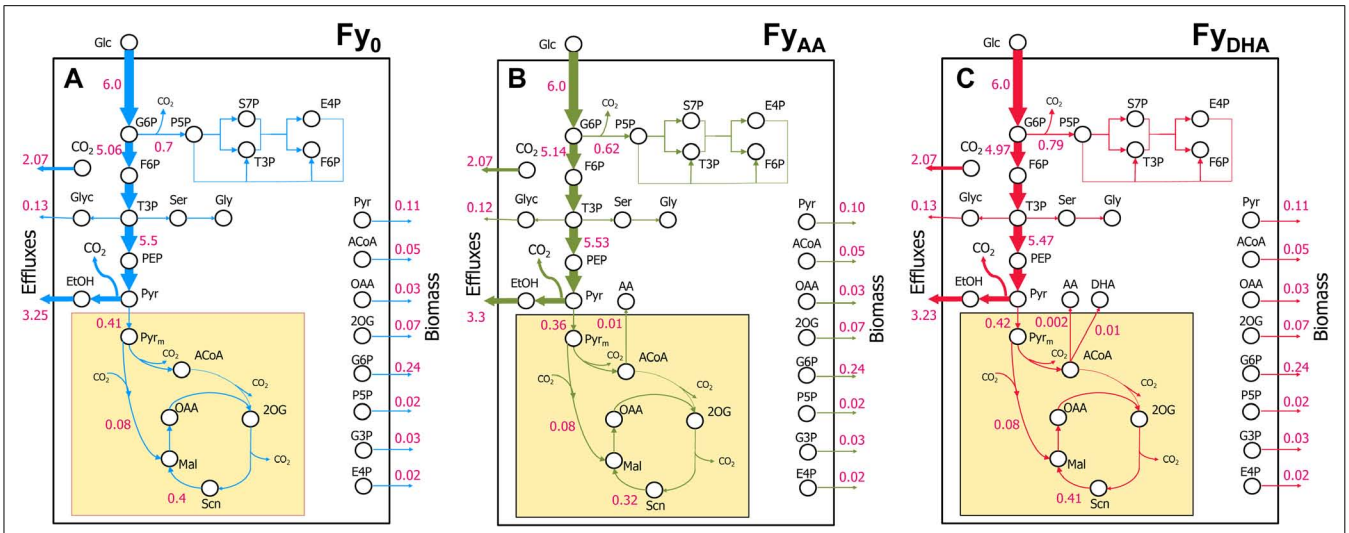


FIGURE 6 | Flux distributions obtained by ^{13}C MFA for the three engineered yeast strains. (A) Fy_0; **(B)** Fy_{AA}; **(C)** Fy_{DHA}. All three strains show similar flux distributions with a substantial fraction of glucose carbon shunted to ethanol and glycerol production. All strains exhibited large fluxes through glycolysis and negligible or low fluxes through both the oxidative PPP and the TCA cycle.

Table 2 | Genetic engineering targets determined by comparing ExPa analysis with ^{13}C MFA.

	ExPa C	ExPa D
DHA yield	0.18	0.11
Biomass yield	0.03	0.06
NADPH supplied by	ME2	PPP
Gene overexpression in strain Fy_{DHA} necessary to emulate this ExPa	1. *YBR221C, YER178W, YFL018C, YGR193C, YNL071W* (pyruvate dehydrogenase) 2. *YKL029C* (NADPH-malic enzyme ME2) 3. *YBR218C, YGL062W* (pyruvate carboxylase)	1. *YBR221C, YER178W, YFL018C, YGR193C, YNL071W* (pyruvate dehydrogenase) 2. *YNL241C* (glucose-6-phosphate dehydrogenase) 3. *YGR043C, YLR354C* (transaldolase in PPP) 4. *YBR117C, YPR074C* (transketolase in PPP)
Gene knockdown in strain Fy_{DHA} necessary to emulate this ExPa	1. *YBR145W, YGL256W, YOL086C* (alcohol dehydrogenase)	1. *YBR145W, YGL256W, YOL086C*(alcohol dehydrogenase) 2. *YBR196C* (glucose-6-phosphate isomerase)

The characteristics of two key ExPas with reasonable DHA and biomass yields are shown, with the corresponding genetic engineering targets to emulate these ExPas in Fy_{DHA}. Common targets include eliminating ethanol biosynthesis by deleting alcohol dehydrogenase and increasing flux from pyruvate to acetyl-CoA by overexpressing pyruvate dehydrogenase.

the glycolytic flux (∼5.0; Data Sheet 2 in Supplementary Material and **Figure 6**). ME2 may contribute to this flux, although this contribution remains unresolved. However, a comparison with ExPa B or C (**Figures 3B,C**) shows that even a 100% contribution by ME2 to this flux is insufficient to meet NADPH demand.

FBA AND MOMA ANALYSIS OF THE MODIFIED iMM904 GENOME-SCALE MODEL REVEAL MULTIPLE GENETIC INTERVENTION STRATEGIES FOR IMPROVING DHA YIELDS

Flux balance analysis, a complementary approach to ^{13}C MFA, estimates or predicts fluxes using linear programming techniques. It is particularly useful for analysis of metabolism at the genome-scale. MOMA is an extension of the FBA approach that identifies non-trivial gene knockouts toward optimizing a metabolic objective, such as the yield of a product. Its use toward determining gene targets has been reported in multiple successful studies (Alper et al., 2005; Park et al., 2007; Asadollahi et al., 2009; Ranganathan et al., 2012). We performed genome-scale FBA and MOMA analysis by using a modified iMM904 model (Mo et al., 2009). First, we used the fluxes obtained from ^{13}C MFA of the Fy_{DHA} strain for constraining fluxes in the iMM904 model, and then used FBA to predict a flux distribution for this strain. Then, we used MOMA analysis to simulate fluxes in single- and double-gene knockouts

of the Fy_{DHA} strain to study the effects of these knockouts on DHA yields.

We initially observed that even with double-gene knockouts, DHA yields did not improve significantly over those of Fy_{DHA}. This is because MOMA minimizes flux changes between the knocked out and control strains, and the low fluxes of NADPH-supplying reactions in the Fy_{DHA} strain prevented MOMA from identifying knockouts for improving DHA yield. Consequently, we modified the flux distribution of Fy_{DHA} *in silico* such that it had equal fluxes through PPP and glycolysis, but its DHA yield was the same as the measured yield of Fy_{DHA}. We performed subsequent MOMA analysis on this new baseline strain Fy_{DHA}'. Subsequent MOMA analysis found 401 single-gene knockouts with significantly increased DHA flux compared to Fy_{DHA}'. We then used the single-gene knockouts associated with the highest DHA yields to identify double-gene knockouts that would potentially exhibit even higher DHA yields. The MOMA analysis predicted substantially improved DHA yields in these knockouts (Data Sheet 3 in Supplementary Material). In many cases, the increase in DHA yield was accompanied by an expected decrease in biomass yield, because knocking out genes can lead to suboptimal growth as the products of the knocked out genes are no longer available for biomass synthesis. A good gene knockout strategy should improve DHA yields substantially with a modest decrease in biomass growth rate.

The best double-gene knockout strategy predicted by MOMA includes the genes *YKL120W* and *YKL106W*, and improves DHA yield to 4.2 mol % (70-fold over the baseline), while reducing biomass yield by only 2.2-fold compared to Fy_{DHA}'. **Figure 7** presents an interpretation of this strategy. First, FBA predicted that Fy_{DHA}' loses a significant amount of mitochondrial acetyl-CoA to CO_2 through a cyclic, cytosolic pathway that contains most steps of the TCA cycle. Both *YKL120W* and *YKL106W* code for enzymes or transporter proteins in facilitating this cycle. *YKL120W* or *OAC1* codes for an oxaloacetate transporter catalyzing the transport oxaloacetate[c] + H^+[c] ≫ oxaloacetate[m] + H^+[m], where [c] and [m] denote the cytosol and mitochondrion, respectively. *YKL106W* or *AAT1* codes for an aspartate aminotransferase catalyzing the reaction α-ketoglutarate[m] + L-aspartate[m] ≫ L-glutamate[m] + oxaloacetate[m]. Thus, knocking out *YKL120W* and *YKL106W* breaks this cycle while not affecting biomass production, allowing mitochondrial acetyl-CoA to be diverted toward downstream pathways including DHA biosynthesis. Of these two genes, *YKL120W* has a greater effect on reducing flux toward

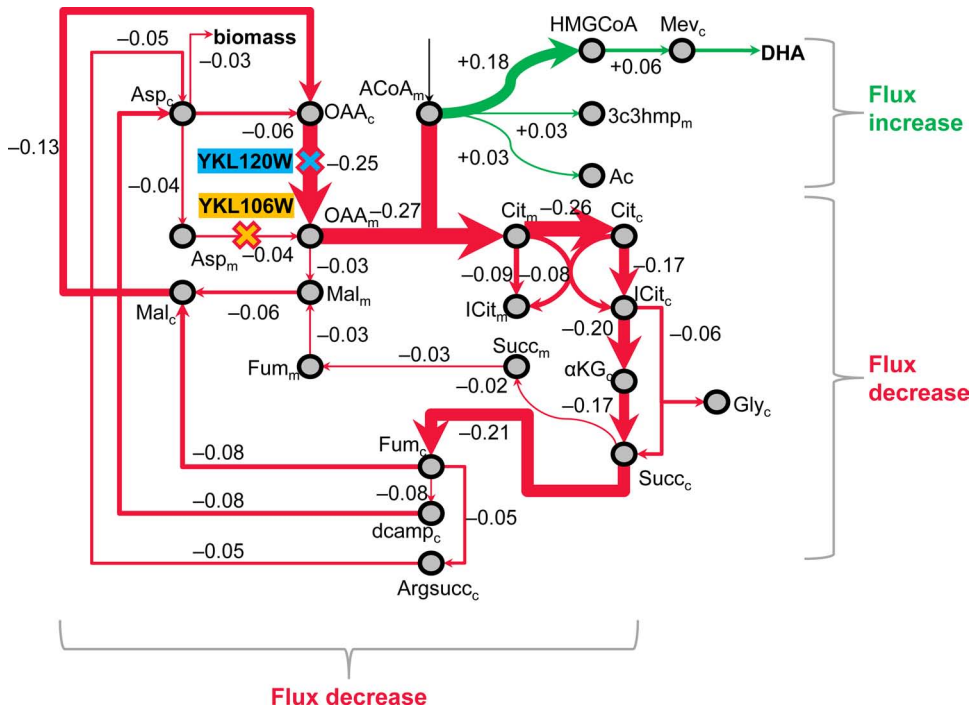


FIGURE 7 | MOMA analysis identifies double-gene knockouts in primary metabolism that funnel carbon toward DHA. As per one MOMA prediction, engineering the Fy_{DHA} strain by knocking out two genes (**1:** *OAC1* or *YKL120W*, which codes for an oxaloacetate transporter and **2:** *AAT1* or *YKL106W*, which codes for an aspartate aminotransferase) should result in 4.2 mol DHA per 100 mol of glucose, which amounts to a ~70-fold increase as compared to our current yield and translates to ~18% of the theoretical yield of DHA from glucose. This metabolic map depicts the mechanism by which these two deletions accomplish the flux redirection. Red arrows indicate decreased flux and green arrows indicate increase in flux relative to our (control) DHA-producing strain; arrow thicknesses and numbers alongside the arrows represent flux differences between the baseline strain Fy_{DHA}' and the double-gene knockout strain are in arbitrary units. On the basis of the ^{13}C isotopomer results, FBA predicted that our control strain loses a significant amount of mitochondrial acetyl-CoA ($ACoA_m$) to CO_2 through a cyclic, cytosolic pathway that contains most steps of the TCA cycle. Knocking out *YKL120W* and *YKL106W* breaks this cycle while not significantly affecting biomass production, thus allowing $ACoA_m$ to be diverted toward downstream pathways including DHA biosynthesis.

mitochondrial oxaloacetate, and also shows up as part of the next best double-gene knockout pair for improving DHA yield (Data Sheet 3 in Supplementary Material).

A limitation of using FBA-type methodologies to predict genetic engineering strategies is their reliance of the assumption of optimized metabolism to predict flux distributions. However, engineered strains may perform sub-optimally. Thus, while FBA is valuable in identifying non-trivial gene targets, it is ambitious to expect the real engineered strain to exhibit yields close to the FBA-predicted yields. Additionally, a recent statistical analysis of previous studies on the production of chemicals by engineered *S. cerevisiae* found that genetic intervention improved product yield only by two- to fourfold in a majority of the 40 studies examined, and that chemicals requiring multiple enzymatic steps were associated with lower yields (Varman et al., 2011). Along the same lines, implementation of the best gene knockout strategy suggested by our study may result in a DHA yield improvement tangibly lower than the predicted 70-fold increase. Very likely, one round of genetic engineering in accordance with FBA predictions may necessitate further rounds of engineering to overcome further bottlenecks to product yield.

CONCLUSION

In summary, we investigated various *in silico* and *in vivo* approaches – ExPa analysis, ^{13}C MFA, FBA, and MOMA analysis – to identify metabolic engineering strategies for improving DHA yield in an engineered yeast strain. Our analyses revealed three major metabolic engineering strategies toward this objective. The first, most obvious strategy is reduction of flux from pyruvate to ethanol and directing this flux toward acetyl-CoA by knocking out fermentative pathways and overexpressing pyruvate dehydrogenase. However, in the absence of further genetic intervention, the available carbon is not likely to be redirected toward the desired pathway. A thorough investigation of the system using detailed ^{13}C MFA, and FBA and MOMA analysis was required to decipher further non-intuitive strategies. A second strategy toward higher DHA yield is the improvement of NADPH availability by overexpressing *ME2* or a rate-limiting step of the oxidative PPP such as *G6PDH2*. Harnessing the oxidative PPP for NADPH supply suffers from the disadvantage that a sixth of the carbon entering this pathway is lost as CO_2; hence, this lowers the yield of DHA significantly. A third strategy is pushing flux from mitochondrial acetyl-CoA toward DHA production. These two strategies are necessary for overcoming the NADPH and mitochondrial acetyl-CoA availability bottlenecks *en route* to DHA biosynthesis. We anticipate that comprehensive analysis of a similar type can be applied to any metabolic system to reveal intuitive and non-intuitive genetic engineering targets for optimizing the production of a target molecule. Such systemic analysis is an important step toward realizing the goal of rational metabolic engineering.

CONTRIBUTIONS

Ashish Misra, Matthew F. Conway, Eddy C. Agbo, and Ganesh Sriram conceived this work; Ashish Misra, Matthew F. Conway, Joseph Johnnie, and Ganesh Sriram designed it. Ashish Misra, Matthew F. Conway, and Joseph Johnnie performed computational analyses; Matthew F. Conway and Ashish Misra performed ^{13}C labeling experiments and ^{13}C MFA. Tabish M. Qureshi performed RT-PCR and DHA assays. Ashish Misra and Ganesh Sriram interpreted the results, prepared data displays, wrote the manuscript and revised the manuscript.

ACKNOWLEDGMENTS

The authors would like to thank Elena Chung and Eli Tung, Department of Chemical and Biomolecular Engineering, University of Maryland, for assistance with experimental analyses. This work was funded by Maryland Industrial Partnerships (MIPS; award no. 4426). Matthew F. Conway was partially supported by a Howard Hughes Medical Institute Undergraduate Research Fellowship from University of Maryland.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Microbiotechnology,_Ecotoxicology_and_Bioremediation/10.3389/fmicb.2013.00200/abstract

REFERENCES

Ahn, W. S., and Antoniewicz, M. R. (2013). Parallel labeling experiments with [1,2-13C]glucose and [U-13C]glutamine provide new insights into CHO cell metabolism. *Metab. Eng.* 15, 34–47. doi: 10.1016/j.ymben.2012.10.001

Alper, H., Jin, Y.-S., Moxley, J. F., and Stephanopoulos, G. (2005). Identifying gene targets for the metabolic engineering of lycopene biosynthesis in *Escherichia coli*. *Metab. Eng.* 7, 155–164. doi: 10.1016/j.ymben.2004.12.003

Asadollahi, M. A., Maury, J., Patil, K. R., Schalk, M., Clark, A., and Nielsen, J. (2009). Enhancing sesquiterpene production in *Saccharomyces cerevisiae* through in silico driven metabolic engineering. *Metab. Eng.* 11, 328–334. doi: 10.1016/j.ymben.2009.07.001

Ba, Q., Zhou, N., Duan, J., Chen, T., Hao, M., Yang, X., et al. (2012). Dihydroartemisinin exerts its anticancer activity through depleting cellular iron via transferrin receptor-1. *PLoS ONE* 7:e42703. doi: 10.1371/journal.pone.0042703

Becker, J., Zelder, O., Häfner, S., Schröder, H., and Wittmann, C. (2011). From zero to hero – design-based systems metabolic engineering of *Corynebacterium glutamicum* for L-lysine production. *Metab. Eng.* 13, 159–168. doi: 10.1016/j.ymben.2011.01.003

Bell, S. L., and Palsson, B. Ø. (2004). Expa: a program for calculating extreme pathways in biochemical reaction networks. *Bioinformatics* 21, 1739 –1740. doi: 10.1093/bioinformatics/bti228

Bertea, C. M., Freije, J. R., van der Woude, H., Verstappen, F. W., Perk, L., Marquez, V., et al. (2005). Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in *Artemisia annua*. *Planta Med.* 71, 40–47. doi: 10.1055/s-2005-837749

Brown, G. D., and Sy, L.-K. (2004). In vivo transformations of dihydroartemisinic acid in *Artemisia annua* plants. *Tetrahedron* 60, 1139–1159. doi: 10.1016/j.tet.2003.11.070

Bruinenberg, P. M., Dijken, J. P. V., and Scheffers, W. A. (1983). A theoretical analysis of NADPH production and consumption in yeasts. *J. Gen. Microbiol.* 129, 953–964. doi: 10.1099/00221287-129-4-953

Burgard, A. P., Pharkya, P., and Maranas, C. D. (2003). Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. *Biotechnol. Bioeng.* 84, 647–657. doi: 10.1002/bit.10803

Chandran, D., Copeland, W. B., Sleight, S. C., and Sauro, H. M. (2008). Mathematical modeling and synthetic biology. *Drug Discov. Today Dis. Models* 5, 299–309. doi: 10.1016/j.ddmod.2009.07.002

Covello, P. S. (2008). Making artemisinin. *Phytochemistry* 69, 2881–2885. doi: 10.1016/j.phytochem.2008.10.001

Eastman, R. T., and Fidock, D. A. (2009). Artemisinin-based combination therapies: a vital tool in efforts to eliminate malaria. *Nat.*

Rev. Microbiol. 7, 864–874. doi: 10.1038/nrmiro2239

Feachem, R. G., Phillips, A. A., Hwang, J., Cotter, C., Wielgosz, B., Greenwood, B. M., et al. (2010). Shrinking the malaria map: progress and prospects. *Lancet* 376, 1566–1578. doi: 10.1016/S0140-6736(10)61270-6

Feng, X., Tang, K.-H., Blankenship, R. E., and Tang, Y. J. (2010). Metabolic flux analysis of the mixotrophic metabolisms in the green sulfur bacterium *Chlorobaculum tepidum*. *J. Biol. Chem.* 285, 39544–39550. doi: 10.1074/jbc.M110.162958

Ferreira, J. F. S., Luthria, D. L., Sasaki, T., and Heyerick, A. (2010). Flavonoids from *Artemisia annua* L. as antioxidants and their potential synergism with artemisinin against malaria and cancer. *Molecules* 15, 3135–3170. doi: 10.3390/molecules15053135

Gao, W., Xiao, F., Wang, X., and Chen, T. (2013). *Artemisinin induces* A549 cell apoptosis dominantly via a reactive oxygen species-mediated amplification activation loop among caspase-9, -8 and -3. *Apoptosis* doi: 10.1007/s10495-013-0857-z [Epub ahead of print].

Grafahrend-Belau, E., Schreiber, F., Koschutzki, D., and Junker, B. H. (2009). Flux balance analysis of barley seeds: a computational approach to study systemic properties of central metabolism. *Plant Physiol.* 149, 585–598. doi: 10.1104/pp.108.129635

Hale, V., Keasling, J., Renninger, N., and Diagana, T. (2007). "Microbially derived artemisinin: a biotechnology solution to the global problem of access to affordable antimalarial drugs," in *Defining and Defeating the Intolerable Burden of Malaria III: Progress and Perspectives,* eds J. Breman, M. Alilio, and N. White (Northbrook: American Society of Tropical Medicine and Hygiene).

Hynes, M. J., and Murray, S. L. (2010). ATP-citrate lyase is required for production of cytosolic acetyl coenzyme A and development in *Aspergillus nidulans*. *Eukaryot. Cell* 9, 1039–1048. doi: 10.1128/EC.00080-10

Kindermans, J.-M., Pilloy, J., Olliaro, P., and Gomes, M. (2007). Ensuring sustained ACT production and reliable artemisinin supply. *Malar. J.* 6, 125. doi: 10.1186/1475-2875-6-125

Kwon, D.-H., Kim, M.-D., Lee, T.-H., Oh, Y.-J., Ryu, Y.-W., and Seo, J.-H. (2006). Elevation of glucose 6-phosphate dehydrogenase activity increases xylitol production in recombinant *Saccharomyces cerevisiae*. *J. Mol. Catal. B Enzym.* 43, 86–89. doi: 10.1016/j.molcatb.2006.06.014

Lenihan, J. R., Tsuruta, H., Diola, D., Renninger, N. S., and Regentin, R. (2008). Developing an industrial artemisinic acid fermentation process to support the cost-effective production of antimalarial artemisinin-based combination therapies. *Biotechnol. Prog.* 24, 1026–1032. doi: 10.1002/btpr.27

Lévesque, F., and Seeberger, P. H. (2012). Continuous-flow synthesis of the anti-malaria drug artemisinin. *Angew. Chem. Int. Ed. Engl.* 51, 1706–1709. doi: 10.1002/anie.201107446

Liu, N. Q., Schuehly, W., von Freyhold, M., and van der Kooy, F. (2011). A novel purification method of artemisinin from *Artemisia annua*. *Ind. Crops Prod.* 34, 1084–1088. doi: 10.1016/j.indcrop.2011.03.023

Llaneras, F., and Picó, J. (2010). Which metabolic pathways generate and characterize the flux space? A comparison among elementary modes, extreme pathways and minimal generators. *J. Biomed. Biotechnol.* 2010, 753904 doi: 10.1155/2010/753904

Mo, M. L., Palsson, B. O., and Herrgård, M. J. (2009). Connecting extracellular metabolomic measurements to intracellular flux states in yeast. *BMC Syst. Biol.* 3:37. doi: 10.1186/1752-0509-3-37

Murphy, T. A., Dang, C. V., and Young, J. D. (2013). Isotopically nonstationary 13C flux analysis of Myc-induced metabolic reprogramming in B-cells. *Metab. Eng.* 15, 206–217. doi: 10.1016/j.ymben.2012.07.008

Nargund, S., and Sriram, G. (2013). Designer labels for plant metabolism: statistical design of isotope labeling experiments for improved quantification of flux in complex plant metabolic networks. *Mol. Biosyst.* 9, 99–112. doi: 10.1039/c2mb25253h

Nissen, T. L., Anderlund, M., Nielsen, J., Villadsen, J., and Kielland-Brandt, M. C. (2001). Expression of a cytoplasmic transhydrogenase in *Saccharomyces cerevisiae* results in formation of 2-oxoglutarate due to depletion of the NADPH pool. *Yeast* 18, 19–32. doi: 10.1002/1097-0061(200101)18:1

O'Meara, W. P., Mangeni, J. N., Steketee, R., and Greenwood, B. (2010). Changes in the burden of malaria in sub-Saharan Africa. *Lancet Infect. Dis.* 10, 545–555. doi: 10.1016/S1473-3099(10)70096-7

Orth, J. D., Thiele, I., and Palsson, B. O. (2010). What is flux balance analysis? *Nat. Biotechnol.* 28, 245–248. doi: 10.1038/nbt.1614

Paddon, C. J., Westfall, P. J., Pitera, D. J., Benjamin, K., Fisher, K., McPhee, D., et al. (2013). *High-level semi-synthetic production of the potent antimalarial artemisinin.* Nature advance online publication. Available at: http://www.nature.com/nature/journal/vaop/ncurrent/full/nature12051.html [accessed April 14, 2013].

Palsson, B. O. (2006). *Systems Biology: Properties of Reconstructed Networks.* New York: Cambridge University Press.

Papini, M., Nookaew, I., Uhlén, M., and Nielsen, J. (2012). *Scheffersomyces stipitis*: a comparative systems biology study with the Crabtree positive yeast *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 11, 136. doi: 10.1186/1475-2859-11-136

Park, J. H., Lee, K. H., Kim, T. Y., and Lee, S. Y. (2007). Metabolic engineering of *Escherichia coli* for the production of l-valine based on transcriptome analysis and in silico gene knockout simulation. *Proc. Natl. Acad. Sci. U.S.A.* 104, 7797–7802. doi: 10.1073/pnas.0702609104

Pharkya, P., Burgard, A. P., and Maranas, C. D. (2004). OptStrain: a computational framework for redesign of microbial production systems. *Genome Res.* 14, 2367–2376. doi: 10.1101/gr.2872004

Price, R. N., and Douglas, N. M. (2009). Artemisinin combination therapy for malaria: beyond good efficacy. *Clin. Infect. Dis.* 49, 1638–1640. doi: 10.1086/647947

Ranganathan, S., Suthers, P. F., and Maranas, C. D. (2010). Optforce: an optimization procedure for identifying all genetic manipulations leading to targeted overproductions. *PLoS Comput. Biol.* 6: e1000744. doi: 10.1371/journal.pcbi.1000744

Ranganathan, S., Tee, T. W., Chowdhury, A., Zomorrodi, A. R., Yoon, J. M., Fu, Y., et al. (2012). An integrated computational and experimental study for overproducing fatty acids in *Escherichia coli*. *Metab. Eng.* 14, 687–704. doi: 10.1016/j.ymben.2012.08.008

Ro, D.-K., Paradise, E. M., Ouellet, M., Fisher, K. J., Newman, K. L., Ndungu, J. M., et al. (2006). Production of the antimalarial drug precursor artemisinic acid in engineered yeast. *Nature* 440, 940–943. doi: 10.1038/nature04640

Romano, A. H., and Conway, T. (1996). Evolution of carbohydrate metabolic pathways. *Res. Microbiol.* 147, 448–455. doi: 10.1016/0923-2508(96)83998-2

Rydén, A.-M., Ruyter-Spira, C., Quax, W. J., Osada, H., Muranaka, T., Kayser, O., et al. (2010). The molecular cloning of dihydroartemisinic aldehyde reductase and its implication in artemisinin biosynthesis in *Artemisia annua*. *Planta Med.* 76, 1778–1783. doi: 10.1055/s-0030-1249930

Schellenberger, J., Que, R., Fleming, R. M. T., Thiele, I., Orth, J. D., Feist, A. M., et al. (2011). Quantitative prediction of cellular metabolism with constraint-based models: the COBRA Toolbox v2.0. *Nat. Protoc.* 6, 1290–1307. doi: 10.1038/nprot.2011.308

Schilling, C. H., Letscher, D., and Palsson, B. Ø. (2000). Theory for the systemic definition of metabolic pathways and their use in interpreting metabolic function from a pathway-oriented perspective. *J. Theor. Biol.* 203, 229–248. doi: 10.1006/jtbi.2000.1073

Schilling, C. H., and Palsson, B. O. (2000). Assessment of the metabolic capabilities of *Haemophilus influenzae* Rd through a genome-scale pathway analysis. *J. Theor. Biol.* 203, 249–283. doi: 10.1006/jtbi.2000.1088

Segrè, D., Vitkup, D., and Church, G. M. (2002). Analysis of optimality in natural and perturbed metabolic networks. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15112–15117. doi: 10.1073/pnas.232349399

Sriram, G., Fulton, D. B., Iyer, V. V., Peterson, J. M., Zhou, R., Westgate, M. E., et al. (2004). Quantification of compartmented metabolic fluxes in developing soybean embryos by employing biosynthetically directed fractional 13C labeling, two-dimensional [13C, 1H] nuclear magnetic resonance, and comprehensive isotopomer balancing. *Plant Physiol.* 136, 3043–3057. doi: 10.1104/pp.104.050625

Sriram, G., Rahib, L., He, J.-S., Campos, A. E., Parr, L. S., Liao, J. C., et al. (2008). Global metabolic effects of glycerol kinase overexpression in rat hepatoma cells. *Mol. Genet. Metab.* 93, 145–159. doi: 10.1016/j.ymgme.2007.09.008

Stephanopoulos, G. N., Aristidou, A. A., and Nielsen, J. (1998). *Metabolic Engineering: Principles and Methodologies,* 1st Edn, Waltham: Academic Press.

Sy, L.-K., and Brown, G. D. (2002). The mechanism of the spontaneous autoxidation of dihydroartemisinic acid. *Tetrahedron* 58, 897–908. doi: 10.1016/S0040-4020(01)01193-0

Szyperski, T. (1995). Biosynthetically directed fractional 13C-labeling

of proteinogenic amino acids. An efficient analytical tool to investigate intermediary metabolism. *Eur. J. Biochem.* 232, 433–448. doi: 10.1111/j.1432-1033.1995.tb20829.x

Szyperski, T. (1998). ^{13}C-NMR, MS and metabolic flux balancing in biotechnology research. *Q. Rev. Biophy.* 31, 41–106.

Teoh, K., Polichuk, D., Reed, D., Nowak, G., and Covello, P. (2006). *Artemisia annua* L. *(Asteraceae)* trichome-specific cDNAs reveal *CYP*71AV1, a cytochrome P450 with a key role in the biosynthesis of the antimalarial sesquiterpene lactone artemisinin. *FEBS Lett.* 580, 1411–1416. doi: 10.1016/j.febslet.2006.01.065

Teste, M.-A., Duquenne, M., François, J. M., and Parrou, J.-L. (2009). Validation of reference genes for quantitative expression analysis by real-time RT-PCR in *Saccharomyces cerevisiae*. *BMC Mol. Biol.* 10:99. doi: 10.1186/1471-2199-10-99

Varman, A. M., Xiao, Y., Leonard, E., and Tang, Y. J. (2011). Statistics-based model for prediction of chemical biosynthesis yield from *Saccharomyces cerevisiae*. *Microb. Cell Fact.* 10, 45. doi: 10.1186/1475-2859-10-45

Wallaart, T. E., Pras, N., Beekman, A. C., and Quax, W. J. (2000). Seasonal variation of artemisinin and its biosynthetic precursors in plants of *Artemisia annua* of different geographical origin: proof for the existence of chemotypes. *Planta Med.* 66, 57–62. doi: 10.1055/s-2000-11115

Weathers, P., Elkholy, S., and Wobbe, K. (2006). Artemisinin: the biosynthetic pathway and its regulation in *Artemisia annua*, a terpenoid-rich species. *In Vitro Cell. Dev. Biol. Plant* 42, 309–317. doi: 10.1079/IVP2006782

Westfall, P. J., Pitera, D. J., Lenihan, J. R., Eng, D., Woolard, F. X., Regentin, R., et al. (2012). Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. *Proc. Natl. Acad. Sci. U.S.A.* 109, E111–E118. doi: 10.1073/pnas.1110740109

Wiback, S. J., and Palsson, B. O. (2002). Extreme pathway analysis of human red blood cell metabolism. *Biophys. J.* 83, 808–818. doi: 10.1016/S0006-3495(02)75210-7

Wiechert, W. (2001). 13C metabolic flux analysis. *Metab. Eng.* 3, 195–206. doi: 10.1006/mben.2001.0187

World Health Organization (2010). *World Malaria Report 2010*. WHO. Available at: http://www.who.int/malaria/world_malaria_report_2010/en/index.html [accessed April 14, 2013].

Zhang, Y., Nowak, G., Reed, D. W., and Covello, P. S. (2011). The production of artemisinin precursors in tobacco. *Plant Biotechnol. J.* 9, 445–454. doi: 10.1111/j.1467-7652.2010.00556.x

Zhang, Y., Teoh, K. H., Reed, D. W., Maes, L., Goossens, A., Olson, D. J. H., et al. (2008). The molecular cloning of artemisinic aldehyde Δ11(13) reductase and its role in glandular trichome-dependent biosynthesis of artemisinin in *Artemisia annua*. *J. Biol. Chem.* 283, 21501–21508. doi: 10.1074/jbc.M803090200

Zhang, Y. J., Gallis, B., Taya, M., Wang, S., Ho, R. J. Y., and Sasaki, T. (2013). pH-responsive artemisinin derivatives and lipid nanoparticle formulations inhibit growth of breast cancer cells in vitro and induce down-regulation of HER family members. *PLoS ONE* 8:e59086. doi: 10.1371/journal.pone.0059086

Zheng, Y., and Sriram, G. (2010). Mathematical modeling: bridging the gap between concept and realization in synthetic biology. *J. Biomed. Biotechnol.* 2010, 541609. doi: 10.1155/2010/541609

Zhu, C., and Cook, S. P. (2012). A concise synthesis of (+)-artemisinin. *J. Am. Chem. Soc.* 134, 13577–13579. doi: 10.1021/ja3061479

Conflict of Interest Statement: Eddy C. Agbo, Anne M. Derrick and Bao Lige are employed with Fyodor Biotechnologies Corp., which has financial interest in the commercialization of antimalarial therapeutics or their precursors. The other authors have no conflict of interest.

Received: 25 April 2013; accepted: 25 June 2013; published online: 26 July 2013.
Citation: Misra A, Conway MF, Johnnie J, Qureshi TM, Derrick AM, Agbo EC and Sriram G (2013) Metabolic analyses elucidate non-trivial gene targets for amplifying dihydroartemisinic acid production in yeast. Front. Microbiol. 4:200. doi: 10.3389/fmicb.2013.00200
This article was submitted to Frontiers in Microbiotechnology, Ecotoxicology and Bioremediation, a specialty of Frontiers in Microbiology.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 23 September 2013
doi: 10.3389/fmicb.2013.00285

Chemical synthetic biology: a mini-review

Cristiano Chiarabelli, Pasquale Stano and Pier Luigi Luisi*

Science Department, University of Roma Tre, Rome, Italy

Edited by:
Weiwen Zhang, Tianjin University, China

Reviewed by:
Martin Siemann-Herzberg, University Stuttgart, Germany
Jiangxin Wang, Arizona State University, USA
Lei Chen, Tianjin University, China

***Correspondence:**
Pier Luigi Luisi, Science Department, University of Roma Tre, Viale Guglielmo Marconi 446, 00146 Rome, Italy
e-mail: luisi@mat.ethz.ch

Chemical synthetic biology (CSB) is a branch of synthetic biology (SB) oriented toward the synthesis of chemical structures alternative to those present in nature. Whereas SB combines biology and engineering with the aim of synthesizing biological structures or life forms that do not exist in nature – often based on genome manipulation, CSB uses and assembles biological parts, synthetic or not, to create new and alternative structures. A short epistemological note will introduce the theoretical concepts related to these fields, whereas the text will be largely devoted to introduce and comment two main projects of CSB, carried out in our laboratory in the recent years. The "Never Born Biopolymers" project deals with the construction and the screening of RNA and peptide sequences that are not present in nature, whereas the "Minimal Cell" project focuses on the construction of semi-synthetic compartments (usually liposomes) containing the minimal and sufficient number of components to perform the basic function of a biological cell. These two topics are extremely important for both the general understanding of biology in terms of function, organization, and development, and for applied biotechnology.

Keywords: liposomes, minimal cell, protein folding, random sequence, RNA stability, synthetic biology, synthetic cells

INTRODUCTION

Synthetic biology (SB) is one of the most attractive new research areas in biology, and traditionally deals with the bioengineering of new forms of life (generally, but not only, unicellular organisms), which do not exist in nature (Endy, 2005; Andrianantoandro et al., 2006). This is done with the aim of taking the control of their genetic–metabolic network in a predictable way, so that a specific chemical can be produced (for pharmaceutical or biofuel applications), or to use them as biosensors, or for other specific pre-defined purposes. As it happened with the publication of the first self-reproducing organism created by implanting a synthetic genome in a pre-existing cell (Gibson et al., 2010), ethical issues are often related to the bioengineering of natural living systems, especially as soon as the technical progresses allow the manipulation of ever-complex organisms.

On the other hand, there is a second "soul" of SB. The second soul, as we have defined in other articles (Luisi, 2007; Chiarabelli et al., 2009, 2012), and in a book (Luisi and Chiarabelli, 2011), has to do with the idea of synthesizing in the lab biological structures which do not exist in nature, and without the use of gene manipulation – but rather with the tools of simple chemistry manipulation. We have dubbed this second line of thought as "chemical synthetic biology" (CSB). CSB is concerned with the synthesis of chemical structures such as proteins, nucleic acids, vesicular forms, and others which do not exist in nature. This is done for two reasons: first, CSB approaches allow understanding the roots of biological function and organization, because it allows to test hypotheses about the reasons according to which biology works. Second, as for SB, it might have a tremendous impact on biotechnology, generating an entire set of tools for nanomedicine, diagnostic, drug/gene delivery, bioengineering, biosensoring, etc., based on artificial molecules. Examples of CSB approaches span from searching for nucleic acid alternatives, such as furanose-based DNA (Bolli et al., 1997), peptide-nucleic acids (PNAs; Egholm et al., 1993) and their conjugates (Alajlouni and Seleem, 2013), to the novel work on synthetic genetic polymers (XNA) capable of heredity and evolution (Pinheiro et al., 2012), and to synthetic genetic codes (Wong and Xue, 2011); and, on the other hand, to proteins composed by random amino acid sequences (Chiarabelli et al., 2006a,b), or from a subset of amino acids (Doi et al., 2005), or other unnatural building blocks, or by combinatorial approaches (Urvoas et al., 2012). In addition to these examples of CSB of "parts," there is a flourishing research on CSB of "systems," and in particular that one focused on the construction of synthetic cells (Luisi et al., 2006b), whose achievement represents the most ambitious goal of CSB.

In this mini-review we will focus on two CSB projects that we are currently developing, namely, the "Never Born RNAs (NBRNA)/proteins" [Never Born Biopolymers (NBB)] and the "Minimal Cell." Due to space limitation we cannot present several interesting and original reports on related subjects, and we simply recapitulate our main results and the comments on them, which have been published in previous reviews (Luisi et al., 2006b; Chiarabelli et al., 2009, 2012), with the addition of some most recent advancements, when available. The NBB project is related to the question why and how the existing protein or RNA structures have been selected out, with the underlying question whether they have something very particular from the structural or thermodynamic point of view (for example, the folding). The Minimal Cell project is focused on the construction of cell models having the minimal and sufficient number of components to be defined as living. For this purpose, liposomes are used as shell membranes, and attempts are made to introduce in the interior a minimal genome. The current state-of-the-art in this research is

the synthesis of functional protein inside liposomes, and the study of the corresponding artificial cell reactivity. Before presenting in more details these two projects, however, a short paragraph on general epistemic considerations on SB and CSB is presented.

SHORT EPISTEMIC REMARKS

Theoretical thinking is too often neglected in modern biotechnological education programs, and contemporary researches seldom consider their studies from the epistemic viewpoint. Quite specific epistemic features characterize SB and CSB approaches. Classical SB, funded on the concept of bioengineering, is a *teleological* enterprise. In fact, as when an engineer plans an aircraft, a synthetic biologist plans (and later builds) a genetically modified organism for a specific purpose. This is generally done by removing, adding, or exchanging biological parts of an organism. Although *reductionism* can be guessed as a guiding principle for such operation, actually, in order to work, the new modules must be *integrated* with the other pre-existing biochemical networks, so that a *systemic* thinking is required. Remarkably, whereas teleology dominates SB, *teleonomy* features natural biological evolution.

On the other hand, CSB often faces dichotomies as *contingency* and *determinism*. For example, the study of random proteins for determining whether natural proteins are the only possible function-bearing structures, answer the question whether life – as we know it – has followed a deterministic path or it is rather a product of contingent, punctual, historical unrepeatable events. The philosophical implication of constructing synthetic living cells from non-living parts deals instead with the concepts of *emergent* properties – those properties that cannot be reduced to the properties of the parts composing the system. In addition, this issue directly brings about the problems connected with the deep understanding of "what is life," its definition, and its recognition. In this respect, the Maturana–Varela concepts of *autopoiesis* and *cognition* (Varela et al., 1974) constitute a powerful and elegant theoretical framework. More detailed epistemological considerations can be found in Luisi (2011).

NEVER BORN BIOPOLYMERS

The idea on the NBB moves from the observation that the extant collection of proteins and RNA molecules, currently present in living organisms, are only a minor part of the all theoretical possible sequences (de Duve, 2002; Luisi, 2003; Xia and Levitt, 2004; Chiarabelli et al., 2006a,b, 2011; Dryden et al., 2008; LaBean et al., 2011). For example, the number of possible different peptides of 50 amino acid residues synthesized using the 20 natural amino acids is equal to 20^{50}, i.e., 10^{65}. The difference between the number of possible proteins and the number of those actually present in living organisms is comparable to the difference existing between a grain of sand and the entire Sahara desert (Luisi, 2006). The number of possible different proteins becomes even greater if we take into account the living organisms, where the average length of proteins is much greater (Lipman et al., 2002). In the same way these considerations can be broadened to the RNAs.

On the basis of this observation, the question whether a functionality is a common feature or a rare result of natural selection is of the utmost importance to elucidate the role of biopolymers in the origin of Life and to fully exploit its biological potential. As an obvious consequence, it has been decided to explore the protein and RNA sequence spaces in search of random molecules presenting a stable fold, by assays set up in our laboratory (Chiarabelli et al., 2006b; De Lucrezia et al., 2006b; Anella et al., 2011).

In order to be proteins, amino acid chains have to be folded; the Never Born Proteins (NBP) project is directed toward the discrimination between folded and unfolded chains in a random ensemble of synthetic polypeptides. The approach involves a "total randomization" with no bias toward any given structural or functional property, leading almost necessarily to novel proteins not present in nature, the NBP (Chiarabelli et al., 2006b; Luisi et al., 2006a). The experimental procedure developed to pursue this goal is based on the concept that folded polypeptides are more protected against digestion by a protease than unfolded ones. The first attempts to combine a library-production method with the selection of folded protein by proteolysis belong to Kristensen and Winter's (1998). In that case the starting point has been the selection of variants with improved properties from known extant proteins. The originality of our procedure, with respect to literature, lies in the library itself, being made by *de novo* completely random sequences not present in nature and in the insertion of a specific short fixed sequence within a total random polypeptide sequence to create the library. Such short sequence is specifically recognized, so that folded peptides have less chances to be enzymatically digested than unfolded ones. The NBP selection method links the proteolytic resistance with the selective recovery, useful to get new folded peptides (**Figure 1**). With our approach, a library of 50 residues long random peptides has been produced and screened (**Figure 1**, bottom), and the work demonstrates that the selection of folded random proteins is feasible (Chiarabelli et al., 2006b, 2009, 2012; Luisi and Chiarabelli, 2011). Preliminary circular dichroism studies further support our conclusions based on limited digestion. Furthermore, because the NBP Project born on the basic idea to test whether the extant proteins have no extraordinary physical properties at all, it is reasonable to say that the natural proteins could have been selected by chance among an enormous number of possibilities of quite similar compounds. They came out by "chance," and it happened that they were capable of fostering cellular life.

Confirming the idea that protein fold is a general feature of relatively long polypeptide chains there are a lot of additional studies. Ribosomal display, which allow the screening, selection, and evolution of functional proteins, was used by Hanes and Plückthun (1997) to investigate antibody sequences variations, by maintaining however the full antigene-binding capacity. Recent directions in ribosome display technologies are illustrated by Plückthun (2012). Yomo and colleagues (Yamauchi et al., 2002) investigated the possibility of selecting an active enzyme by *in vitro* evolution starting from the very limited number of 10 random sequences, showing that a significant fraction of all possible sequences may have functions, at least binding activity, using a simple selection method applied to relatively short random proteins (about 140 amino acids). In a second experiment, starting from the same library as before, they also demonstrated the evolvability of both

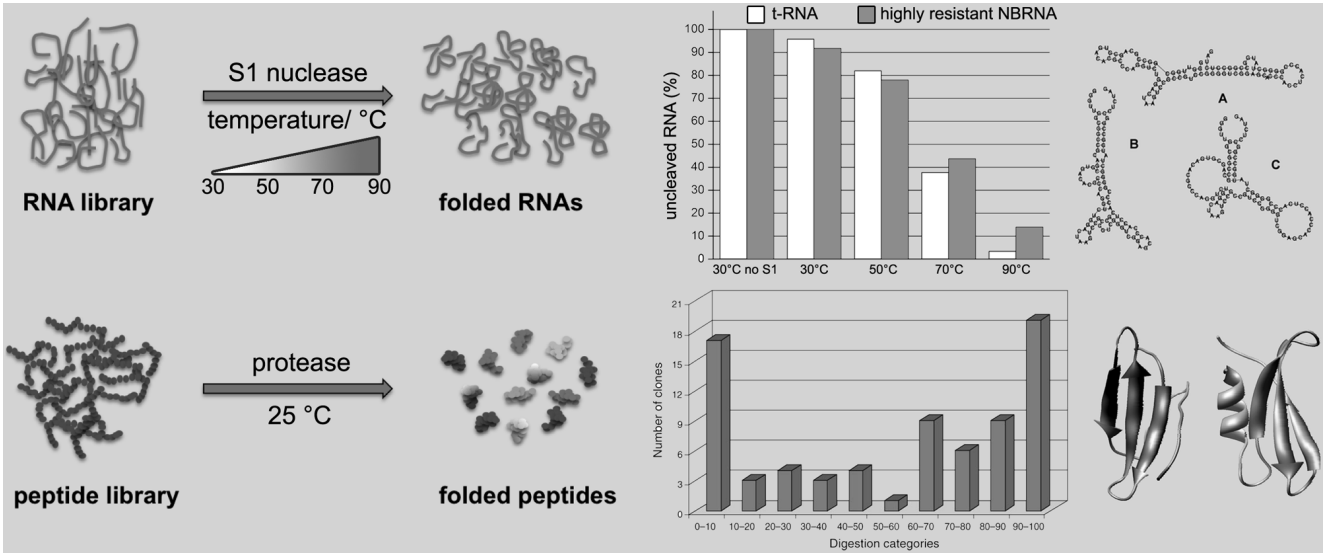


FIGURE 1 | Schematic representation of the random RNAs and peptides selection procedures. An RNA library, containing folded and unfolded (or not well-folded) sequences, is treated with S1 nuclease at various temperatures. In the corresponding graphic the residual amount (%) of a tRNA as control (white bars) in comparison to a highly resistant random RNA (NBRNA; gray bars) at different temperatures. The corresponding secondary structure predictions for the NBRNA at different temperatures are shown (A: $24 \leq T \leq 63°C$; B: $64.5 \leq T \leq 72°C$; C: $74 \leq T \leq 91°C$). Only folded RNA sequences are preserved, whereas the other are digested. Similarly, a peptide library, containing folded and unfolded (or not well-folded) sequences, is treated with a protease. Only folded peptide sequences are recovered, whereas the unfolded one are digested. In the graphic [reproduced from Chiarabelli et al. (2006b), with the permission of Wiley] the tested peptides are divided in categories according to the different levels of resistance against enzymatic digestion shown by each peptide sequence; for example, class 0–10 displays a digestion between 0 and 10%. Two tertiary structure predictions examples of folded resistant NBP are shown.

a single soluble arbitrary sequence toward a structure with a biological function, for example molecular folding and recognition (Hayashi et al., 2003), and a insoluble random polypeptide toward a soluble one (Ito et al., 2003). Importantly, a well-structured molecular fold is a pre-requisite for more interesting catalytic functions.

Even the idea to use a binary pattern of polar/non-polar amino acid scheme periodicity (Kamtekar et al., 1993; Wei et al., 2003; Hecht et al., 2004) or a subset of amino acids (glutamine, leucine, and arginine; Davidson et al., 1995) to construct *de novo* libraries with stable three-dimensional structures demonstrate that the frequency of folding in random libraries is higher than expected, and that it is possible to select proteins with desired characteristics. Unfortunately, in the last years we do not have more examples on the study of totally random proteins with respect to folding and structure analysis.

Novel reports have highlighted the role of combinatorial design to produce and select 102 residues long proteins with capability of sustaining *E. coli* growth (Fisher et al., 2011), as well as the importance of *in silico* approaches for the exploration of the sequence space (Prymula et al., 2009; Cossio et al., 2010; De Lucrezia et al., 2012).

In a similar way as described for the NBP has been possible to investigate the random RNA (NBRNA) characteristics. Knowing the firm relation between structure and function of biological molecules we decided to precede the random RNAs functional exploration with some structural studies using the RNA Foster (RNA folding stability test). The assay allows to determine the presence and thermal stability of secondary domains in RNA molecules by coupling enzymatic digestion with temperature denaturation (**Figure 1**, top). The Foster is capable to quantitatively determine the fraction of folded RNAs in function of temperature (De Lucrezia et al., 2006a). It employs a specific nuclease (S1; Vogt, 1973) to cleave at different temperatures single-strand RNA sequences, monitoring the presence of double-stranded domains and indirectly any possible structure. In fact, folded RNAs are more resistant to S1 nuclease than unfolded ones, namely the latter are degraded faster than the former. In addition, we exploited the capability of nuclease S1 to work over a broad range of temperatures to probe RNA secondary domain stability at different conditions. In fact, an increase in temperature destabilizes the RNA fold, inducing either global or local unfolding. Consequently, the RNA becomes susceptible to nuclease attack and is readily degraded (De Lucrezia et al., 2006b; Anella et al., 2011). In few words the most stable sequences at high temperature will be those with a more stable secondary and possibly tertiary structure.

So far, the most general result of our studies lies in the demonstration that RNAs have the capacity to fold into compact secondary structures, even in absence of selective pressure (Anella et al., 2011). This confirm our hypothesis that molecules involved in nowadays life do not have exclusive features as far as the ability to adopt a stable fold is concerned.

Unexpectedly, one of the sequence analyzed has a stability even higher than a tRNA, used as control, at 70°C, with an approximately melting temperature higher than 80°C. These results are

absolutely outstanding and can be used to provide directions and suggestions for further studies concerning the functional properties of RNAs, in the early evolution scenario as well. In our laboratory we are trying to study more in detail the structure characteristic of NBRNA using more complex libraries and secondary and tertiary structure predictions as well as spectroscopic methods.

SEMI-SYNTHETIC MINIMAL CELLS

When we look at modern living cells it is difficult not to remain astonished by the beautiful complexity of thousands of intricate genetic–metabolic complexes occurring in the tiny cellular environment, and we ask how this complexity was originated by spontaneous generation and later shaped by the evolution. However, there was a time when cells were much simpler that modern one, and still being "alive," i.e., capable of self-maintenance, self-reproduction, and with the capacity of evolve. How can we study such simple and primitive cells? These biological entities do not exist any more in nature, and therefore the synthetic (constructive) approach – typical of CSB – is the only way to get insights into the physical and chemical constraints that ruled the emergence of early cells in ancient times. However, any attempt to construct a complex system such as a living – yet primitive – cell needs the knowledge of the minimal biological organization that characterizes life. In this context, the theory of autopoiesis, introduced in the 1970s by Humberto Maturana and Francisco Varela (Varela et al., 1974), is of great help. Autopoiesis (self-production) says that a living cell is a physical object (composed by reactive molecules), which: (1) distinguishes itself from the environment thank to a well-defined semi-permeable boundary, (2) encloses networks of reactions that transform the precursors available in the environment in the same molecules that form the reaction network, (3) despite the continuous turnover of its molecular constituents, the autopoietic system maintain its own identity in terms of (dynamic and spatial) organization. Thanks to these theoretical guidelines, can we build minimal autopoietic cells in the laboratory? The research project called "Minimal Cell" aims at answering this question in several ways. One possibility is the construction of minimal cells basing on allegedly primitive molecules, such as fatty acids, ribozymes, simple primitive peptides. A simple ribozyme-in-liposome model has been proposed (Szostak et al., 2001), but this route, although fascinating, is hampered by the very limited reactivity displayed by known ribozymes, and by the lack of viable catalytic short peptides (despite some notable cases, such as the Ser-His catalysis, see Gorlero et al., 2009; Wieczorek et al., 2012; Adamala and Szostak, 2013), and notwithstanding the instead rather rich and well-characterized properties of fatty acid vesicles (Walde et al., 1994; Berclaz et al., 2001; Stano et al., 2006; Zhu and Szostak, 2009). Another possibility deals with totally synthetic systems, based for example on polymers, PNAs, elaborated transition metal catalysts, etc. (Rasmussen et al., 2004), aiming at creating a totally synthetic minimal cell. Water-in-oil emulsion droplets, where compartmentalized reactions can be carried out, as pioneered by Tawfik and Griffiths (1998), are also alternative synthetic systems to build cellular models, but they lack of the semi-permeable lipid bilayer that characterizes biological cells.

We believe that a third way is actually the most fruitful one, because it has been successfully used to shed light on the emergence primitive cells, and at the same time it promises remarkable applications in biotechnology (Jewett and Forster, 2010), nanomedicine (Leduc et al., 2007), drug delivery, and in modern bio-chem based information/communication technologies (ICTs; Stano et al., 2012). This is the "semi-synthetic" approach (Luisi et al., 2006b; Stano et al., 2011), consisting in the construction of cell-like structures by encapsulating the minimal number of available DNA, RNA, proteins inside liposomes (**Figure 2**). Currently this approach stems from the convergence of liposome technology and cell-free technology and it is investigated by several groups, as witnessed by the richness of the recently published original work (Budin et al., 2012; Hamada and Yoshikawa, 2012; Maeda et al., 2012; Kobori et al., 2013; Lentini et al., 2013; Nourian and Danelon, 2013). Several important concepts and technical issues play essential roles in semi-synthetic minimal cells, such as (1) life as an emergent property, (2) the minimal genome, (3) the production of functional water-soluble and membrane proteins, (4) the conditions for core-and-shell self-reproduction, (5) "spontaneous" versus "directed" preparation methods, and many others.

For example, several studies revealed that the essential housekeeping functions of living cells are encoded in a minimal number of genes, around 200 (Gil et al., 2004), as derived from comparative genomic analysis carried out on the smallest living organisms (often intracellular parasites or symbionts). About one half of the minimal genome is devoted to the specification of proteins and RNAs dedicated to the protein synthesis, without doubts the most important process, together the nucleic acid replication and the lipid synthesis (but consider that the latter two processes are only possible thank to enzymes). It is therefore not quite surprising that the current state-of-the-art in minimal cell research focuses on protein synthesis inside liposomes. Several advancements have been reported from the time of the pioneer report from Oberholzer et al. (1999). In particular, proteins like the green fluorescent protein, β-galactosidase, Qβ-replicase, α-hemolysin, and T7 RNA polymerase have been successfully synthesized inside liposomes, according to a variety of experimental design, aimed at functionalizing synthetic cells with pores, RNA-replication, two-steps genetic cascades, etc. (for a review, see Stano et al., 2011). Most of the more recent studies are carried out by incorporating – inside liposomes – of the minimal number (ca. 80) of transcription/translation macromolecules, i.e., the PURE system (Shimizu et al., 2001). The issue of lipid production has been faced by reconstituting the "lipid-salvage-pathway" inside liposomes, a four-steps mini-metabolic route that converts glycerol-3-phosphate into phosphatidylcholine (Schmidli et al., 1991; Kuruma et al., 2009). The low yield associated to the involvement of the membrane enzymes that catalyze these transformations has prevented efficient lipid production, so that the direct observation of a spontaneous growth and division due to intraliposome lipid production is still missing.

There are, however, novel and intriguing advancements in exploring the emergence of minimal cells from separated

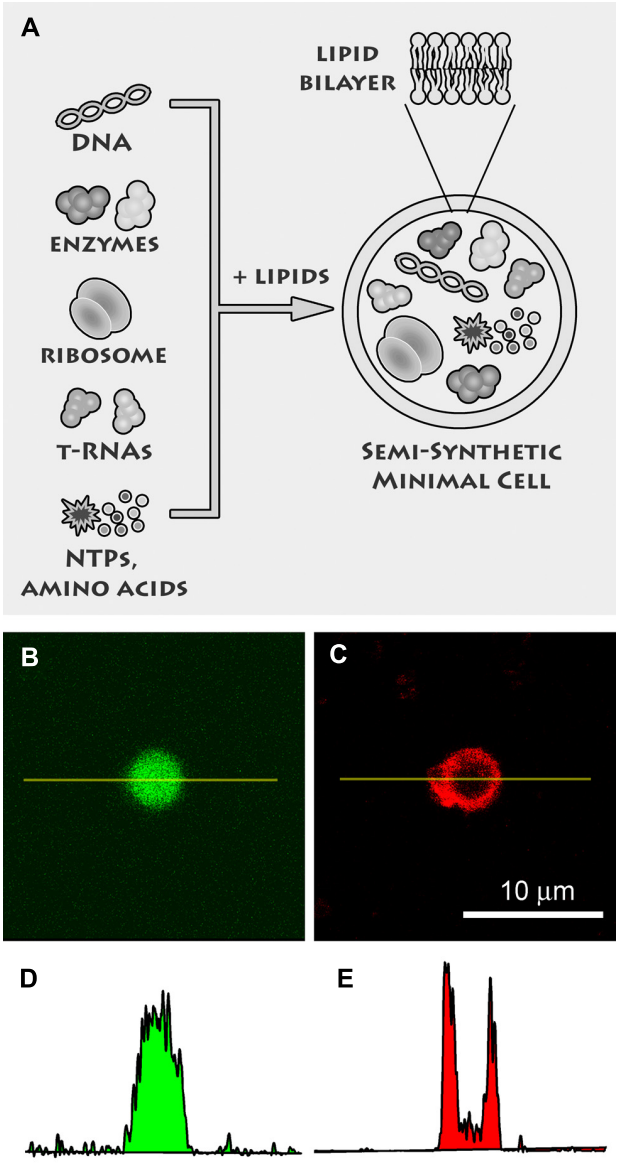


FIGURE 2 | Semi-synthetic minimal cells. (A) Semi-synthetic minimal cells are based on the encapsulation of the minimal number of biological molecules, like DNA, ribosomes, tRNAs, enzymes, amino acids, nucleotides, etc., inside lipid vesicles. Currently it is possible to encapsulate the whole transcription–translation machinery so that proteins are synthesized inside the synthetic cell. The goal of research is the self-reproduction of the minimal cells thanks to the simultaneous (and possibly coupled) production of internal and membrane components. Reproduced from Chiarabelli et al. (2009) with the permission of Elsevier. **(B)** Confocal fluorescence image of a liposome, prepared by the droplet transfer method, which contains the whole transcription–translation machinery (*E. coli* cell extracts) for producing a protein from the corresponding DNA sequence. In this case, the enhanced green fluorescence protein (eGFP) was synthesized, as evidenced by the green fluorescence. **(C)** The liposome membrane were made visible after Trypan blue addition, which binds to phospholipid bilayers and becomes red fluorescent upon 543 nm excitation. **(D,E)** Quantitation of fluorescence along the yellow lines in **(B,C)** gives typical bell-shaped and U-shaped profiles, respectively, for water-soluble (eGFP) and membrane-bound (Trypan blue) fluorochromes.

components. In fact, we recently investigated in details the physics of solute encapsulation inside liposomes spontaneously formed by lipid self-assembly – a key event for the emergence of primitive cells. Driven by our intriguing observations on the success of solute encapsulation and protein synthesis inside 200 nm (diameter) vesicles (Souza et al., 2009), we asked what are the mechanisms that allow the capture of very large number of solutes inside a closed lipid compartment. By using ferritin, ribosomes, and ribopeptidic complexes, we revealed the true intraliposome solute distribution by direct counting the number of entrapped macromolecules via cryo-transmission electron microscopy (Luisi et al., 2010; Souza et al., 2011, 2012). Interestingly, although the majority of liposomes were "empty" or contain the expected number of solutes, we found about 0.1% of liposomes containing a very high number of solutes, exceeding by at least one order of magnitude the expected concentration. In other words, liposomes can spontaneously concentrate macromolecular solutes in their cavity, providing a mechanism for the emergence of cellular metabolism even starting from a diluted solution. In fact, this mechanism might provide a rational explanation on the onset of intraliposome complex reactions that cannot occur in bulk phase due to extreme dilution. We have recently assayed such realistic scenario by using the synthesis of green fluorescent protein synthesis as a metabolic model (Stano et al., 2013).

CONCLUDING REMARKS

An additional dimension has been added to SB, namely the CSB approach, based on the concept of producing biological structures alternative to the natural ones, by using chemical and biochemical technology.

In this mini-review we have shortly commented two of our projects in CSB, namely the "Never Born Biopolymers" and the "Minimal Cells" projects. The corresponding investigations are based, respectively, on the exploration of peptide- and RNA-libraries, and on the encapsulation of solutes inside lipid vesicles. These approaches give a rather rich variety of novel forms and corresponding novel ideas, which may be relevant for understanding how biological systems are constructed and works, as well as for potentially new biotechnological applications. For example, NBRNAs might provide insights into the so-called RNA world hypothesis, but also provide sequences with unexpected biological effects. NBRNAs might become novel therapeutical agents. Minimal cells, when properly designed, might find applications in advanced drug delivery approaches, and would not only serve as model or primitive cells. CSB is a large field in which basic science and applicative research combine together, and we are confident that it will bring about significant advancements.

ACKNOWLEDGMENTS

This work was derived from our recent involvement in the Sixth Framework EU Program (SYNTHCELLS: Approaches to the Bioengineering of Synthetic Minimal Cells 043359), HFSP (RGP0033/2007-C), ASI (I/015/07/0), PRIN2008 (2008FY7RJ4); and further expanded thanks to networking initiatives as SynBioNT(UK), and the COST Systems Chemistry action (CM0703).

REFERENCES

Adamala, K., and Szostak, J. W. (2013). Competition between model protocells driven by an encapsulated catalyst. *Nat. Chem.* 5, 634. doi: 10.1038/nchem.1700

Alajlouni, R. A., and Seleem, M. N. (2013). Targeting *Listeria* monocytogenes rpoA and rpoD genes using peptide nucleic acids. *Nucleic Acid Ther.* 23, 363–367. doi: 10.1089/nat.2013.0426

Andrianantoandro, E., Basu, S., Karig, D. K., and Weiss, R. (2006). Synthetic biology: new engineering rules for an emerging discipline. *Mol. Syst. Biol.* 2, 2006.0028. doi: 10.1038/msb4100073

Anella, F., De Lucrezia, D., Chiarabelli, C., and Luisi, P. L. (2011). Stability studies on random folded RNAs (Never Born RNAs), implications for the RNA World. *Chem. Biodivers.* 8, 1422–1432. doi: 10.1002/cbdv.201100224

Berclaz, N., Müller, M., Walde, P., and Luisi, P. L. (2001). Growth and transformation of vesicles studied by ferritin labeling and cryotransmission electron microscopy. *J. Phys. Chem. B* 105, 1056–1064. doi: 10.1021/jp001298i

Bolli, M., Micura, R., and Eschenmoser, A. (1997). Pyranosyl-RNA: chiroselective self-assembly of base sequences by ligative oligomerization of tetranucleotide-2′,3′-cyclophosphates (with a commentary concerning the origin of biomolecular homochirality). *Chem. Biol.* 4, 309–320. doi: 10.1016/S1074-5521(97)90074-0

Budin, I., Debnath, A., and Szostak, J. W. (2012). Concentration-driven growth of model protocell membranes. *J. Am. Chem. Soc.* 134, 20812–20819. doi: 10.1021/ja310382d

Chiarabelli, C., Portela Pallares, C., and Quintarelli, A. (2011). "Searching for de novo totally random amino acid sequences," in *Chemical Synthetic Biology*, eds P. L. Luisi and C. Chiarabelli (Chichester: Wiley), 155–174.

Chiarabelli, C., Stano, P., Anella, F., Carrara, P., and Luisi, P. L. (2012). Approaches to chemical synthetic biology. *FEBS Lett.* 586, 2138–2145. doi: 10.1016/j.febslet.2012.01.014

Chiarabelli, C., Stano, P., and Luisi, P. L. (2009). Chemical approaches to synthetic biology. *Curr. Opin. Biotechnol.* 20, 492–497. doi: 10.1016/j.copbio.2009.08.004

Chiarabelli, C., Vrijbloed, J. W., Thomas, R. M., and Luisi, P. L. (2006a). Investigation of de novo totally random biosequences. Part I. A general method for in vitro selection of folded domains from a random polypeptide library displayed on phage. *Chem. Biodivers.* 3, 827–839. doi: 10.1002/cbdv.200690087

Chiarabelli, C., Vrijbloed, J. W., De Lucrezia, D., Thomas, R. M., Stano, P., Polticelli, F., et al. (2006b). Investigation of de novo totally random biosequences. Part II. On the folding frequency in a totally random library of de novo proteins obtained by phage display. *Chem. Biodivers.* 3, 840–859. doi: 10.1002/cbdv.200690088

Cossio, P., Trovato, A., Pietrucci, F., Seno, F., Maritan, A., and Laio, A. (2010). Exploring the universe of protein structures beyond the Protein Data Bank. *PLoS Comput. Biol.* 6:e1000957. doi: 10.1371/journal.pcbi.1000957

Davidson, A. R., Lumb, K. J., and Sauer, R. T. (1995). Cooperatively folded proteins in random sequence libraries. *Nat. Struct. Biol.* 2, 856–864. doi: 10.1038/nsb1095-856

de Duve, C. (2002). *Life Evolving: Molecules, Mind, and Meaning*. New York: Oxford University Press.

De Lucrezia, D., Franchi, M., Chiarabelli, C., Gallori, E., and Luisi, P. L. (2006a). Investigation of de novo totally random biosequences, part III: RNA Foster: a novel assay to investigate RNA folding structural properties. *Chem. Biodivers.* 3, 860–868.

De Lucrezia, D., Franchi, M., Chiarabelli, C., Gallori, E., and Luisi, P. L. (2006b). Investigation of de novo totally random biosequences, part IV: folding properties of de novo, totally random RNAs. *Chem. Biodivers.* 3, 869–877. doi: 10.1002/cbdv.200690090

De Lucrezia, D., Slanzi, D., Poli, I., Polticelli, F., and Minervini, G. (2012). Do natural proteins differ from random sequences polypeptides? Natural vs. random proteins classification using an evolutionary neural network. *PLoS ONE* 7:e36634. doi: 10.1371/journal.pone.0036634

Doi, N., Kakukawa, K., Oishi, Y., and Yanagawa, H. (2005). High solubility of random-sequence proteins consisting of five kinds of primitive amino acids. *Protein Eng. Des. Sel.* 18, 279–284. doi: 10.1093/protein/gzi034

Dryden, D. T. F., Thomson, A. R., and White, J. H. (2008). How much of protein sequence space has been explored by life on Earth? *J. R. Soc. Interface* 5, 953–956. doi: 10.1098/rsif.2008.0085

Egholm, M., Buchardt, O., Christensen, L., Behrens, C., Freier, S. M., Driver, D. A., et al. (1993). PNA hybridizes to complementary oligonucleotides obeying the Watson–Crick hydrogen-bonding rules. *Nature* 365, 566–568. doi: 10.1038/365566a0

Endy, D. (2005). Foundations for engineering biology. *Nature* 438, 449–453. doi: 10.1038/nature04342

Fisher, M. A., McKinley, K. L., Bradley, L. H., Viola, S. R., and Hecht, M. H. (2011). De novo designed proteins from a library of artificial sequences function in *Escherichia coli* and enable cell growth. *PLoS ONE* 6:e15364. doi: 10.1371/journal.pone.0015364

Gibson, D. G., Glass, J. I., Lartigue, C., Noskov, V. N., Chuang, R. Y., Algire, M. A., et al. (2010). Creation of a bacterial cell controlled by a chemically synthesized genome. *Science* 329, 52–56. doi: 10.1126/science.1190719

Gil, R., Silva, F. J., Pereto, J., and Moya, A. (2004). Determination of the core of a minimal bacteria gene set. *Microbiol. Mol. Biol. Rev.* 68, 518–537. doi: 10.1128/MMBR.68.3.518-537.2004

Gorlero, M., Wieczorek, R., Adamala, K., Giorgi, A., Schininà, M. E., Stano, P., et al. (2009). Ser-His catalyses the formation of peptides and PNAs. *FEBS Lett.* 583, 153–156. doi: 10.1016/j.febslet.2008.11.052

Hamada, T., and Yoshikawa, K. (2012). Review: cell-sized liposomes and droplets: real-world modeling of living cells. *Materials* 5, 2292–2305. doi: 10.3390/ma5112292

Hanes, J., and Plückthun, A. (1997). In vitro selection and evolution of functional proteins by using ribosome display. *Proc. Natl. Acad. Sci. U.S.A.* 94, 4937–4942. doi: 10.1073/pnas.94.10.4937

Hayashi, Y., Sakata, H., Makino, Y., Urabe, I., and Yomo, T. (2003). Can an arbitrary sequence evolve towards acquiring a biological function? *J. Mol. Evol.* 56, 162–168. doi: 10.1007/s00239-002-2389-y

Hecht, M. H., Das, A., Go, A., Bradley, L. H., and Wei, Y. (2004). De novo proteins from designed combinatorial libraries. *Protein Sci.* 13, 1711–1723. doi: 10.1110/ps.04690804

Ito, Y., Kawama, T., Urabe, I., and Yomo, T. (2003). Evolution of an arbitrary sequence in solubility. *J. Mol. Evol.* 58, 196–202. doi: 10.1007/s00239-003-2542-2

Jewett, M. C., and Forster, A. C. (2010). Update on designing and building minimal cells. *Curr. Opin. Biotechnol.* 21, 697–703. doi: 10.1016/j.copbio.2010.06.008

Kamtekar, S., Schiffer, J. M., Xiong, H., Babik, J. M., and Hecht, M. H. (1993). Protein design by binary patterning of polar and nonpolar amino acids. *Science* 262, 1680–1685. doi: 10.1126/science.8259512

Kobori, S., Ichihashi, N., Kazuta, Y., and Yomo, T. (2013). A controllable gene expression system in liposomes that includes a positive feedback loop. *Mol. Biosyst.* 9, 1282–1285. doi: 10.1039/c3mb70032a

Kristensen, P., and Winter, G. (1998). Proteolytic selection for protein folding using filamentous bacteriophages. *Fold. Des.* 3, 321–328. doi: 10.1016/S1359-0278(98)00044-3

Kuruma, Y., Stano, P., Ueda, T., and Luisi, P. L. (2009). A synthetic biology approach to the construction of membrane proteins in semisynthetic minimal cells. *Biochim. Biophys. Acta* 1788, 567–574. doi: 10.1016/j.bbamem.2008.10.017

LaBean, T. H., Butt, T. R., Kauffman, S. A., and Schultes, E. A. (2011). Protein folding absent selection. *Genes* 2, 608–626. doi: 10.3390/genes2030608

Leduc, P. R., Wong, M. S., Ferreira, P. M., Groff, R. E., Haslinger, K., Koonce, M. P., et al. (2007). Towards an in vivo biologically inspired nanofactory. *Nat. Nanotechnol.* 2, 3–7. doi: 10.1038/nnano.2006.180

Lentini, R., Forlin, M., Martini, L., Del Bianco, C., Spencer, A. C., Torino, D., et al. (2013). Fluorescent proteins and in vitro genetic organization for cell-free synthetic biology. *ACS Synth. Biol.* doi: 10.1021/sb400003y [Epub ahead of print].

Lipman, D. J., Souvorov, A., Koonin, E. V., Panchenko, A. R., and Tatusova, T. A. (2002). The relationship of protein conservation and sequence length. *BMC Evol. Biol.* 2:20. doi: 10.1186/1471-2148-2-20

Luisi, P. L. (2003). Contingency and determinism. *Philos. Trans. R. Soc. Lond. A* 361, 1141–1147. doi: 10.1098/rsta.2003.1189

Luisi, P. L. (2006). *The emergence of life. From chemical origins to synthetic biology*. Cambridge: Cambridge University Press. doi: 10.1017/CBO9780511817540

Luisi, P. L. (2007). Chemical aspects of synthetic biology. *Chem. Biodivers.* 4, 603–621. doi: 10.1002/cbdv.200790053

Luisi, P. L. (2011). "The synthetic approach in biology: epistemological notes for synthetic biology," in *Chemical Synthetic Biology*, eds P. L. Luisi and C. Chiarabelli (Chichester: Wiley), 343–362. doi: 10.1002/9780470977873.ch14

Luisi, P. L., Allegretti, M., Souza, T., Steineger, F., Fahr, A., and Stano, P.

(2010). Spontaneous protein crowding in liposomes: a new vista for the origin of cellular metabolism. *Chembiochem* 11, 1989–1992. doi: 10.1002/cbic.201000381

Luisi, P. L., and Chiarabelli, C. (eds) (2011). *Chemical Synthetic Biology*. Chichester: Wiley.

Luisi, P. L., Chiarabelli, C., and Stano, P. (2006a). From the Never Born Proteins to the minimal living cells: two projects in synthetic biology. *Orig. Life Evol. Biosph.* 36, 605–616. doi: 10.1007/s11084-006-9033-6

Luisi, P. L., Ferri, F., and Stano, P. (2006b). Approaches to semi-synthetic minimal cells: a review. *Naturwissenschaften* 93, 1–13. doi: 10.1007/s00114-005-0056-z

Maeda, Y. T., Nakadai, T., Shin, J., Uryu, K., Noireaux, V., and Libchaber, A. (2012). Assembly of MreB filaments on liposome membranes: a synthetic biology approach. *ACS Synth. Biol.* 1, 53–59. doi: 10.1021/sb200003v

Nourian, Z., and Danelon, C. (2013). Linking genotype and phenotype in protein synthesizing liposomes with external supply of resources. *ACS Synth. Biol.* 2, 186–193. doi: 10.1021/sb300125z

Oberholzer, T., Nierhaus, K. H., and Luisi, P. L. (1999). Protein expression in liposomes. *Biochem. Biophys. Res. Commun.* 261, 238–241. doi: 10.1006/bbrc.1999.0404

Pinheiro, V. B., Taylor, A. I., Cozens, C., Abramov, M., Renders, M., Zhang, S., et al. (2012). Synthetic genetic polymers capable of heredity and evolution. *Science* 336, 341–344. doi: 10.1126/science.1217622

Plückthun, A. (2012). Ribosome display: a perspective. *Methods Mol. Biol.* 805, 3–28. doi: 10.1007/978-1-61779-379-0_1

Prymula, K., Piwowar, M., Kochanczyk, M., Flis, L., Malawski, M., Szepieniec, T., et al. (2009). In silico structural study of random amino acid sequence proteins not present in nature. *Chem. Biodivers.* 6, 2311–2336. doi: 10.1002/cbdv.200800338

Rasmussen, S., Chen, L., Deamer, D., Krakauer, D. C., Packard, N. H., Stadler, P. F., et al. (2004). Transitions from non-living to living matter. *Science* 303, 963–965. doi: 10.1126/science.1093669

Schmidli, P. K., Schurtenberger, P., and Luisi, P. L. (1991). Liposome-mediated enzymatic synthesis of phosphatidylcholine as an approach to self-replicating liposomes. *J. Am. Chem. Soc.* 113, 8127–8130. doi: 10.1021/ja00021a043

Shimizu, Y., Inoue, A., Tomari, Y., Suzuki, T., Yokogawa, T., Nishikawa, K., et al. (2001). Cell free translation reconstituted with purified components. *Nat. Biotechnol.* 19, 751–755. doi: 10.1038/90802

Souza, T., Stano, P., and Luisi, P. L. (2009). The minimal size of liposome-based model cells brings about a remarkably enhanced biological activity. *Chembiochem* 10, 1056–1063. doi: 10.1002/cbic.200800810

Souza, T., Stano, P., Steiniger, F., D'Aguanno, E., Altamura, E., Fahr, A., et al. (2012). Encapsulation of ferritin, ribosomes, and ribo-peptidic complexes inside liposomes: insights into the origin of metabolism. *Orig. Life Evol. Biosph.* 42, 421–428. doi: 10.1007/s11084-012-9303-4

Souza, T., Steiniger, F., Stano, P., Fahr, A., and Luisi, P. L. (2011). Spontaneous crowding of ribosomes and proteins inside vesicles: a possible mechanism for the origin of cell metabolism. *Chembiochem* 12, 2325–2330. doi: 10.1002/cbic.201100306

Stano, P., Carrara, P., Kuruma, Y., Souza, T., and Luisi, P. L. (2011). Compartmentalized reactions as a case of soft-matter biotechnology: synthesis of proteins and nucleic acids inside lipid vesicles. *J. Mater. Chem.* 21, 18887–18902. doi: 10.1039/c1jm12298c

Stano, P., D'Aguanno, E., Bolz, J., Fahr, A., and Luisi, P. L. (2013). A remarkable self-organization process as the origin of primitive functional cells. *Angew. Chemie.* doi: 10.1002/anie.201306613

Stano, P., Rampioni, G., Carrara, P., Damiano, L., Leoni, L., and Luisi, P. L. (2012). Semi-synthetic minimal cells as a tool for biochemical ICT. *Biosystems* 109, 24–34. doi: 10.1016/j.biosystems.2012.01.002

Stano, P., Wehrli, E., and Luisi, P. L. (2006). Insights on the oleate vesicles self-reproduction. *J. Phys. Condes. Matter* 18, S2231–S2238. doi: 10.1088/0953-8984/18/33/S37

Szostak, J. W., Bartel, D. P., and Luisi, P. L. (2001). Synthesizing life. *Nature* 409, 387–390. doi: 10.1038/35053176

Tawfik, D. S., and Griffiths, A. D. (1998). Man-made cell-like compartments for molecular evolution. *Nat. Biotechnol.* 16, 652–656. doi: 10.1038/nbt0798-652

Urvoas, A., Valerio-Lepiniec, M., and Minard, P. (2012). Artificial proteins from combinatorial approaches. *Trends Biotechnol.* 30, 512–520. doi: 10.1016/j.tibtech.2012.06.001

Varela, F., Maturana, H. R., and Uribe, R. B. (1974). Autopoiesis: the organization of living system, its characterization and a model. *Biosystems* 5, 187–196. doi: 10.1016/0303-2647(74)90031-8

Vogt, V. M. (1973). Purification and further properties of single-strand-specific nuclease from *Aspergillus oryzae*. *Eur. J. Biochem.* 33, 192–200. doi: 10.1111/j.1432-1033.1973.tb02669.x

Walde, P., Wick, R., Fresta, M., Mangone, A., and Luisi, P. L. (1994). Autopoietic self-reproduction of fatty acid vesicles. *J. Am. Chem. Soc.* 116, 11649–11654. doi: 10.1021/ja00105a004

Wei, Y., Liu, T., Sazinsky, S. L., Moffet, D. A., Pelczer, I., and Hecht, M. H. (2003). Stably folded de novo proteins from a designed combinatorial library. *Protein Sci.* 12, 92–102. doi: 10.1110/ps.0228003

Wieczorek, R., Doerr, M., Chotera, A., Luisi, P. L., and Monnard, P. A. (2012). Formation of RNA phosphodiester bond by histidine-containing dipeptides. *Chembiochem* 14, 217–223. doi: 10.1002/cbic.201200643

Wong, J. T. F., and Xue, H. (2011). "Synthetic genetic codes as the basis of synthetic life," in *Chemical Synthetic Biology*, eds P. L. Luisi, and C. Chiarabelli (Chichester: Wiley), 177–199.

Xia, Y., and Levitt, M. (2004). Funnel-like organization in sequence space determines the distributions of protein stability and folding rate preferred by evolution. *Proteins* 55, 107–114. doi: 10.1002/prot.10563

Yamauchi, A., Nakashima, T., Tokuriki, N., Hosokawa, M., Nogami, H., Arioka, S., et al. (2002). Evolvability of random polypeptides through functional selection within a small library. *Protein Eng.* 15, 619–626. doi: 10.1093/protein/15.7.619

Zhu, T. F., and Szostak, J. W. (2009). Coupled growth and division of model protocell membranes. *J. Am. Chem. Soc.* 131, 5705–5713. doi: 10.1021/ja900919c

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 05 June 2013; accepted: 04 September 2013; published online: 23 September 2013.

Citation: Chiarabelli C, Stano P and Luisi PL (2013) Chemical synthetic biology: a mini-review. Front. Microbiol. 4:285. doi: 10.3389/fmicb.2013.00285

This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology.