CONFLICT AND COOPERATION IN MICROBIAL SOCIETIES

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CONFLICT AND COOPERATION IN MICROBIAL SOCIETIES

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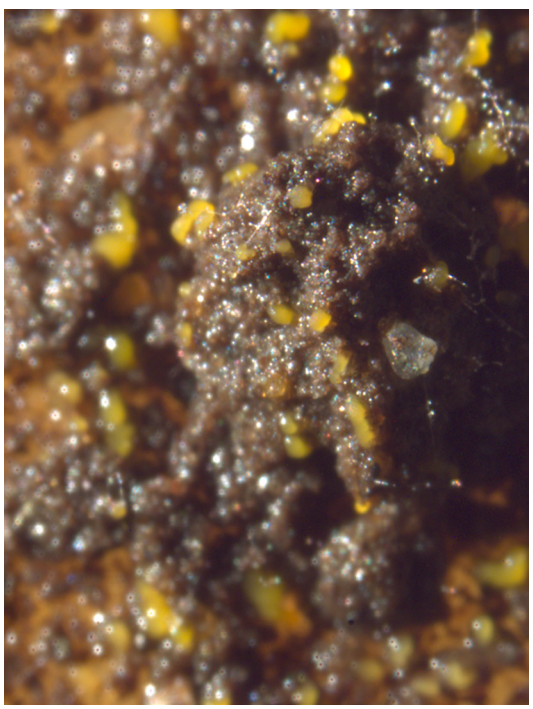

Myxobacteria fruiting bodies growing on small soil aggregates.
Figure by Ana E. Escalante

The most evident aspect of biodiversity is the variety of complex forms and behaviors among organisms, both living and extinct. Comparative molecular and physiological studies show that the evolution of complex phenotypic traits involves multiple levels of biological organization (i.e. genes, chromosomes, organelles, cells, individual organisms, species, etc.). Regardless of the specific molecular mechanisms and details, the evolution of different complex biological organizations share a commonality: cooperation and conflict among the parts of the biological unit under study. The potential for conflict among parts is abundant. How then do complex systems persist, given the necessity of cooperative behavior for their maintenance, when the potential for conflict occurs across all levels of biological organization?

In this Research Topic and eBook we present ideas and work on the question, how coexistence of biological components at different levels of organization persists in the face of antagonistic, conflicting or even exploitative behavior of the parts? The goal of this topic is in presenting examples of cooperation and conflict at different levels of biological organization to discuss the consequences that this "tension" have had in the diversification and emergence of novel phenotypic traits. Exemplary cases are studies investigating: the evolution of genomes, formation of colonial aggregates of cells, biofilms, the origin and maintenance of multicellular organisms, and the stable coexistence of multispecies consortia producing a cooperative product.

Altogether, we hope that the contributions to this Research Topic build towards mechanistic knowledge of the biological phenomenon of coexistence in the face of conflict. We believe that knowledge on the mechanisms of the origin and evolutionary maintenance of cooperation has implications beyond evolutionary biology such as novel approaches in controlling microbial infections in medicine and the modes by studies in synthetic biology are conducted when designing economically important microbial consortia.

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EDITORIAL
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Editorial: Conflict and Cooperation in Microbial Societies

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Keywords: conflict, cooperation, spatial structure, diversity, cheating, antagonistic interactions, mutualistic interactions, consortia

Editorial on the Research Topic

Conflict and Cooperation in Microbial Societies

The most evident aspect of biodiversity is the variety of complex forms and behaviors among organisms, both living and extinct. Comparative molecular and physiological studies show that the evolution of complex phenotypic traits involves multiple levels of biological organization (i.e., genes, chromosomes, organelles, cells, individual organisms, species, etc.). Regardless of the specific molecular mechanisms and details, the evolution of different complex biological organizations share a commonality: cooperation (e.g., mutualisms) and conflict (e.g., antagonistic behavior) among the parts of the biological unit under study, so how do complex systems persist, given the necessity of cooperative behavior for their maintenance, when the potential for conflict occurs across all levels of biological organization?

In this Research Topic we are particularly interested in presenting ideas and work on the manifestation of different complex phenotypes that are based on cooperative or conflicting interactions of the component parts. Across these different systems, we see examples of cooperation and conflict at different levels of biological organization and discuss the consequences that this "tension" has had in the diversification and emergence of novel or complex phenotypic traits.

Cooperation among genes and among individual multicellular organisms (e.g., communities) are the extremes of different levels of biological organization in which cooperation has important roles in the origin and maintenance of complex phenotypic traits that range from the organization and size of genomes (Martínez-Cano et al.) to the maintenance of the microbiomes of animals (e.g., Loudon et al.). These models provide experimental data about the origin and maintenance of cooperative phenotypes and the role of cooperation in the evolution of biological complexity. In this Research Topic we present contributions that include all this range of biological complexity and that explore the mechanisms controlling the emergence and maintenance of diversity via cooperation in the face of conflict. We organized the contributions of this Research Topic in order of the level of biological organization that the studies refer to. We start with genome evolution (Martínez-Cano et al.), then continue with different examples of interactions among microorganisms from virus (Williams et al.) to bacteria, experimental and theoretical, and we finalize with an example of a multiple interaction system including animals, bacteria and fungi (Loudon et al.).

Martínez-Cano et al. present an analysis of experimental data on the processes behind genome reduction in endosymbiotic prokaryotes. Their work indicates that natural selection on cooperation or complementation of genomes (of the host and endosymbiont) may be behind this reduction which challenges other studies, based on comparative genomics, that have attributed this reduction to neutral processes (drift).

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Mora van Cauwelaert et al. present a complementary view on the processes behind the emergence of multicellularity. Although the authors recognize the role of cooperative behavior, they discuss explicitly the role of other mechanisms such as physicochemical aspects of the environment in the emergence of complex phenotypes, such as multicellular aggregates in the early stages of the evolution of multicellularity.

A group of articles (Allison et al.; Kovács; Escalante et al.) discuss the role of spatial structure in the maintenance of cooperative behavior. Allison et al. focus their contribution in an experimental model of *Pseudomonas fluorescens* and the extracellular production of enzymes, a cooperative behavior that can only persist if spatial structure is imposed to the populations, in this way controlling the spread of cheater genotypes. Escalante et al. review studies on microbial population biology and identify that depending on the type of cooperative behavior (transitive or non-transitive), spatial structure may or may not favor the persistence of the cooperative behavior and the ecological diversity of the systems. Finally, Kovács, in a perspective article presents similar reflections on the role of spatial structure in the maintenance of mutualistic interactions and in turn of diversity.

Ponce-Soto et al. investigate experimentally the role of nutrient availability in the interactions and resulting diversity in microbial populations in natural environments. Their results suggest an important participation of environmental resource availability in shifting ecological strategies from more antagonistic (i.e., antibiotic resistance) when resources are limited to less so, maybe cooperative (i.e., biofilm formation), when the environment is enriched with nutrients. Moreover, in a complementary article, Aguirre-von-Wobeser et al. investigate theoretically the cost of production of antagonistic molecules in nutrient limited environments (oligotrophic) as a mechanistic exploration of interaction shifts in natural environments. Finally, Zapién-Campos et al. investigate through a mathematical model the dynamics of antagonistic interactions based on previously published experimental data, their results show that spatial structure emerge among the less antagonistic strains (more cooperative) as aggregates, which may be in accordance with the relevance of spatial structure in the emergence and maintenance of complex phenotypes and ecological diversity (see Allison et al.; Kovács; Escalante et al.).

Chubiz et al. study the mechanistic basis of cooperative and conflicting interactions at the genome-metabolite level by *in silico* knocking-out genes and testing the resulting metabolic interactions. Their findings highlight the importance of connecting genomic-metabolic studies to the context of interactions when looking for ecological stability or the mechanistic basis of the persistence of ecological diversity.

The last contribution of the research topic is presented by Loudon et al. showing the emergence of phenotypic traits at the microbial community level through the cooperative behavior of two bacterial populations in the production of antifungal metabolites in frogs' skin.

The maintenance of diversity and cooperation in the face of conflict has puzzled evolutionary biology for many years and in this topic we present new data and ideas that add to the mechanistic knowledge of the underlying processes. Mechanistic knowledge of any system or biological phenomenon allows for informed and effective manipulation or intervention of such. Thus, understanding the mechanisms of the origin and evolutionary maintenance of cooperation has implications far beyond evolutionary biology. Our research topic articles have the potential of impacting, for example, approaches in controlling microbial infections in medicine, as well as modes by which studies in synthetic biology are conducted when designing economically important microbial consortia.

AUTHOR CONTRIBUTIONS

AE and MT contributed equally to the proposal and, organization of the Research Topic, as well as to the writing of the Editorial.

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Evolution of small prokaryotic genomes

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As revealed by genome sequencing, the biology of prokaryotes with reduced genomes is strikingly diverse. These include free-living prokaryotes with ∼800 genes as well as endosymbiotic bacteria with as few as ∼140 genes. Comparative genomics is revealing the evolutionary mechanisms that led to these small genomes. In the case of free-living prokaryotes, natural selection directly favored genome reduction, while in the case of endosymbiotic prokaryotes neutral processes played a more prominent role. However, new experimental data suggest that selective processes may be at operation as well for endosymbiotic prokaryotes at least during the first stages of genome reduction. Endosymbiotic prokaryotes have evolved diverse strategies for living with reduced gene sets inside a host-defined medium. These include utilization of host-encoded functions (some of them coded by genes acquired by gene transfer from the endosymbiont and/or other bacteria); metabolic complementation between co-symbionts; and forming consortiums with other bacteria within the host. Recent genome sequencing projects of intracellular mutualistic bacteria showed that previously believed universal evolutionary trends like reduced G+C content and conservation of genome synteny are not always present in highly reduced genomes. Finally, the simplified molecular machinery of some of these organisms with small genomes may be used to aid in the design of artificial minimal cells. Here we review recent genomic discoveries of the biology of prokaryotes endowed with small gene sets and discuss the evolutionary mechanisms that have been proposed to explain their peculiar nature.

Keywords: reductive genome evolution, endosymbiosis, minimal genome size, streamlining evolution, Black Queen Hypothesis, Muller's ratchet, robustness-based selective reduction, symbionelle

INTRODUCTION

Darwin proposed an externalist theory of evolution where organisms provide the raw material and the environment selects (Gould, 2002). The outcome of this process is a fine adjustment of organisms to the environment. The evolution of prokaryotes with reduced genomes is not an exception to this Darwinian principle. Host-associated bacteria and archaea evolved the smallest genomes in nature other than those of organelles and viruses. The rationale of this pattern is simple. Prokaryotes living in a protected and chemically rich medium can afford losing more genes than those coping with the vagaries of a free-living lifestyle (Morowitz, 1993). On the other hand, different lineages of free-living bacteria, most of them in marine environments, evolved reduced genomes likely by the direct action of natural selection (Giovannoni et al., 2014).

WHAT IS THE MINIMAL GENOME SIZE FOR EXTANT FREE-LIVING PROKARYOTES?

Previous surveys indicated that free-living prokaryotes had no less than ∼1,300 genes (Islas et al., 2004; Podar et al., 2008; Delaye et al., 2010). However, recent metagenomic sequencing suggests that there are free-living Actinobacteria with approximately 800 genes. This was discovered at the Mediterranean Sea and the bacteria were named "*Candidatus* Actinomarina minuta" (Ghai et al., 2013). Surprisingly, it is also one of the smallest cells with a cell volume of only ∼0.013 μm^3. If further sequencing of its complete genome confirms this estimate (and it is very likely that it will do), it will sensibly change our knowledge about the minimum number of genes a cell needs to survive in present free-living conditions, in a similar fashion than the discovery of "*Candidatus* Carsonella ruddii" shook our belief of the minimal gene set required for cells in 2006 (Nakabachi et al., 2006; McCutcheon and Moran, 2012). Meanwhile, as reviewed below, there exists a diversity of lineages of free-living prokaryotes that converged to approximately 1,300 genes despite their varying phylogenetic origins and nutritional strategies.

Nowadays, *Methanothermus fervidus* with a genome coding for 1,311 proteins and 50 RNA genes, stands as the free-living archaeon (that does not grow associated to another cell) with the smallest sequenced genome. This organism is a methanogen and was isolated from an anaerobic Icelandic spring (Anderson et al., 2010). As mentioned above, other groups of free-living

Abbreviations: AM, arbuscular mycorrhizal; AVG, anti-virulence gene; dN/dS, non-synonymous versus synonymous substitutions; HGT, horizontal gene transfer; IS, insertion sequence; MGEs, mobile genetic elements; Trp, tryptophan; UGA_{stop}, UGA stop-coding codon; UGA_{trp}, UGA tryptophan-coding codon.

prokaryotes evolved similar genome sizes, several of them from marine environments.

For instance, α-proteobacteria from clade SAR11, which is the most abundant group of heterotrophic bacteria in the oceans, are endowed with genomes ranging from 1,321 to 1,541 protein-coding genes (Grote et al., 2012). Among them, is "*Candidatus* Pelagibacter ubique" HTCC1062, which is one of the most studied members of clade SAR11 and is an oligotroph with 1,354 protein-coding genes that generates energy by respiration (Giovannoni et al., 2005).

Another group of marine prokaryotes that evolved similar genome sizes are the β-proteobacteria from clade OM43. Specifically strains HTCC2181 and HIMB624 that have 1,377 and 1,381 protein-coding genes respectively (Giovannoni et al., 2008; Huggett et al., 2012). These are marine and freshwater bacteria that live heterotrophically by using methylated compounds as carbon sources (Giovannoni et al., 2008; Huggett et al., 2012).

Contrasting with the previously mentioned heterotrophs whose smallest genomes have ∼1,300 genes, photoautotrophic free-living bacteria have larger genomes. For instance, some strains from *Prochlorococcus marinus*, the most abundant photosynthetic organism on Earth, have genomes coding for as few as 1,716 protein-coding genes (Dufresne et al., 2003; Rocap et al., 2003; Scanlan et al., 2009).

And finally, non-marine bacteria with small genomes include the mollicute *Acholeplasma laidlawii*, which is a saprophyte and opportunistic parasite found in a wide variety of environments and has a genome coding for 1,380 protein-coding genes (Windsor et al., 2010; Lazarev et al., 2011); the lactobacilli *Weissella koreensis* KACC 15510 which is a heterotroph that participates in the fermentation of kimchi (a representative Korean fermented food) and has a genome coding for 1,335 predicted protein-coding sequences (Lee et al., 2011); the dehalorespiration *Dehalococcoides* sp. BAV1 with a genome coding for 1,371 protein-coding genes and a member of the Chloroflexi (He et al., 2003; Löffler et al., 2013); and the Chrenarchaeon *Desulfurococcus mucosus* O7/1 with its ability for sulfur respiration with a genome with 1,371 protein-coding genes (Wirth et al., 2011).

Why do different lineages of free-living cultivable bacteria have genomes with no less than 1,300 genes? One possible explanation is that extant biotic and abiotic environments exert a selective pressure against simpler cells, therefore imposing an ecological limit on the minimum complexity necessary for a cell to survive (Gould, 1996). The idea is that free-living cells with fewer genes are outcompeted by cells with a more complete genetic arsenal, unless associated to other organisms. However, intuitive this idea is, still requires experimental validation.

However, it is important to take into consideration that it is possible that our sample of genome sequences from cultivable organisms does not accurately represents the distribution of genome sizes that exist on nature (Giovannoni et al., 2014). Additionally, as suggested by metagenomic data, there may exist a whole biodiversity of uncultivable bacteria with genomes with less than 1,300 genes, as seems to be the case of "*Ca.* Actinomarina minuta" (Ghai et al., 2013).

In this direction, a note of caution regarding the limit of ∼1,300 genes for free-living prokaryotes is given by *Lactobacillus fermentum* CECT 5716, a hetero-fermentative lactic acid bacterium inhabiting human mucosal surfaces and breast milk. This bacterium has a genome with 1,109 protein-coding genes (Jiménez et al., 2010). And, although this organism would be classified as a symbiont because it is naturally associated to humans, it grows well under laboratory conditions (Jiménez, personal communication) thus blurring the distinction between free-living and host-associated microorganisms. The discovery of "*Ca.* Actinomarina minuta," as well as the existence of *L. fermentum* CECT 5716, indicates that in the near future we will probably discover free-living bacteria with smaller genomes.

DRIVERS OF GENOME REDUCTION AMONG FREE-LIVING PROKARYOTES

As we will describe below, different mechanisms have been proposed to account for genome reduction among free-living prokaryotes (**Figure 1**; Mira et al., 2001; Dufresne et al., 2005; Giovannoni et al., 2005; Marais et al., 2008; Morris et al., 2012).

THE STREAMLINING HYPOTHESIS

Genome reduction by a process known as streamlining, in which smaller genomes are favored directly by selection as a way to cellular economization, is perhaps the most popular explanation (Mira et al., 2001; Dufresne et al., 2005). According to this hypothesis, natural selection directly favors genome reduction in free-living prokaryotes living in low-nutrient environments (Mira et al., 2001; Grote et al., 2012). This claim is based on the basic idea that superfluous genes are eliminated because they confer a fitness cost to the bacterium. This is especially effective in large population sizes. The reasoning is as follows: very large population sizes render negligible the effect of genetic drift and more importantly, render highly efficient the process of natural selection. Then, when a fitness-increasing deletion occurs it is quickly fixed in the population, especially under high selective pressures such as low-nutrient environments where small genomes evolve as a way to economize on matter and energy for cell maintenance (Dufresne et al., 2005; Koskiniemi et al., 2012).

For instance, streamlining was suggested to be responsible of genome reduction in the high-light adapted marine cyanobacteria *P. marinus* MED4 and the low-light adapted *P. marinus* SS120 (Dufresne et al., 2003, 2005). According to the streamlining hypothesis the following characteristics are consistent with natural selection acting to economize cellular metabolism. First, the small G+C content of these genomes (∼30 to 36%) contributes to fewer requirements for phosphorus and nitrogen for DNA synthesis which are scarce in the environment where *P. marinus* MED4 lives (Dufresne et al., 2005). Second, the small cell volume of *P. marinus* SS120 (∼0.1 μm^3) is suggested to improve photosynthetic efficiency by reducing self-shading and enhancing nutrient uptake by increasing the surface-to-volume ratio for the cell, and also it is in itself an adaptation that has in turn exerted an evolutionary pressure for a smaller genome (Dufresne et al., 2003). This follows the logic that a smaller bacterial cell has a smaller volume and can only contain a small amount of DNA, otherwise too much of the internal space is devoted to DNA storing and

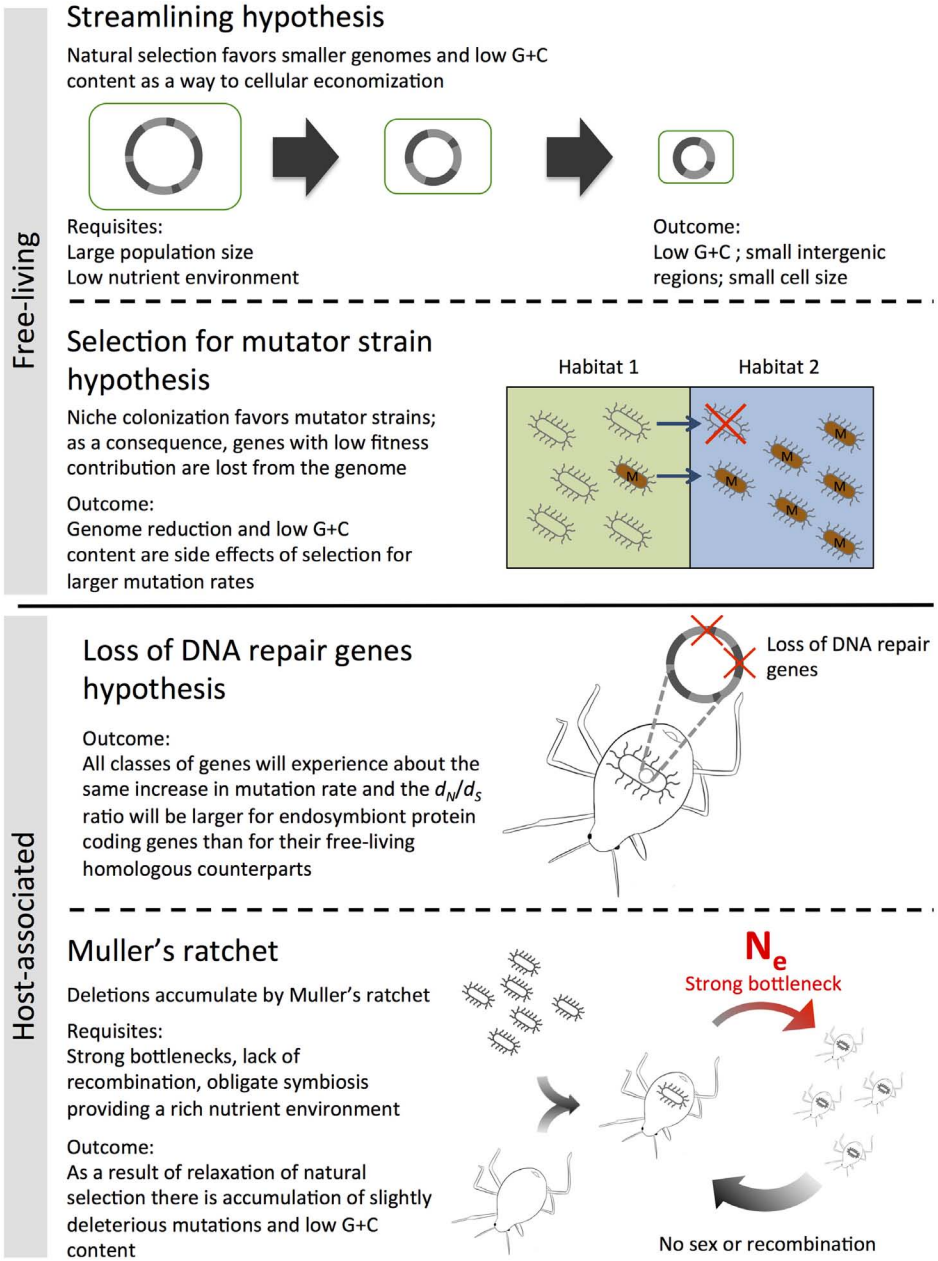


FIGURE 1 | Streamlining and Muller's ratchet hypotheses are commonly used to explain genome reduction in free-living and host-associated bacteria respectively. Alternative hypotheses include "selection for mutator strains" and "loss of DNA repair genes."

the remaining volume would not be sufficient for other cellular components (Trevors and Masson, 2011). Cell volume has a wide range of ~0.013 to 400 μm^3, however, a cell is considered small if its volume is less than 0.6 μm^3 (Koch, 1996; Ghai et al., 2013). And third, the streamlining hypothesis suggests that the effects of economization are observable as an increase in fitness.

Therefore, genome reduction is believed to have had a favorable effect on fitness in *Prochlorococcus* species. In the oceanic ecosystem, the diversity of photosynthetic prokaryotes is mostly represented by two genera: *Prochlorococcus* and *Synechococcus*. While *Synechococcus* are ubiquitous owning to their flexibility and adaptability to various marine environments, the *Prochlorococcus* have had an apparently better ecological success in oligotrophic areas where the conditions are more stable (Partensky et al., 1999). The success of *Prochlorococcus* is believed to be due to the differential distribution across the vertical axis of the water column of specialized ecotypes which are genetically and physiologically distinct populations distributed in accordance with the quality of light (Scanlan et al., 2009).

Genome streamlining was suggested also to explain reductive genome evolution in the case of the α-proteobacterium "*Ca.* Pelagibacter ubique" HTCC1062 and other members from the SAR11 clade (Giovannoni et al., 2005; Grote et al., 2012). As described above, this proposal is also based on several features of its streamlined genome. For instance, "*Ca.* Pelagibacter ubique" was reported to have a median space size between coding genes of only three nucleotides, the smallest among then analyzed genomes. In addition, no pseudogenes, phage genes or recent gene duplications were found (Giovannoni et al., 2005). And similar to the case of *P. marinus* MED4 and SS120, it was suggested that the small cell size of "*Ca.* Pelagibacter ubique" (0.019–0.039 μm^3) evolved by natural selection. In this case based on a theory proposed by Button (1991). Accordingly to this theory, selection optimized surface-to-volume ratio so that the capacity of the cytoplasm to process substrates matches transport rates (Giovannoni et al., 2005; Steindler et al., 2011).

The β-proteobacterias from Clade OM43 are another lineage where genome reduction by streamlining was suggested. As in the two cases described above, the small proportion of non-coding DNA in the reduced genome of strain HTCC2181 was interpreted as evidence of streamlining selection (Giovannoni et al., 2008). And as in the case of the strains MED4 and SS120 from *P. marinus* and in "*Ca.* Pelagibacter ubique," the β-proteobacteria HIMB624 also has small cell size of about 0.1–0.3 μm wide and 0.6–1.8 μm long (Huggett et al., 2012).

Finally, streamlining could be suggested also for "*Ca.* Actinomarina minuta." Its small genome is contained in an incredible small cell (0.013 μm^3). The median length of its intergenic sequences is of only three bases, the same as of "*Ca.* Pelagibacter ubique." "*Ca.* Actinomarina minuta" lives in aquatic environments where nutrients are scarce (Ghai et al., 2013).

Supporting the streamlining hypothesis, Koskiniemi et al. (2012) found that under laboratory conditions, selection can drive genome reduction. In order to do this, they devised a method that would report large deletions in the genome of *Salmonella enterica*. When they measured fitness against the wild type, they observed that several mutant strains showed an increase in fitness and concluded that fitness increases were common following deletions on specific genomic loci. Additionally, they performed a serial passage experiment and observed that selection could be a significant driver of gene loss. They suggested that in naturally occurring populations, fixation of deletion could occur very fast.

ACCELERATED RATES OF PROTEIN EVOLUTION

Returning to *P. marinus* strains MED4 and SS120, accelerated rates of protein evolution has been observed in these cyanobacteria (Dufresne et al., 2005). This is similar to what is observed in symbiotic bacteria with reduced genomes (McCutcheon and Moran, 2012). However, the cause of this acceleration in free-living bacteria seems to be different. According to the streamlining hypothesis, this is a consequence of an increase in the mutation rate due to the loss of repair genes and not a direct consequence of selection. Supporting this hypothesis is the fact that both strains lack the *ada* gene, which encodes 6-*O*-methylguanine-DNA methyltransferase among other repair genes (Dufresne et al., 2005). In addition, the lack of this gene can lead to G:C to A:T transversions which, as discussed above, can be adaptive in a low phosphorus environments (Mackay et al., 1994; Dufresne et al., 2005). Nevertheless, differing from the marine picocyanobacteria described above, "*Ca.* Pelagibacter ubique" codes for the DNA repair enzyme 6-*O*-methylguanine-DNA methyltransferase while showing a G+C content as low as 29% (Giovannoni et al., 2005). This suggests that the loss of this enzyme is not a necessary prerequisite to evolve high levels of A+T and that the direct action of selection favoring high levels of A+T could be the cause.

INCREASED RATE OF MUTATION HYPOTHESIS

An alternative explanation for genome reduction has been proposed which includes the previously mentioned accelerated rates of protein evolution for *P. marinus* strains (Marais et al., 2008). This explanation suggests that genome reduction occurs as a byproduct when an increased mutation rate becomes advantageous, like in the cases of novel niche colonization. And indeed, *P. marinus* MED4 and SS120 colonized high-light and low-light niches of the water column respectively between 150 and 80 million years ago (Dufresne et al., 2005).

The argument is as follow. According to classical population genetic models, the fate of an allele is determined by selection if the product of the effective population size (N_e) by the coefficient of selection (s) is larger than one (i.e., $N_e\, s > 1$); and is determined by genetic drift if it is smaller than one: $N_e\, s < 1$ (**Figure 2**; Gillespie, 1998). However, this model applies only when the mutation rate (μ) is negligible (Marais et al., 2008).

When mutation rate is not negligible and taken into account, the equilibrium frequency of the fittest allele becomes (1 μ/s) if $\mu < s$, and 0 if $\mu > s$. This is according to a simple model developed by Eigen (1971). Therefore, when the mutation rate is larger than the selection coefficient, the fittest allele will have an equilibrium frequency of 0 and therefore, will be lost in the population (Marais et al., 2008).

As mention above, high mutation rates can be advantageous when bacteria colonize new habitats. In natural populations some strains often develop increased mutation rates compared to the wild type due to the loss of repair genes. These strains are called mutator strains. Accordingly, mutator strains were selected during novel niche colonization by *P. marinus* MED4 and SS120. These increased μ over s, and favored the loss of genes that have only modest contribution to fitness thus reducing the genome (Marais et al., 2008).

In agreement with this hypothesis, the proteins from MED4 and SS120 *Prochlorococcus* have similar sizes to their homologs from *Prochlorococcus* with larger genomes. This is contrary to what would be expected if natural selection directly favored genome minimization as predicted by the streamlined theory (Marais et al., 2008). However, the same pattern can be accounted by the streamlining hypothesis if natural selection has not been strong enough to select for smaller proteins.

THE BLACK QUEEN HYPOTHESIS

Our discussion of the mechanisms of genome reduction would not be complete if we did not include a recent illuminating proposal known as the Black Queen Hypothesis (BQH; Morris et al.,

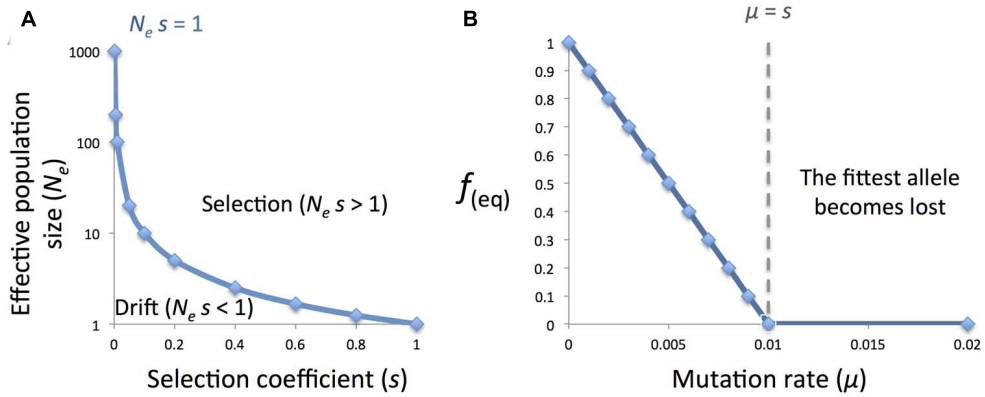


FIGURE 2 | Population genetic models. (A) The fate of an allele is determined by natural selection if the product of the effective population size (N_e) and the coefficient of selection (s) is larger than one, and by genetic drift otherwise; **(B)** however, when mutation is taken into account, the equilibrium frequency (f_{eq}) of the fittest allele is 0 when the mutation rate (μ) is larger than the selection coefficient (s).

2012). The BQH introduces gene loss and thus genomic reduction as a community-dependent adaptive event. In order for genomic reduction to occur according to the BQH there are three main components required in a community: a public good (PG), a helper and beneficiary organisms. A PG is a function or product that is energetically or nutritionally expensive to do or make, and that it is required and accessible by the whole community and not only by the producing organism. A helper organism is an organism capable of producing the PG and whether actively or passively is capable of leaking it to all the community. A beneficiary is an organism which utilizes the PG but becomes incapable of producing it itself. Genome reduction occurs in the beneficiary, and accordingly, there must be a selective advantage to lose the function and thus, the genes that code for it. Importantly, the benefit of losing the function is frequency-dependent, thus once the function becomes too scarce it is no longer advantageous to lose it, such that the presence of helpers is guaranteed in the population and permits the function to remain active for all the community (**Figure 3**; Morris et al., 2012).

As pointed out by Morris et al. (2012) and Sachs and Hollowell (2012), *Prochlorococcus* strains cannot grow axenically in its own habitat. This is because these bacteria lack the gene for catalase-peroxidase to eliminate hydrogen peroxide that is produced by photooxidation up to toxic levels by sunlight. For *Prochlorococcus* (a beneficiary) to grow under these conditions, it is necessary the presence of other bacteria (a helper) that code for catalase-peroxidase (a PG) to detoxify the environment. This is possible because hydrogen peroxide is permeable, thus the helper organisms catalyze the removal of hydrogen peroxide and reduces natural concentrations below the toxic level in marine environments (Morris et al., 2012).

Another example is that of highly reduced bacteria "*Ca.* Pelagibacter ubique." Similarly as the above, this bacterium could not grow on artificial media until recently, when Carini et al. (2013) were capable of uncovering the mystery behind this organism amazing nutritional strategy. They found that this bacterium uses a balanced supply of organic matter, which included methionine, glycine, and pyruvate. These could be replaced by other metabolites, which included some common osmolytes. As such, they suggested that "*Ca.* Pelagibacter ubique" had evolved to efficiently utilize various low molecular weight metabolites of phytoplankton origin produced in low but continuous concentrations (Carini et al., 2013). Other suggested examples of genetic loss by BQH are related to nitrogen fixation, inorganic nutrient acquisition and biofilm matrix deposition but is likely that the list is much longer (Morris et al., 2012). Therefore, reductive genome evolution has to be re-analyzed at the light of the BQH. The community-dependent nature of the BQH would also suggest an important role in understanding the evolution of host-associated prokaryotes.

HOST-ASSOCIATED PROKARYOTES WITH REDUCED GENOMES

Symbiosis can be defined as "*an intimate, close association between species in which the large majority or entire life cycle of one species occurs within or in very close association with another*" (Holland and Bronstein, 2008). As we mentioned earlier, symbiotic organisms tend to evolve smaller genomes amongst which host-associated intracellular mutualistic prokaryotes from different phyla have evolved the smallest cellular genomes known (**Table 1**). Although the term symbiosis is sometimes confounded with mutualism, this intimate and close association can be mutualistic or not. For intracellular prokaryotes these relationships are of parasitic, commensal as well as mutualistic nature. In particular, the biology of mutualistic prokaryotes with highly reduced genomes is strikingly diverse. Moreover, recent advances in genome sequencing have revealed novel and unexpected evolutionary trends, as briefly reviewed below.

EARLY OBLIGATED INTRACELLULAR SYMBIOSIS

The transition from free-living to endosymbiotic mutualistic lifestyle was recently studied by comparative genomics of free-living bacteria and their counterparts, living either in a protist or in insects (weevils or aphids; Clayton et al., 2012; Boscaro et al., 2013; Manzano-Marín and Latorre, 2014; Oakeson et al., 2014). These studies showed that in both cases, gene inactivation,

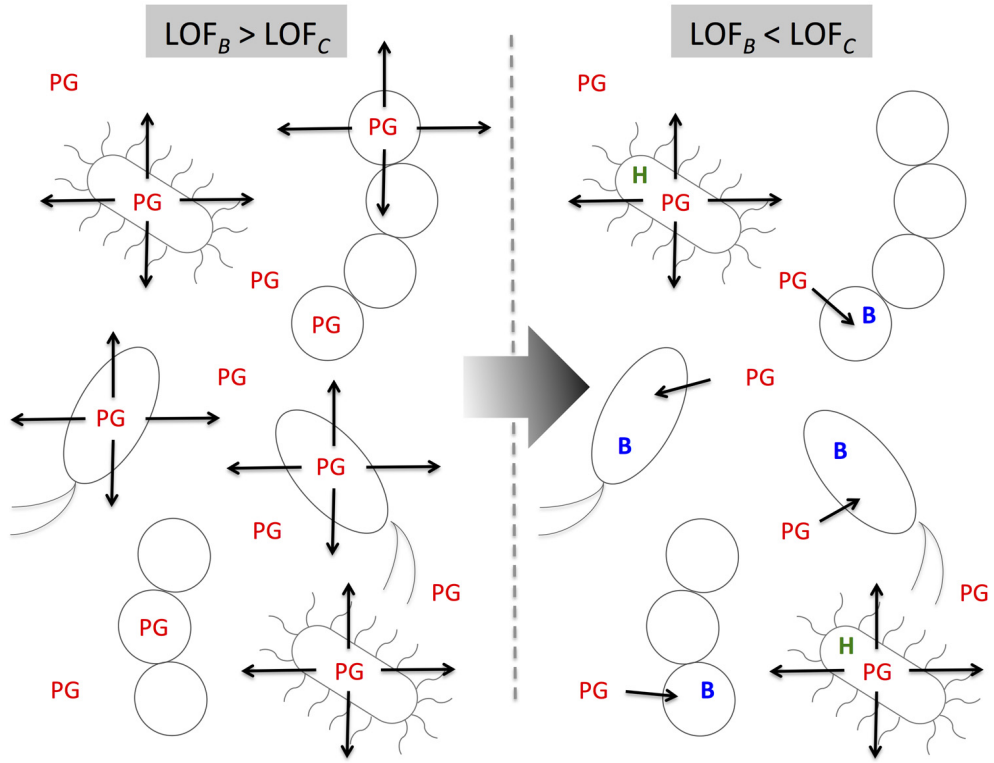


FIGURE 3 | The Black Queen Hypothesis predicts that if there is a community where different species produce an expensive and diffusible public good (PG), the system will evolve toward a scenario where only a few of their members will continue with the production of the PG, only if the benefit of losing the production of the function outweighs the cost of losing it. In such community producers of the PG become helpers (H) and the rest become beneficiaries (B). LOF_B, benefit of losing the function producing the PG; LOF_C, cost of losing the function producing the PG.

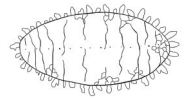
genome rearrangement and loss of some of the repair mechanisms played important roles. However, there were also important differences. In particular, there is an extreme proliferation of MGEs in symbiotic bacteria associated to the early stages of the integration process in insects, but not in the bacteria associated to the protist. This difference was attributed to the fact that in the case of the insect, the population of the bacteria undergoes recursive bottlenecks that lower the efficacy of selection allowing the proliferation of MGEs (Mira et al., 2001). Furthermore, the importance of mobile elements in the transition from free-living to an obligate endosymbiotic state involves their participation in gene inactivation and genome size reduction in recent endosymbiont genomes, as observed from comparative studies between ancient endosymbionts having lost all mobile elements, and related free-living bacteria, with a controlled number of these, presumably by natural selection (Manzano-Marín and Latorre, 2014; Oakeson et al., 2014).

Observational as well as experimental data indicate that genome reduction in host-associated bacteria occurs at a fast rate once the symbiosis is obligate. For instance, it is estimated that the obligate endosymbiont of the rice weevil *Sitophilus oryzae*, "*Candidatus* Sodalis pierantonius" str. SOPE, lost 55% of their genes in just 28,000 years (Oakeson et al., 2014). In agreement with previous observation, experimental evidence shows that under laboratory conditions, similar to those of intracellular bacteria (strong bottlenecks and absence of HGT), genome reduction can occur very rapidly on an evolutionary time scale (Nilsson et al., 2005).

"*Candidatus* Sodalis pierantonius" str. SOPE lives inside the rice weevil's bacteriocytes and has a relatively large genome coding for about 2,309 protein-coding genes and 1,771 pseudogenes. Perhaps the most striking characteristic of the genome of this bacterium is that it is plagued with MGEs, about 18% of the genome consists of ISs. These MGEs have contributed in this organism to genome rearrangement, partial genome duplications, deletogenic rearrangements and to ∼10% of gene inactivation. This is dramatically exemplified by the constant perturbations of the G+C skew in its chromosome that reveals multiple changes in leading versus lagging strand orientation (Oakeson et al., 2014).

Early evolution of intracellular mutualistic symbiosis was also studied in aphids. Manzano-Marín and Latorre (2014) compared the genome of bacteria in different stages of the process of adaptation to intracellular life-style. This comparison included three strains of "*Candidatus* Serratia symbiotica" which represented an early facultative stage ("*Ca.* Serratia symbiotica" from *Acyrthosiphon pisum,* SAp) a later facultative bordering on early obligate stage ("*Ca.* Serratia symbiotica" from *Cinara tujafilina,* SCt), and an co-obligate stage ("*Ca.* Serratia symbiotica" from *Cinara cedri,* SCc). Strain SCc has obligate endosymbiotic characteristics, such as the lack of MGEs, high A+T content

Table 1 | Mutualistic prokaryotes with reduced genomes.

Symbiotic prokaryotes	Taxonomy	Protein-coding genes	RNA-coding genes	Genome size (Kbp)	Host	
Nasuia deltocephalinicola str. NAS-ALF *Sulcia muelleri* str. Sulcia-ALF	β-Proteobacteria Flavobacteriia	137 188	32 35	112 191	*Macrosteles quadrilineatus* (aster leafhopper)	
"*Ca.* Tremblaya princeps" PCVAL "*Ca.* Moranella endobia" PCVAL	β-Proteobacteria γ-Proteobacteria	116 411	20 47	139 538	*Planococcus citri* (citrus mealybug)	
"*Ca.* Hodgkinia cicadicola" Dsem "*Ca.* Sulcia muelleri" SMDSEM	α-Proteobacteria Flavobacteriia	169 242	19 33	144 277	*Diceroprocta semicincta* (cicada)	
"*Ca.* Carsonella ruddii" PV	γ-Proteobacteria	182	31	160	*Pachypsylla venusta* (psyllid)	
"*Ca.* Sulcia muelleri" GWSS *Baumannia cicadellinicola* Hc	Flavobacteriia γ-Proteobacteria	227 595	36 46	246 686	*Homalodisca coagulata* (sharpshooter)	
"*Ca.* Zinderia insecticola" CARI "*Ca.* Sulcia muelleri" CARI	β-Proteobacteria Flavobacteriia	202 246	29 34	209 277	*Clastoptera arizonana* (spittlebug)	
"*Ca.* Walczuchella monophlebidarum" Enterobacterial endosymbiont	Flavobacteriia γ-Proteobacteria	271¶ ns	36¶ ns	309 ns	*Llaveia axin axin* (scale insect "Niij")	
"*Ca.* Carsonella ruddii" CE Secondary endosymbiont of *Ctenarytaina eucalypti*	γ-Proteobacteria γ-Proteobacteria	190 918	31 45	163 1441	*Ctenarytaina eucalypti* (psyllid)	

(Continued)

Table 1 | Continued

Symbiotic prokaryotes	Taxonomy	Protein-coding genes	RNA-coding genes	Genome size (Kbp)	Host	
"*Ca.* Carsonella ruddii" HC Secondary endosymbiont of *Heteropsylla cubana*	γ-Proteobacteria γ-Proteobacteria	192 576	31 44	166 1122	*Heteropsylla cubana* (psyllid)	
"*Ca.* Uzinura diaspidicola" ASNER	Flavobacteriia	227	35	263	*Aspidiotus nerii* Bouché (oleander scale)	
"*Ca.* Portiera aleyrodidarum" BT-QVLC "*Ca.* Hamiltonella defensa" MED	γ-Proteobacteria γ-Proteobacteria	246 1474	38 42	357 1843	*Bemisia tabaci* (Mediterranean whiteflies)	
Buchnera aphidicola BCc *Serratia symbiotica* str. 'Cinara cedri'	γ-Proteobacteria γ-Proteobacteria	362* 672	37 42	422* 1763	*'Cinara cedri'* (cedar aphid)	
Blattabacterium cuenoti Cpu	Flavobacteriia	548*	38	610*	*Cryptocercus punctulatus* (wood roaches)	
"*Ca.* Riesia pediculicola" USDA	γ-Proteobacteria	555*	40	582*	*Pediculus humanus corporis* (body louse)	
Profftella armatura "*Ca.* Carsonella ruddii" DC	β-Proteobacteria γ-Proteobacteria	372* 207	37 31	465* 174	*Diaphorina citri* (Asian citrus psyllid)	
Nanoarchaeum equitans Kin4-M *Ignicoccus hospitalis* KIN4/I	Phylum: Nanoarchaeota Thermoprotei	540 1434	45 53	491 1298	*Ignicoccus hospitalis* KIN4/I	

(Continued)

Table 1 | Continued

Symbiotic prokaryotes	Taxonomy	Protein-coding genes	RNA-coding genes	Genome size (Kbp)	Host	
Ishikawaella capsulata Mpkobe	γ-Proteobacteria	623*	48	755*	*Megacopta punctatissima* (stinkbug)	
Blochmannia vafer BVAF	γ-Proteobacteria	587	42	723	*Camponotus vafer* (ant)	
Wigglesworthia glossinidia endosymbiont of *Glossina morsitans* *Sodalis glossinidius* str. morsitans	γ-Proteobacteria γ-Proteobacteria	618 2516*	44 91	720 4293*	*Glossina morsitans* (tsetse fly)	
"*Ca.* Kinetoplastibacterium oncopeltii" TCC290E	β-Proteobacteria	694	52	810	*Strigomonas oncopelti* (tripanosome)	
"*Ca.* Endolissoclinum faulkneri" L2	α-Proteobacteria	1498	48	1481	*Lissoclinum patella* (tunicate)	
"*Ca.* Endomicrobium sp. Rs-D17"	Phylum: Elusimicrobia	776*	48	1149*	*Trichonympha agilis* (termite protist)	
"*Ca.* Azobacteroides pseudotrichonymphae genomovar CFP2"	Bacteroidia	852*	42	1225*	*Pseudotrichonympha grassii* (termite protist)	
Wolbachia endosymbiont TRS of *Brugia malayi*	α-Proteobacteria	805	37	1080	*Brugia malayi* (filarial nematode)	

(Continued)

Table 1 | Continued

Symbiotic prokaryotes	Taxonomy	Protein-coding genes	RNA-coding genes	Genome size (Kbp)	Host	
"*Ca.* Vesicomyosocius okutanii" Ha	γ -Proteobacteria	937	38	1022	*Calyptogena okutanii* (deep-sea clam)	
"*Ca.* Ruthia magnifica" str. Cm	γ-Proteobacteria	976	41	1161	*Calyptogena magnifica* (giant clam)	
Cyanobacterium sp. UCYN-A	Subclass: Oscillatoriophycideae	1199	42	1440	Prymnesiophyte (unicellular eukaryote)	–
"*Ca.* Midichloria mitochondrii" IricVA	α-Proteobacteria	1211	38	1184	*Ixodes ricinus* (tick)	
Polynucleobacter necessarius subsp. necessarius STIR1	β-Proteobacteria	1279[†]	44	1560	*Euplotes aediculatus* (ciliated protist)	

The information of genes and gene size was obtained from Genbank. The class is shown in taxonomy unless specified. "ns": values unknown from not-sequenced organism. "": Values include the gene content and genomic size of strain specific plasmids. "¶": Values as reported in the original article (Rosas-Pérez et al., 2014). "[†] ": Value as reported in the original article (Boscaro et al., 2013). Host drawings are kindly provided by Sofia Delaye.*

and no genetic redundancy. However, it possesses large intergenic regions, remnants of ancient pseudogenes still not degraded (Lamelas et al., 2011a). On the other hand strain SCt, while being phylogenetically and genomically very closely related to the facultative *SA*p strain (Burke and Moran, 2011), it shows a variety of metabolic, genetic and architectural features which point toward this endosymbiont being one step closer to an obligate intracellular life-style. By studying the genome rearrangements and the impact that MGEs have had on the genome architecture of these two *Serratia* endosymbionts (SCt and SAp), it was determined that those genes belonging to IS families have been the key factor promoting massive rearrangements. These MGEs have also mediated inactivation in various genes, sometimes creating long stretches of inactivated proteins in tandem (Manzano-Marín and Latorre, 2014).

In the case of bacteria associated to the protist, the genome sequences of free-living and symbiotic strains of the β-proteobacteria *Polynucleobacter necessarius* were compared. The free-living strain is a common inhabitant of lentic freshwater ecosystems, while the symbiotic strain lives as an intracellular symbiont of the ciliated protist *Euplotes aediculatus*. These strains diverged very recently, as shown by their similarity at the level of the 16S rRNA which is >99% (Boscaro et al., 2013). The genome of the free-living strain contains 2,088 protein-coding genes (Meincke et al., 2012) and is itself a reduced genome (Boscaro et al., 2013). The genome of the symbiotic strain contains 1,279 protein-coding genes and is mostly a subset of the free-living strain (i.e., only 105 genes are not shared with its free-living relative). The symbiotic strain also contains between 231 and 460 pseudogenes. Of course, the ultimate cause of this genome reduction is the endosymbiotic lifestyle of *P. necessarius*. However, the proximal cause of the genome reduction is less well understood. Furthermore, the metabolic bases of the obligate symbiosis between the bacteria and the ciliate are not known. However, it was suggested that *P. necessarius* complements some metabolic deficiency in *E. aediculatus* (Boscaro et al., 2013). Nevertheless, it was suggested that genome reduction in the symbiotic strain was caused by illegitimate recombination and loss of mismatch repair genes. In addition it was suggested that the early loss of the gene coding for the translesion DNA polymerase exerted further evolutionary pressure for a smaller genome and favored polyploidy, which is one of the main differences between both strains such that the symbiotic strain contains several nucleoids, each one containing one copy of the genome (Boscaro et al., 2013).

As exemplified by *P. necessarius*, rapid gene loss can occur in the absence of proliferation of MGEs. The endosymbiotic strain of *P. necessarius* has already lost over 40% of its coding capacity with 13–18% still observable as pseudogenes. This loss occurred in the absence of MGEs, and as mentioned above, the lack of MGEs in *P. necessarius* has been attributed to a larger population size relative to that of "*Ca.* Sodalis pierantonius" str. SOPE (Boscaro et al., 2013).

In addition to the previously mentioned examples, the lack of proliferation of MGEs in the case of *P. necessarius* contrast with other obligate symbiosis. These include *Burkholderia rhizoxinica* the endosymbiont of the fungus *Rhizopus microsporus* with 6% of their encoded proteins similar to transposases (Lackner et al., 2011a); the γ1 symbiont of the marine oligochaeta *Olavius algarvensis* that has a genome coding for 20% of transposases (Woyke et al., 2006); NoAz, the extracellular mutualistic endosymbiotic cyanobacteria of the water-fern *Azolla filiculoides* with ∼600 IS elements (Ran et al., 2010); and the facultative symbiont of the whitefly "*Candidatus* Cardinium hertigii" possessing ∼200 MGEs (Santos-Garcia et al., 2014).

Proliferation of MGEs can occur also in the absence of massive genome rearrangement, as shown in the case of the intracellular bacteria "*Candidatus* Amoebophilus asiaticus" that maintains a regular G+C skew along its genome despite that 24% of its genes code for MGEs (Schmitz-Esser et al., 2010, 2011). Nevertheless, this last comparison has to be taken with caution since "*Ca.* Amoebophilus asiaticus" has not adapted recently to the intracellular lifestyle, has received several genes by HGT and as a parasite is under different evolutionary pressures than mutualistic endosymbionts (Schmitz-Esser et al., 2010).

PARADOXICALLY LARGE G+C CONTENT IN TWO HIGHLY REDUCED GENOMES

As in the case of free-living bacteria with reduced genomes, host obligated bacteria with reduced genomes also show large levels of A+T content (Moran, 2003; Moya et al., 2008; Delaye et al., 2010). It is hypothesized that the loss of repair enzymes due to genome reduction, in combination with a reduced efficacy of natural selection, and a universal G:C to A:T mutational bias in bacteria, is responsible of the observed trend (Hershberg and Petrov, 2010; Hildebrand et al., 2010; Van Leuven and McCutcheon, 2012).

However, there are notable exceptions to the above rule. The cicada *Diceroprocta semicincta* contains in its bacteriome two symbiotic bacteria, the α-Proteobacteria "*Candidatus* Hodgkinia cicadicola" and the Flavobacteria "*Candidatus* Sulcia muelleri" (McCutcheon et al., 2009a,b). The genome of "*Ca.* Hodgkinia cicadicola" is in itself a paradox for molecular evolutionary theory because it has relatively large G+C content (∼58%) despite having an extremely small genome (∼144 kbp). This high G+C content is not due to biased gene conversion, since this bacterium lacks repair enzymes. Also, the high G+C content is not due to an A:T to G:C mutational bias since it is known that "*Ca.* Hodgkinia cicadicola" suffers from the same G:C to A:T mutational pressure universally present in bacteria (Van Leuven and McCutcheon, 2012).

One possibility that was suggested to explain this unexpectedly large G+C content is that the demography of its host *D. semicincta* inflates the population size of "*Ca.* Hodgkinia cicadicola" making natural selection more efficient to counter balance the G:C to A:T mutational bias (Van Leuven and McCutcheon, 2012). However, it is not clear what benefit could confer single G:C over A:T polymorphisms in these bacteria. Other possibilities are selection for better DNA replication and/or DNA packing on C+G rich genomes (Hershberg and Petrov, 2010). A similar situation is found in "*Candidatus* Tremblaya princeps," symbiont of the citrus mealybug *Planococcus citri*, which also shows an extremely reduced genome of ∼139 kbp and a relatively high G+C content (McCutcheon and von Dohlen, 2011; López-Madrigal et al., 2013b).

UNEXPECTED LOSS OF GENOMIC STABILITY

During the early stages of reduction, genome architecture is quite unstable as discussed above. Many rearrangements occur over relatively short periods of time. As an example of this, the comparison of the genome architectures of six free-living *Serratia* and three "*Ca.* Serratia symbiotica" endosymbionts, showed surprising amounts of genomic rearrangements suffered between the three *S. symbiotica* lineages (Manzano-Marín and Latorre, 2014).

However, as the endosymbiosis evolves toward the last stages of reduction, genomic stability increases to a stalemate such that gene order conservation was considered one of the hallmarks of the genomes of obligate mutualistic bacteria. This was first noticed among genomes of *Buchnera aphidicola* from different strains with more than 50 million years of divergence. Initially, this high degree of conservation in gene order was attributed to absence of the *recA* gene in these bacteria. Its product, RecA, is the key enzyme in homologous recombination repair (Shigenobu et al., 2000; Tamas et al., 2002; van Ham et al., 2003).

Homologous recombination is a high-fidelity DNA repair mechanism for double strand break. The broken DNA is processed at the ends by several possible pathways producing a 3′-tailed duplex onto which RecA is loaded. RecA is an ATP-dependent multifunctional enzyme, which has recombinase activity. RecA assembles into a filament and then sequesters the template double stranded DNA, where it looks for the homologous loci, exchanges DNA strands and forms joints between recombining molecules which allows the recombination and repair of the broken chromosome (Spies, 2013; Wigley, 2013).

However, the hypothesis that genome stasis is the result of loss of *recA* lost support when it was found that genome sequences from other endosymbionts like *Blattabacterium*, *Carsonella,* and *Wigglesworthia* showed similar levels of synteny conservation despite coding for *recA* (reviewed in Sloan and Moran, 2013). Additionally, recent genome sequencing projects showed that lack of repair and recombination genes may not be the cause of genome stability. "*Candidatus* Portiera aleyrodidarum" the primary endosymbiont of whiteflies (*Bemisia tabaci*) shows genome structural polymorphisms (i.e., lack of synteny) despite lacking *recA* and having one of the most reduced repair and recombination gene sets. These polymorphisms are demonstrated to be present even within bacteria inhabiting individual hosts and likely within individual bacterial cells. The presence of such structural polymorphisms was attributed to recombination events between large intergenic regions and repetitive elements that in turn are maintained by gene conversion (Sloan and Moran, 2012b, 2013).

A similar case of loss of genome stability was found in "*Ca.* Tremblaya princeps." This bacterium shows a 7,032 bp region flanked by inverted repeats that is found in both orientations in the population. And similar to "*Ca.* Portiera aleyrodidarum," this bacterium also codes for a highly reduced set of DNA replication, recombination and repair enzymes (McCutcheon and von Dohlen, 2011; López-Madrigal et al., 2013b).

NOVEL HYPOTHESIS TO EXPLAIN THE REASSIGNMENT OF STOP TO Trp CODON

The obligate endosymbionts "*Candidatus* Nasuia deltocephalinicola," "*Candidatus* Zinderia insecticola," and "*Ca.* Hodgkinia cicadicola" evolved an alternative genetic code in which the codon UGA is reassigned from coding to stop (UGA_{stop}) to code for tryptophan (UGA_{Trp}). This codon reassignment has been observed also in mycoplasmas and some mitochondrial genomes (McCutcheon et al., 2009a; Bennett and Moran, 2013).

The evolution from UGA_{stop} to UGA_{Trp} has been explained with the "capture" hypothesis (**Figure 4**). According to this model, all UGA codons mutate first to its synonymous codon UAA in A+T rich genomes. This change does not affect protein length or fitness. Then, when UGA re-appears through mutation, it is free to be "captured" by an amino acid, in this case Trp. The fact that almost all reassignments of the UGA_{stop} to UGA_{Trp} evolved in A+T rich genomes supports this hypothesis (Osawa and Jukes, 1989).

Nonetheless, "*Ca.* Hodgkinia cicadicola" has a relatively high G+C content of about 58.4% making the "capture" hypothesis an unlikely explanation for this phenomenon. Therefore, a new hypothesis was proposed to explain the reassignment of UGA_{stop} to UGA_{Trp} in this bacterium. The new hypothesis proposes that the loss of translational release factor RF2 (encoded by *prfB*) that recognizes UGA as a stop codon triggered the evolution of this rearrangement (McCutcheon et al., 2009a). According to this scenario, the loss of *prfB* caused that some proteins were translated with an extended sequence. These "extended" proteins were not necessarily lethal, perhaps just slightly deleterious. This is supported by experiments showing that extended proteins can increase fitness under stress conditions in yeast (Halfmann et al., 2012). Since symbiotic bacteria from insects are subject to recurrent bottlenecks, these slightly deleterious mutations become fixed by genetic drift under a Mullerian ratchet process. Then, once they are fixed, natural selection favored replacement of UGA_{Stop} codons with functional UAA or UAG, thus restoring the original size of proteins. This in turn allows UGA to be captured by tRNA-Trp (McCutcheon et al., 2009a).

CLUES TO EARLY LIFE?

The genome of "*Candidatus* Riesia pediculicola" the symbiont of the body louse *Pediculus humanus corporis*, codes for what seems to be a minimal tRNA decodification set (Kirkness et al., 2010). This endosymbiont lost all the enzymes that modify the tRNA body, and kept only those genes that make modifications of the anticodon-stem-loop which are essential for mRNA decoding. Therefore, it was suggested that the minimal tRNA decodification set of this bacterium could resemble that of very ancient cells that existed during the early evolution of life on Earth (Kirkness et al., 2010). In support of this hypothesis, pseudouridine synthase A (encoded by *truA*), which is the enzyme responsible of the formation of pseudouridine at positions 38, 39, and 40 in the anticodon stem loop of tRNAs was suggested to be present in the last common ancestor of all extant life, or *cenancestor* (Ouzounis et al., 2006). However, at this moment, there is no evidence that the rest of the enzymes of the tRNA codification set of "*Ca.* Riesia pediculicola" are as ancient as *truA*. Nevertheless, this tRNA modification set exemplifies how simpler cellular systems can work. Which is highly relevant for synthetic biology approaches to the minimal cell. Whether other

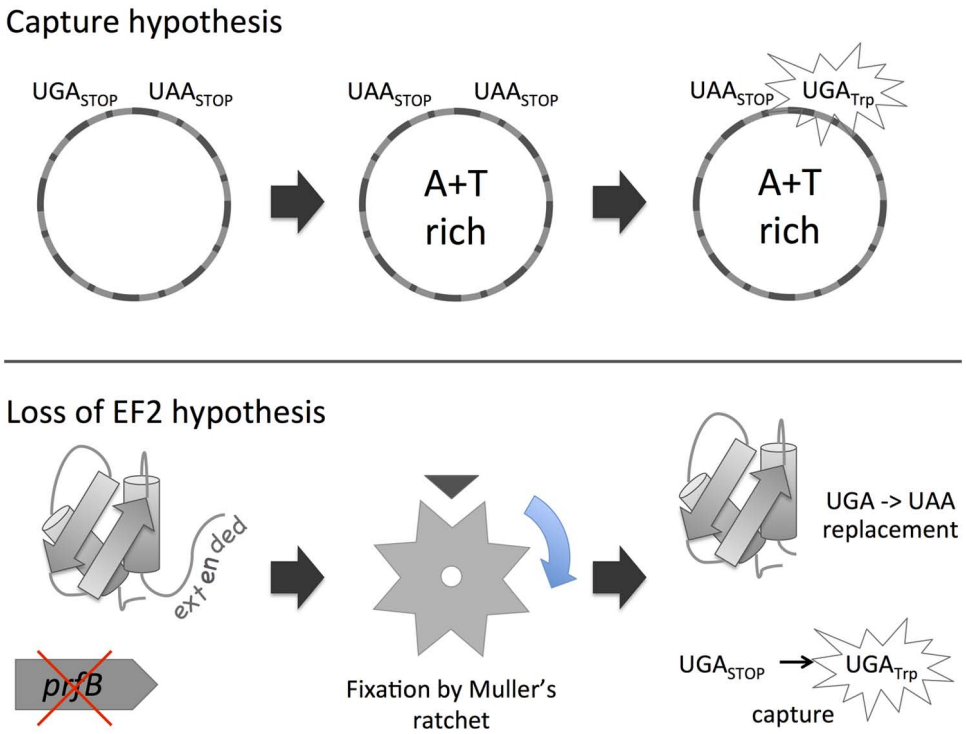


FIGURE 4 | The reassignment of stop codon (UGA$_{stop}$) to code for tryptophan (UGA$_{Trp}$) is explained using the "capture hypothesis." However, the large G+C content in "*Ca.* Hodgkinia cicadicola" makes the capture hypothesis unlikely in this organism. Instead, it is hypothesized that the lose of translational release factor RF2 triggered the evolution of this rearrangement.

symbionts offer clues to early life on Earth still has to be carefully discussed.

THE ROLE OF HORIZONTAL GENE TRANSFER IN THE EVOLUTION OF INTRACELLULAR SYMBIOSIS

One of the most striking peculiarities of host-associated bacteria with reduced genomes is how these organisms perform their symbiotic function and all the necessary process to maintain themselves with such a reduced gene set. There are at least three non-mutually exclusive possibilities (McCutcheon and von Dohlen, 2011). On the first place, modifications of some genes coded in the reduced genome could allow the endosymbiont to cope with the loss of otherwise essential genes; second, the presence of complementary genes in the genomes of co-symbionts (if any) may compensate for gene losses in the endosymbiont; and third, genes coded in the genome of the host compensate for gene losses in the genome of the endosymbiont. From an evolutionary point of view, this last group of genes could be of host origin, or originally from the endosymbiont and transferred to the host, or horizontally transferred from unrelated organisms not participating in the symbiosis to the host genome or its endosymbionts (McCutcheon, 2010; McCutcheon and von Dohlen, 2011).

Horizontal gene transfer is one of the main forces in prokaryotic evolution (Zhaxybayeva and Doolittle, 2011). Recent discoveries show that HGT has played a role in the evolution of some obligate mutualistic symbiosis. For instance, "*Ca.* Carsonella ruddii" the obligate symbiont from the psyllid *Pachypsylla venusta* has one of the smallest genomes with 213 genes and ∼160 kbp. This organism lives in the absence of other co-symbionts (Nakabachi et al., 2006). And as expected for such a small genome, a detailed analysis of its gene content indicated that several functions considered essential for a cell are missing (Tamames et al., 2007), raising the question of how these bacteria accomplish its symbiotic function. A recent transcriptomic analysis showed that the biosynthesis of essential and non-essential amino acids is performed collaboratively by the symbiont and by genes expressed in the bacteriocytes some of them of bacterial origin, and at least one of them directly acquired from "*Ca.* Carsonella ruddii" (Sloan et al., 2014).

Similarly, the genome of the pea aphid (*A. pisum*) does contain genes of bacterial origin that are highly expressed in the bacteriocytes and likely participate in the symbiosis with *B. aphidicola* Aps (Nikoh and Nakabachi, 2009; The International Aphid Genomics Consortium, 2010). These functional genes in *A. pisum* were acquired from bacteria other than its primary endosymbiont *B. aphidicola* Aps (Nikoh et al., 2010). Noteworthy, it was recently shown that the protein RplA4, coded by one of these genes, is targeted to the cytoplasm of the *B. aphidicola* Aps (Nakabachi et al., 2014). A finding that has been interpreted as blurring the distinction between endosymbionts and organelles (McCutcheon and Keeling, 2014). In addition, the only genes of *B. aphidicola* Aps origin in the genome of *A. pisum* are two highly truncated pseudogenes (Nikoh et al., 2010). Furthermore, experimental evidence has shown that the biosynthesis of some of the essential amino acids provided by *B.*

aphidicola is performed partially by host enzymes (Russell et al., 2013).

Another case is found in the citrus mealybug *P. citri* where at least six distinct lineages of bacteria contributed with horizontally transferred genes to its nucleus. These genes code for protein products that complement the biosynthesis of essential amino acids, vitamins and peptidoglycan in their endosymbionts "*Ca.* Tremblaya princeps" and "*Candidatus* Moranella endobia" (Husnik et al., 2013).

Also, HGT contributed to the acquisition of toxicity in other symbiotic systems. The genome of "*Candidatus* Profftella armatura" the symbiont of the Asian citrus psyllid (*Diaphorina citri*) acquired by HGT genes for the synthesis of cytotoxic polyketides. In this tripartite symbiosis, "*Ca.* Profftella armatura" produces the polyketides, while another bacterium from the genus *Carsonella* provides the host with essential amino acids (Nakabachi et al., 2013).

The recent genome sequencing of the filarial nematode *Brugia malayi* showed that approximately 10.6% of the genome of its symbiont, *Wolbachia* wBM has been transferred to the eukaryotic genome. Interestingly, there is evidence that some of the genes coded in these regions are transcribed in particular stages of the life cycle of the nematode suggesting functionality. However, their role in symbiosis still has to be determined (Ioannidis et al., 2013).

And finally, the synthesis of essential amino acids in symbiont harboring trypanosomes is carried in part by genes of bacterial origin coded in the genome of the protist (Alves et al., 2013). These data clearly shows that HGT is an important force in the evolution of some intracellular symbiosis (Sloan et al., 2014).

However, not all the symbiosis shows evidences of HGT. The genome sequence of the body louse *P. h. corporis* does not appear to contain any genes of prokaryotic origin, indicating no transfer from its endosymbiont "*Ca.* Riesia pediculicola" strain USDA, nor other bacteria (Kirkness et al., 2010). Similarly, "*Candidatus* Endolissoclinum faulkneri" a defensive symbiont that also produces cytotoxic polyketes and that inhabits *Lissoclinum patella*, a colonial filter-feeding tunicate, does not seem to have acquired this capacity through HGT (Kwan et al., 2012).

BIOCHEMICAL COMPLEMENTARITY AND CONVERGENT EVOLUTION OF CO-RESIDENT SYMBIONTS

In cases where there are more than one species of obligate mutualistic symbiont, the biosynthesis of relevant metabolites for the host often requires the participation of enzymes that are coded in both co-symbionts and in some cases, as reviewed above, in the insect host (McCutcheon and von Dohlen, 2011). Furthermore, there are some occasions where different symbiotic systems conformed by two co-symbionts have converged independently to the same division of labor regarding the biosynthesis of amino acids that are provided to their hosts.

In this sense *B. aphidicola* BCc, from the aphid *C. cedri*, requires the co-symbiont "*Ca.* Serratia symbiotica" to provide Trp to its host. In this symbiotic system, the first two genes of Trp biosynthesis (*trpEG*) are located in a plasmid in *B. aphidicola* BCc, while the rest of genes (*trpDCBA*) are located on the main chromosome of "*Ca.* Serratia symbiotica" (**Figure 5**; Pérez-Brocal et al., 2006; Gosalbes et al., 2008). The same phenomena occurs in the symbiotic system of the psyllid *Heteropsylla cubana*, where one of

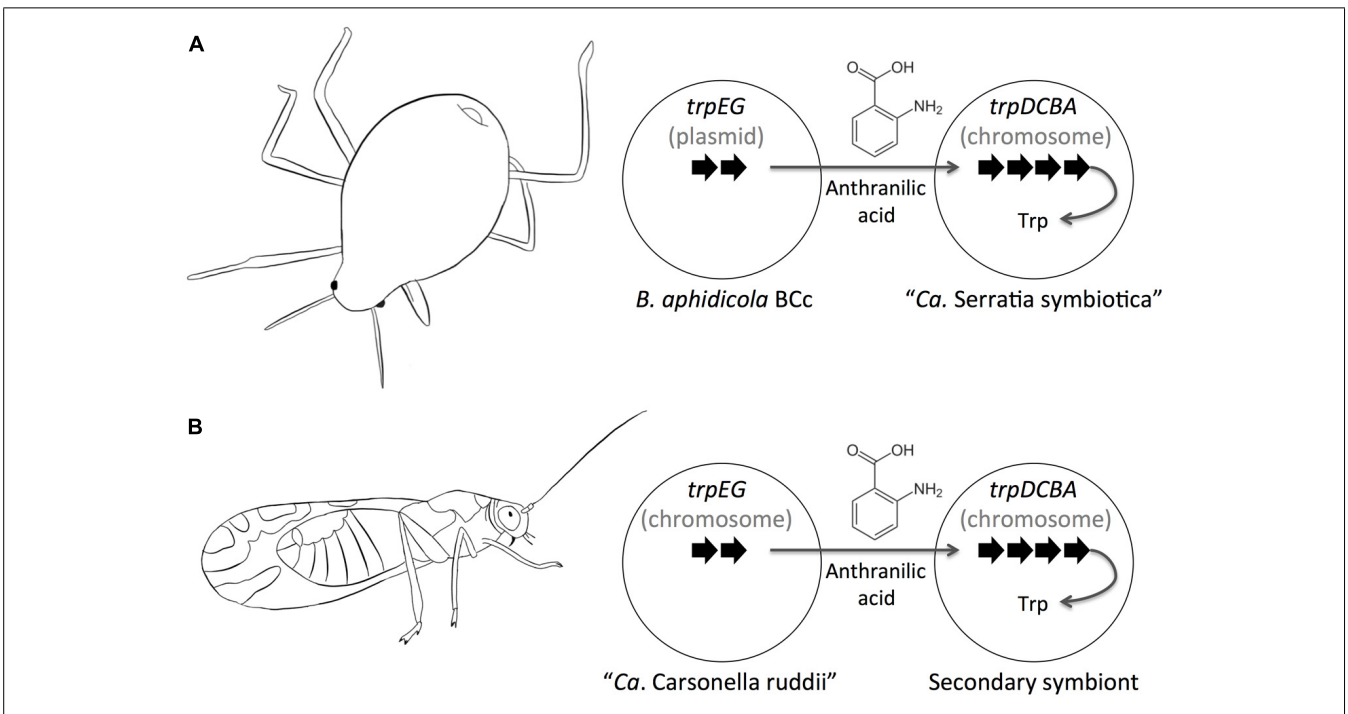


FIGURE 5 | Convergent evolution of Trp biosynthesis. The biosynthesis of Trp is performed cooperatively by two different endosymbionts in the aphid *Cinara cedri* **(A)** and in the psyllid *Heteropsylla cubana* **(B)**. Strikingly, the same division of labor evolved in both systems.

the symbionts (tentatively classified as a secondary symbiont) lost nearly all genes for the biosynthesis of essential amino acids except those of the (*trpDCBA*) operon, however, complementing the Trp biosynthetic capabilities of "*Ca.* Carsonella ruddii" HC (Sloan and Moran, 2012a). As exemplified above, the secondary symbiont of *H. cubana* and "*Ca.* Serratia symbiotica" have both evolve convergently to code for the same genes required for the biosynthesis of Trp (*trpDCBA*).

A similar biosynthesis of Trp is observed in the case of *B. aphidicola* BCt, the symbiont of the aphid *C. tujafilina*, where the first two genes of of Trp biosynthesis (*trpEG*) are also located in a plasmid together with the structural genes for leucine synthesis. However, differing from *B. aphidicola* BCc, the biosynthesis of Trp does not occur cooperatively between two symbionts since the rest of the genes of Trp biosynthesis are located in the main chromosome of *B. aphidicola* BCt (Gil et al., 2006; Lamelas et al., 2011b).

Another remarkable case of metabolic complementarity is provided by the co-symbionts *Baumannia cicadellinicola* and "*Ca.* Sulcia muelleri" Hc from the xylem-feeding glassy-winged sharpshooter *Homalodisca coagulata*. For instance, *B. cicadellinicola* is able to make cysteine from homoserine and coenzyme A from 2-ketovaline, but is unable to make homoserine and 2-ketovaline. And "*Ca.* Sulcia muelleri" Hc is able to make homoserine from aspartate and 2-ketovaline by the valine biosynthetic pathway. This kind of complementarity extends also to the biosynthesis of fatty acids, where *B. cicadellinicola* has all the genes necessary for the synthesis of fatty acids except for *fabF*, which in turn is encoded in "*Ca.* Sulcia muelleri" Hc. However, it is not clear how these molecules are transported across membranes since "*Ca.* Sulcia muelleri" Hc possesses few transporters (McCutcheon et al., 2009b).

Additionally, "*Ca.* Sulcia muelleri" Hc provides its host with eight of the ten essential amino acids (arginine, phenylalanine, tryptophan, lysine, threonine, isoleucine, leucine, and valine) required by the hosts, while *B. cicadellinicola* produces the remaining two which are methionine and histidine. At the same time, the same scheme of amino acid provision by co-symbionts is found in the cicada *D. semicincta*. Both, *D. semicincta* and *H. coagulata* share the endosymbiont "*Ca.* Sulcia muelleri" which provides the same eight essential amino acids. However, *H. coagulata* and *D. semicincta* differ in their co-symbiont accompanying "*Ca.* Sulcia muelleri." While *H. coagulata* contains *B. cicadellinicola*, *D. semicincta* hosts "*Ca.* Hodgkinia cicadicola." This suggest that *B. cicadellinicola* and "*Ca.* Hodgkinia cicadicola" have evolved convergently to provide methionine and histidine to their host (Wu et al., 2006; McCutcheon et al., 2009b).

It is important to note that, as expected from convergent evolution, there are metabolic differences between *B. cicadellinicola* and "*Ca.* Hodgkinia cicadicola" particularly in the biosynthesis of methionine. While *B. cicadellinicola* uses the cobalamin (vitamin B12)-independent version of methionine synthase, "*Ca.* Hodgkinia cicadicola" uses the cobalamin-dependent version of the enzyme. Both bacteria differ as well on the vitamin and cofactor biosynthetic capabilities (McCutcheon et al., 2009b).

In a similar fashion, the spittlebug *Clastoptera arizonana* contains in its bacteriome "*Ca.* Sulcia muelleri" CARI and "*Ca.* Zinderia insecticola" (McCutcheon and Moran, 2010). And as in the cases described above, both kinds of bacteria are needed to provide with the ten essential amino acids to their host. However, in this case "*Ca.* Sulcia muelleri" CARI cannot make Trp. Instead, this amino acid is synthetized by "*Ca.* Zinderia insecticola" in addition to methionine and histidine (McCutcheon and Moran, 2010).

Finally, the phloem-feeding aster leafhopper (ALF) *Macrosteles quadrilineatus* contains in separated cells within its bacteriome the symbionts "*Ca.* Sulcia muelleri" ALF and "*Ca.* Nasuia deltocephalinicola" ALF (Bennett and Moran, 2013). The genome of "*Ca.* Sulcia muelleri" ALF with 190,733 bp and 188 protein-coding genes is the smallest among sequenced genomes from this genus of bacterial symbionts. And the genome of "*Ca.* Nasuia deltocephalinicola" ALF with 112,091 bp and 137 protein-coding genes is the smallest bacterial genome sequenced so far. Similarly as above, "*Ca.* Sulcia muelleri" ALF codes for the genes necessary to synthetize the same eight essential amino acids and "*Ca.* Nasuia deltocephalinicola" ALF codes for the genes required to produce methionine and histidine. (Bennett and Moran, 2013).

It seems that "*Ca.* Sulcia muelleri" infected the ancestor of a large group of sap-feeding insects (Auchenorrhyncha) including the sharpshooter and cicada >260 million years ago (McCutcheon and Moran, 2007). This is clearly seen by the fact that the genomes of "*Ca.* Sulcia muelleri" from *D. semicincta* and *H. coagulata* are almost collinear despite having diverged several million years ago (McCutcheon et al., 2009b).

Interestingly, *Nasuia* and *Zinderia* appear to be sister clades, which suggested the existence of an ancient lineage of β-proteobacterial endosymbionts hosted at least since the divergence of Cicadomorpha from Fulgoroidea 200 million years ago which are the only two clades in the suborder Auchenorrhyncha. This also suggests the loss and latter independent acquisition of *B. cicadellinicola* and "*Ca.* Hodgkinia cicadicola" in the lineage leading to the sharpshooter and the cicada, respectively. The exchange in hosted symbionts was hypothesized to correlate with the new nutritional needs related to the diversification in the host diet (Bennett and Moran, 2013).

Similarly, metabolic complementation is suggested for the amino acid biosynthesis in the flavobacterium "*Ca.* Walczuchella monophlebidarum" where it was observed that many missing genes and pseudogenes in "*Ca.* Walczuchella monophlebidarum" were present in the γ-proteobacterial (Enterobacteriaceae) co-symbiont (Rosas-Pérez et al., 2014). Similarly as observed in the co-speciation between "*Ca.* Sulcia muelleri" and sap-feeding insects, co-speciation was observed between Flavobacteria and several scale insects but not so with the Enterobacteriaceae symbiont suggesting a similar trend in metabolic complementarity (Rosenblueth et al., 2012).

Metabolic complementarity is also observed in the obligate symbionts of the Asian citrus psyllid *D. citri* which are "*Ca.* Carsonella ruddii" DC and "*Ca.* Profftella armatura." The metabolic capacities of both symbionts are largely non-redundant. For instance, the genome of "*Ca.* Profftella armatura" encodes 16 genes for coenzyme transport and metabolism, including those

for the synthesis of riboflavin and biotin, and the genome of "*Ca.* Carsonella ruddii" DC completely lacks these genes (Nakabachi et al., 2013).

Another extraordinary case of metabolic convergence is provided by *Blattabacterium* strain BGe that is the primary endosymbiont of the German cockroach *Blattella germanica*, and the carpenter ant endosymbionts from the genus *Blochmannia* spp. (López-Sánchez et al., 2009). While *Blattabacterium* strain BGe is a member of the Bacteroidetes, *Blochmannia* spp. belongs to Proteobacteria. Despite the different phylogenetic origin of these bacteria, they resemble each other at the broad level of functional gene categories. A situation not found when *Blattabacterium* is compared with other insect endosymbionts like *Wolbachia* sp. and *Sulcia muelleri*. It seems that both bacteria converged due to the omnivorous diets of their hosts (Feldhaar et al., 2007; Patiño-Navarrete et al., 2014).

Finally, and perhaps one of the most striking examples of complementary is provided by one of the most extreme cases of symbiosis in nature. "*Ca.* Tremblaya princeps," a prokaryote with one of the smallest genomes, that as described above, lives inside the bacteriocytes of the mealybug *P. citri* and contains itself the bacteria "*Ca.* Moranella endobia" (McCutcheon and von Dohlen, 2011; López-Madrigal et al., 2013b). In "*Ca.* Tremblaya princeps" most of its genes are devoted to RNA metabolism, the assembly of iron–sulfur [Fe–S] clusters and to the partial biosynthesis of some essential amino acids. However, its genome does not code for any complete pathway. Therefore, "*Ca.* Tremblaya princeps" seems to depend for almost all basic functions on the coding capacities of "*Ca.* Moranella endobia" and likely from host-encoded proteins. In fact, it was proposed that "*Ca.* Tremblaya princeps" acquires the necessary cell components to function by sporadic cell lysis of "*Ca.* Moranella endobia" (McCutcheon and von Dohlen, 2011). The distribution of coded tRNAs in the genomes of "*Ca.* Tremblaya princeps" and "*Ca.* Moranella endobia" supports this hypothesis (López-Madrigal et al., 2013b). However, immunohistochemistry assays with polyclonal antibodies to identify the location of the channel protein MscL coded only by "*Ca.* Moranella endobia" and GroEL coded by both bacteria, did not find evidence of massive and constitutive protein movement from the cytoplasm of "*Ca.* Moranella endobia" to the cytoplasm of "*Ca.* Tremblaya princeps" (López-Madrigal et al., 2013a).

GENOME REDUCTION IN BACTERIAL SYMBIONTS OF FUNGI, A RELATIVELY UNEXPLORED WORLD

Bacteria and fungi are commonly co-inhabitants of a large variety of niches on Earth. Both groups of microorganisms are the major colonizers of terrestrial and aquatic environments, and both have been revealed as intracellular guests of eukaryotic hosts (Bonfante and Genre, 2008; Moran et al., 2008; Gibson and Hunter, 2010). Despite the fact that bacterial-fungal interactions are ubiquitous and relevant for the industry, agriculture and medicine (Frey-Klett et al., 2011), intracellular bacterial symbionts of fungi remain largely unexplored.

Bacteria living inside fungal cells were first well documented in the AM fungus *Geosiphon pyriformis*. The symbiosis of this fungus with the filamentous cyanobacteria *Nostoc puntiforme* is the only known example of fungal endocyanosis to date. Interestingly, this intracellular symbiosis is cyclical, which means that the incorporation of free-living cyanobacteria within the fungal cytosol occurs periodically and only when the cyanobacteria are in the appropriate developmental state (Mollenhauer et al., 1996; Wolf and Schüßler, 2005). This cyclical transmission resembles the one that exists between plants and rhizobia, and plants and mycorrhizal fungi. *N. puntiforme* has the capability of establishing symbiosis not only with *Geosiphon*, but also with gymnosperm cycad *Macrozamia* sp. (Rippka and Herdman, 1992). Despite its endosymbiotic nature, the genome size of *N. puntiforme* is quite large (8.9 Mb), where 29% of the predicted genes seemed to be unique to this free-living and symbiotic cyanobacteria (Meeks et al., 2001).

Besides the endocyanosis, *G. pyriformis* harbors other type of intracellular bacteria, which were previously referred to as "bacterial-like organisms." Recent molecular studies on these bacterial-like organisms have revealed that these endofungal bacteria belong to a monophyletic clade of the *Mollicutes*, which form a sister group to *Mycoplasmatales* and *Entoplasmatales* (Naumann et al., 2010). These obligate and hereditable bacterial symbionts are widespread in several lineages of AM fungi including *Archeosporales*, *Diversisporales* and *Glomerales*, which suggests that this symbiosis preceded the diversification of AM fungi and thus, that it may be as old (about 400 million years) as the symbiosis of AM fungi with plants (Naumann et al., 2010). Currently, no genomic information about these diverse *Mollicutes*-related symbionts is available. Furthermore, and largely due to their unculturability, their characteristics, functional roles and capacities remain cryptic.

Among AM fungi, the best-studied system in terms of their bacterial endosymbionts is the AM fungus *Gigaspora margarita* BEG34. Pioneering studies on this AM fungus clearly showed that it harbored an intrahyphal, gram-negative and vertically transmitted β-proteobacteria from the *Burkholderiaceae* family named "*Candidatus* Glomeribacter gigasporarum" (Bianciotto et al., 1996, 2003, 2004). In this relationship, the fungus is an obligate symbiont of plants, but facultative with respect to its bacterial symbiont (Desirò et al., 2014b). Studies made with cured/endobacterium-free fungal spores indicated that *G. margarita* BEG34 can survive, without its bacterial partner, albeit showing signs of a diminished ecological fitness (Lumini et al., 2007). However, "*Ca.* Glomeribacter gigasporarum," although it can be extracted from the fungus, it remains unculturable in laboratory settings (Salvioli et al., 2008). It has been shown that in several strains of *G. margarita* both, the gram-positive *Mollicutes*-related bacteria as well as the gram-negative "*Ca.* Glomeribacter gigasporarum," may be present (Desirò et al., 2014b).

The genome of "*Ca.* Glomeribacter gigasporarum" revealed that these endobacterium possess a reduced genome (1.72 Mbp) with a relatively high G+C content of 54.82%, and a notorious dependency on its host for carbon, phosphorus and nitrogen as well as energy which is likely obtain from transporting and degrading amino acids (Ghignone et al., 2012). Phylogenetically, "*Ca.* Glomeribacter gigasporarum" closest relative is the also endofungal bacteria *B. rhizoxinica*, but analyses based on the metabolic pathways and their completion grouped this bacterium closer to

some other endosymbionts of insects like "*Candidatus* Hamiltonella defensa," *Wolbachia* spp. and *Wigglesworthia glossinidia*, suggesting that both fungal and insect intracellular symbionts had undergone convergent evolution (Ghignone et al., 2012).

A contrasting bacterial–fungal symbiosis was reported on *R. microsporus*, a zygomycete fungus, which was known as the causal agent of rice seedling blight thanks to the production of the toxin and potent antitumor agent rhizoxin (Gho et al., 1978; Takahashi et al., 1987). Partida-Martinez and Hertweck (2005) demonstrated that rhizoxin is not produced by the fungus, but by intracellular living bacteria from the Genus *Burkholderia*. The successful isolation and cultivation of the bacteria without its fungal host provided direct evidence of the bacterial origin of rhizoxin and their derivatives (Partida-Martinez and Hertweck, 2005, 2007; Scherlach et al., 2006), and paved also the way for establishing a model for bacterial-fungal symbioses. All endosymbiotic bacteria associated to toxinogenic *R. microsporus* formed a defined cluster among *Burkholderia* and were later taxonomically defined as *B. rhizoxinica* and *Burkholderia endofungorum* (Partida-Martinez et al., 2007b), the latter being the type strain which besides rhizoxin, is able to produce the ciclopeptide toxin rhizonin A (Partida-Martinez et al., 2007a). Strikingly, it was also demonstrated that cured, that is *R. microsporus* strains in which the bacteria has been eliminated by antibiotic treatment, are unable to form asexual sporangia and sporangiospores, meaning that the fungal host depends on endobacteria not only for the production of "mycotoxins," but also for its asexual reproduction. These experiments also confirmed that bacteria are transmitted vertically, as single bacterial cells were encapsulated in fungal spores, warranting the maintenance of the symbiosis along generations (Partida-Martinez et al., 2007c).

Further evolutionary studies on the *Rhizopus–Burkholderia* symbiosis have suggested that it is not as antique as the one between the AM fungi *G. margarita* and "*Ca.* Glomeribacter gigasporarum" (Castillo and Pawlowska, 2010), and that it likely emerged as a shift from parasitism to mutualism, as mucoromycotina and some branches of the Ascomycota independently evolved rhizoxin resistant mechanisms before the establishment of the symbiosis (Schmitt et al., 2008).

Analyses of the first published endofungal genome of the type strain of *B. rhizoxinica* revealed a relatively reduced genome of 3.75 Mbp. These analyses suggested that *B. rhizoxinica* is in an early phase of endosymbiosis, as many pseudogenes, MGEs and transposons are present in its genome (Lackner et al., 2011a,b). Interestingly, and in contrast to other symbiotic systems which are also predicted to be in an early phase of evolution such as the chromatophore in *Paulinella chromatophora* (Nowack et al., 2008), the genome of *B. rhizoxinica* has shown that around 30.8% of its coding genes had no homology to any other organisms sequenced to date. This means that *B. rhizoxinica*'s genome is not simply a subset of genes derived from free-living *Burkholderia* spp. (Lackner et al., 2011a).

Despite this moderate genome reduction and evidence of a genome in transition, it is striking the great dependency of both partners to each other. These facts prompt questions such as: how fast can intracellular mutualisms between bacteria and fungi be established? Which molecular mechanisms had contributed to this dependency? Under which circumstances would the *Burkholderia* endosymbionts evolve to a completely reduced genome close to the status of an organelle? Are host switching and/or population effective size contributing to genome stability in this symbiosis?

Very recently, the discovery of *Mollicutes*-related endobacteria not only in Glomeromycota, but also inside several strains of *Endogone* (Desirò et al., 2014a), a fungal genus belonging to Mucoromycotina which forms intimate associations with the earliest groups of land plants, has raised further questions about the ecological and evolutionary relevance of endofungal bacterial symbionts in the establishment of the symbiosis of plants with fungi and ultimately, in the establishment in land by plants (Bidartondo et al., 2011). All these reports lend support to the idea that bacterial endosymbiosis in fungi is ancient and may have started within ancestral fugal members characterized by coenocytic mycelium (Desirò et al., 2014a).

Certainly, further comprehensive molecular studies from the aforementioned as well as other bacterial–fungal symbiosis known to date will shed light on the evolutionary patterns of genome reduction and stability in these systems. Recent reports on intracellular gram-negative bacteria producing *N*-homoserine lactones in the zygomycete *Mortierella alpina* A-178 (Kai et al., 2012), together with diverse bacteria associated to endophytic fungi of the Ascomycetes (Hoffman and Arnold, 2010), and the endobacterial communities associated with the ectomycorrhizal fungus *Laccaria bicolor* (Bertaux et al., 2005) are expanding the universe of close interactions between bacteria and fungi, enabling a deeper understanding of the commonalities and differences between these symbioses and the best known bacteria-insect models. Moreover, some genome projects from fungi and bacteria engaged in intracellular symbioses had been recently undertaken as indicated in the website of the Department of Energy Joint Genome Institute (Nordberg et al., 2014). Thus, the consequences of such endosymbioses may be soon evaluated from both sides of the partnership.

DRIVERS OF GENOME REDUCTION IN HOST-ASSOCIATED BACTERIA

The process of genome reduction in host-associated bacteria is largely determined by the intracellular environment in which they live. Specifically, it is reasoned that genes unnecessary for living in intracellular conditions are not maintained by selection and lost along evolution. The process of genome reduction has been documented and seem to follow common trends from one stage of reduction to the next (Toft and Andersson, 2010). However, the mechanisms that drive these changes are far from established. In this section, we will review some of the more prominent and recent hypothesis about what drives genome reduction in host-associated bacteria.

Currently one of the most prominent and widely accepted hypotheses to explain genome reduction is based on the process known as the Muller's ratchet, which states that in populations undergoing constant bottlenecks and no recombination, genome reduction occurs through the accumulation of slightly deleterious mutations (Moran, 1996; McCutcheon and Moran, 2012). Under these conditions selection fails to retain genes which then, by the constant accumulation of mutations, become inactive and

are eventually deleted from the genome. As a result, several of the typical characteristics of these genomes, like their large A+T content or their small genomes, reflect known mutational biases (i.e., G:C to A:T mutations and deletions over insertions) rather than adaptations evolved by selection (Moran, 2003; Moya et al., 2008; McCutcheon and Moran, 2012).

In agreement with this hypothesis, theoretical studies suggest that proteins in *Buchnera* are less stable as a consequence of accumulating slightly deleterious mutations over large periods of time (van Ham et al., 2003). Additionally, proteomic studies demonstrate that the chaperonin GroEL is one of the most abundant proteins in *Buchnera* (Poliakov et al., 2011). As such, it is believed that GroEL plays a central role by stabilizing an otherwise unstable proteome (Moran, 1996; Fares et al., 2002). Furthermore, it has been shown that GroEL has suffered substitutions due to positive natural selection in two important functional regions of the protein which were suggested to be involved in the optimization of the ability to bind and prevent inappropriate folding of GroEL in *Buchnera* spp. and Flavobacteria endosymbionts (Fares et al., 2005). Also supporting this hypothesis, the pattern of nonsynonymous (d_N) versus synonymous (d_S) substitutions (d_N/d_S) among 42 pairs of closely related bacteria is consistent with genetic drift driving the process of genome reduction. Accordingly, d_N/d_S is consistently larger in organisms with smaller genomes (Kuo et al., 2009).

However, other processes have been suggested to explain the evolution of reduced genomes. For instance, Itoh et al. (2002) suggested that the acceleration of molecular evolution experienced in these genomes is due to a general increase in the mutation rate rather than to the Muller's ratchet mechanism. This hypothesis is based on: (a) the fact that the genomes of obligate mutualistic bacteria often lack DNA repair genes; (b) Muller's ratchet hypothesis is not consistent with the fact that the genomes of mutualistic endosymbionts, like those of *Buchnera* spp. have 100s of millions of years of existence; and (c) the pattern of acceleration of molecular evolution of *Buchnera* spp. proteins is consistent with increase of mutation rate and not with relaxation of purifying selection. In fact, the authors propose that loss of DNA repair genes is one of the necessary prerequisites to evolve a reduced genome. This hypothesis resembles the one proposed by Marais et al. (2008) which also suggest that the increased mutation rate causes genomic reduction in free-living bacteria. However, lack of repair genes coupled with recurrent bottlenecks and no recombination sets the conditions for evolution by Muller's ratchet.

Whatever the cause of the acceleration of the rate of evolution is, the hypothesis that these bacteria accumulate slightly deleterious mutations has to explain how these organisms manage to survive despite millions of years of existence. In this sense, compensatory evolution has to be part of the answer which suggest that a mutation may be compensated by a second mutation which returns the system to a working state (Kern and Kondrashov, 2004; Fares et al., 2005; McCutcheon and Moran, 2012). An example of which is the afore mentioned chaperon overexpression which help the organism to tolerate more mutations by lowering the threshold of the free energy necessary to fold properly compensating for the introduction of destabilizing mutations and making the system more robust (Gros and Tenaillon, 2009).

Importantly, the Muller's ratchet hypothesis, although very compelling in the latter stages of reduction, falls short in the early stages where symbionts lack many of the prerequisites for this process to occur. Such as in facultative pathogens, which due to their ability to return to a free-living state can evade bottlenecks and have larger population sizes. In addition, the early stages of reduction in pathogens are characterized by acquisition of genes by HGT as well as their rapid modification by recombination (Toft and Andersson, 2010).

Traditionally, genome reduction in host-associated bacteria has been linked to this view of evolution based on the relaxed or neutral selection coupled to genetic drift, since it better explains the presence of non-functional DNA such as ancient pseudogenes and intergenic regions commonly found in these bacteria (Mira et al., 2001; Dutta and Paul, 2012; McCutcheon and Moran, 2012). However, a recent shift in this view has started to appear in the form of empirical evidence (Koskiniemi et al., 2012; Lee and Marx, 2012; D'Souza et al., 2014), and new hypothesis which view genome reduction of host-associated bacteria as a selection-based process, at least on its early stages (Mendonça et al., 2011; Bliven and Maurelli, 2012; Morris et al., 2012). Here we will review a few of these hypotheses.

The first is an interesting novel hypothesis which suggests selective gene loss based on the loss of robustness in predictable environment. In this case, robustness is defined as the ability of an organism to withstand harsh and variable environments as well as coping with internal changes and perturbations in the inner workings of the cell. The hypothesis predicts that under predictable environments such as the interior of the cell, this robustness is not required and thus, genomic reduction would be observed. In correlation with this, the authors found empirical evidence of the existence of a selective drive to retain protein family diversity by sacrificing redundancy of functions. Here, redundancy of function, being a form of robustness. In other words, reduced genomes tend to have more protein families but each family tends to have very few members. Thus the authors suggest that the probability of losing a gene is higher if multiple copies of redundant genes exist, but very small if the function is unique. This also indicates that only those paralogs with similar functions will be lost. Finally, they suggest that other forms of robustness such as network redundancy may be similarly affected. For instance, the protein family composed of the transketolases TktA (EC2.2.1.1), TktB (EC 2.2.1.1), and the 1-deoxyxylulose-5-phosphate synthase Dsx (EC2.2.1.7) in *Escherichia coli* provides an example of this. The transketolases are 99% identical to each other but only 29% with respect to Dsx. In *B. aphidicola,* only one transketolase and one 1-deoxyxylulose-5-phosphate synthase remain (Mendonça et al., 2011).

Although originally proposed for free-living organisms, the BQH may also play an important role in genome reduction of host-associated bacteria. A study by D'Souza et al. (2014) showed that 76% of 949 sequenced bacteria were auxotrophic for at least one of 25 different metabolites needed for growth (20 amino acids, 3 vitamins, and 2 nucleosides), of which endosymbiotic bacteria where the most commonly observed auxotrophs (91% of endosymbiotic bacteria where auxotrophs for at least one

of the 25 metabolites as opposed to 85% for free-living and 64% gut-inhabiting bacteria). Additionally, they observed that when supplemented with the metabolite, auxotroph strains of *E. coli* and *Acinetobacter baylyi* showed a significant increase in fitness as compared to the wild type. The selective advantage depended on the concentration of the metabolite, the metabolite in question and the absence or presence of a competitor (D'Souza et al., 2014).

And finally, another hypothesis that supports selection as the driver to reduction is the AVG hypothesis. This theory, proposed for pathogens, states that once a pathogen colonizes a new niche, its new role as a pathogen may be hindered by the expression of genes present and required in its previous environment. In order to better adapt and fulfill its role as a pathogen, these AVGs are selected against and end up inactivated or deleted. This theory is based on the concept of antagonistic pleiotropy, which states that the same gene may have adverse fitness in different environments. And thus, the AVG hypothesis may be considered not only for pathogens but also for other forms of symbiosis. An example of an AVG gene is that of *speG* in *Shigella* species. This gene codes for a spermidine acetyltranferase that generates *N*-acetylspermidine from spermidine. The loss of this gene prevents spermidine metabolism and allows for high levels of this compound in the cell. High spermidine concentration is correlated to higher survival to oxidative stress, which is of particular importance for *Shigella* spp. since part of it life cycle includes being swallowed by macrophages, and withstanding severe oxidative stress. Thus, the loss of this gene confers a higher fitness (Bliven and Maurelli, 2012).

CONCLUSION

In Monterroso (1959) the Latin-American writer Augusto Monterroso wrote one of the smallest stories on Spanish language called "The dinosaur": "*Cuando despertó, el dinosaurio aún estaba allí.*" An approximate English translation would be "When he awoke, the dinosaur was still." The story is composed of two parts separated by a coma. In the first one, a tacit subject awakes. In the second one, the subject realizes that a dinosaur, an explicit subject, "was still." Despite of its small size, all the elements of a story (i.e., character, setting, plot, conflict and theme) are present in these just seven words, although some of these elements are implicit and left to the imagination of the reader.

Similarly, in the case of host-associated bacteria, extreme genome reduction is possible by metabolic and functional integration with the host and with other co-symbionts. And in the case of free-living bacteria, the BQH suggest that selection will favor the loss of those genes that code for expensive functions that are anyway provided as PGs by other species (Morris et al., 2012). In both cases the outcome is dependence between different cellular lineages. And, as in the case of Augusto Monterroso's story, in which some parts of the story are left implicit to the reader, parts of the functions required by the cells are performed outside their boundaries.

What cellular status do prokaryotes with extremely reduced genomes deserve? Perhaps the symbionelle concept is part of the answer (Reyes-Prieto et al., 2014). The symbionelle concept was constructed to accommodate those cases of endosymbionts that fail to reach a minimal gene set (Gil et al., 2004). They possess evolved genomes with so few genes that they are not able to perform the three basic functions of present day cells without the presence of a host and/or other co-symbionts, and so, represent a new category. These symbionelles present evolutionary convergence with organelles exhibiting clear and important similarities and distinctions, although each evolved in completely different evolutionary scenarios, where organelles evolved before multicellular life and symbionelles distinctly throughout insect evolution. (Reyes-Prieto et al., 2014).

A concluding remark is made by Wolf and Koonin (2013), in which they suggest that genome reduction is the dominant form of evolution in a two phase genomic model where a short phase of abrupt increase in complexity, and thus genomic size, permits innovation while a long phase defined by genomic reduction allows for adaptation. Once again, demonstrating the importance of dependence as a form of adaptation born from symbiotic interactions. And finally, as pointed out by (Sloan and Moran, 2013), the biology of obligate intracellular mutualistic bacteria offers the opportunity to study the evolutionary process acting on different levels of biological organization. Thus, the development of a multilevel theory of causation stands at the frontier of evolutionary theory (Gould, 2002).

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Repeatable Population Dynamics among Vesicular Stomatitis Virus Lineages Evolved under High Co-infection

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Parasites and hosts can experience oscillatory cycles, where the densities of these interacting species dynamically fluctuate through time. Viruses with different replication strategies can also interact to produce cyclical dynamics. Frequent cellular co-infection can select for defective-interfering particles (DIPs): "cheater" viruses with shortened genomes that interfere with intracellular replication of full-length (ordinary) viruses. DIPs are positively selected when rare because they out-replicate ordinary viruses during co-infection, but DIPs are negatively selected when common because ordinary viruses become unavailable for intracellular exploitation via cheating. Here, we tested whether oscillatory dynamics of ordinary viruses were similar across independently evolved populations of vesicular stomatitis virus (VSV). Results showed identical cyclical dynamics across populations in the first 10 experimental passages, which transitioned to repeatable dampened oscillations by passage 20. Genomic analyses revealed parallel molecular substitutions across populations, particularly novel mutations that became dominant by passage 10. Our study showed that oscillatory dynamics and molecular evolution of interacting viruses were highly repeatable in VSV populations passaged under frequent co-infection. Furthermore, our data suggested that frequent co-infection with DIPs caused lowered performance of full-length viruses, by reducing their population densities by orders of magnitude compared to reproduction of ordinary viruses during strictly clonal infections.

Keywords: cheating behavior, co-evolution, defective interfering particle, ecological dynamics, experimental evolution, RNA virus, virus ecology

INTRODUCTION

Evolutionary ecology concerns interactions between biological populations and their biotic and abiotic environments. Viruses necessarily interact with host organisms (Hurst, 2000), whose metabolic functions are required for completing virus replication. However, less-appreciated aspects of virus evolutionary ecology are the relationships between individual viruses, and the role of these interactions in driving virus evolution. Viruses can indirectly affect the fitness and evolutionary fate of other viruses through interactions with the host immune system, such as cross reactivity where one infecting virus species causes the immune system to also recognize and target

related viruses. In contrast, direct virus–virus interactions are possible when multiple viruses co-infect the same host cell, such as a host bacterium or an individual cell within a multi-cellular eukaryotic host. Here, one virus can directly lower the fitness of a co-infecting virus by sequestering intracellular proteins essential for virus genome replication, thus interfering with the co-infecting strain's reproductive success (Huang and Baltimore, 1970; Horiuchi, 1983; Depolo et al., 1987; Turner and Chao, 1999; Thompson and Yin, 2010; Ojosnegros et al., 2011).

When direct ecological interactions occur, these can lead to short-term or long-term dynamics; classic examples include dynamics between parasites and their hosts (Fine and Clarkson, 1982; Potts et al., 1984) and between predators and their prey (MacLulick, 1937). These community dynamics are often driven by resource limitation and availability, principally when one community member uses another as a resource (e.g., predators consuming prey, parasites utilizing hosts) or when community members compete for the same resources. Resource competition can select for differing resource-use strategies, where a classic example is evolution of cheating versus non-cheating behavior, when resources are available in a common pool. Here, we define a cheater as possessing a mechanism (e.g., behavioral trait, molecular property) that causes it to experience a reproductive advantage relative to a non-cheater, when utilizing limited resources to create progeny. Although the evolution of cheating appears to be very prevalent in biological systems (Smith, 1982; Turner and Chao, 1999; Travisano and Velicer, 2004), the long- and short-term ecological dynamics of cheater/non-cheater interactions are seldom studied outside of mathematical theory (e.g., game theory), and are therefore poorly understood even in systems where the underlying molecular biology and genetics of cheating have been determined (Huang, 1973; Griffin and Fried, 1975; Huang and Baltimore, 1977; Lazzarini et al., 1981; Velicer et al., 1991). Do independently evolving but otherwise identical populations show equivalent dynamics when cheater genotypes evolve? Experimental evolution studies in microbes demonstrate that interaction dynamics can be highly repeatable across laboratory microcosms (Turner et al., 1996; Rainey and Travisano, 1998; Rozen et al., 2002). But microbial studies have rarely addressed the repeatability of population dynamics when virus–virus interactions, such as cheating, evolve *de novo* (e.g., Horiuchi, 1983).

To study this problem, we allowed replicate populations of viruses to evolve in the laboratory under conditions where co-infection occurred frequently. These culture conditions favored selection for viral "cheaters" defined as defective-interfering particles (DIPs) that are observed in natural virus populations, and seen in clinical samples (von Magnus, 1951; Lief and Henle, 1956; Huang and Baltimore, 1970; Huang, 1973; Huang and Baltimore, 1977; Horodyski and Holland, 1980; Taylor, 1999; Aaskov et al., 2006). Errors in genome replication can sometimes create virus mutants that have point mutations or deletions that inactivate one or more essential virus genes. However, it is important to distinguish DIPs from other types of deletion mutants, because DIPs are defined to possess two particular properties that allow them to "cheat" when co-infecting cells with ordinary (full-length) variants of the same virus. First, DIPs are 'defective' because these viral genotypes lack proper function in one or more essential virus genes due to a genomic deletion, which prevents a DIP from replicating in a cell unless a full-length virus is also present (Holland et al., 1991). Second, DIPs gain an advantage over otherwise identical full-length viruses during co-infection because they somehow 'interfere' with their replication, hence lowering the expected progeny production of ordinary viruses within the cell. These combined properties define DIPs as 'defective-interfering' and these traits explain why DIPs can lower the fitness (reproductive output) of full-length viruses during co-infection of the same cell. Importantly, we emphasize that an ordinary virus must be present during co-infection in order for a DIP to replicate, and this presence of the full-length virus necessarily 'helps' the DIP genotype. Although by definition an ordinary virus suffers reduced fitness relative to a co-infecting DIP, the rescue effect of the ordinary virus has popularized such full-length variants to be termed 'helper virus' in the literature (e.g., Huang and Baltimore, 1970). From an evolutionary biology standpoint, 'helper virus' can be considered a misnomer because it incorrectly implies that ordinary viruses are selected to beneficially interact as mutualists with DIP genotypes that arise spontaneously in the virus population. Rather, the correct interpretation is that DIPs are spontaneous mutants that parasitize ordinary viruses during co-infection, and in the remainder of the paper we avoid using the classic 'helper virus' terminology to avoid confusion by the reader.

Whereas genome replication errors can cause DIP mutants to spontaneously occur during most (or all) cellular infections (Huang, 1973; Lazzarini et al., 1981; Perrault, 1981), only frequent opportunities for co-infection are expected to foster persistence of DIPs. The reason is that DIPs are unlikely to persist as a pure population because they do not encode all of the functional genes necessary for completion of the virus life cycle within a cell (but see Garcia-Arriaza et al., 2004, for a rare example of multiple DIPs that coexist via complementation). In contrast, frequent co-infection allows spontaneously occurring DIP mutants to persist alongside ordinary virus genotypes, because the latter provide missing essential viral proteins and functions within the cell. Various mechanisms can account for the interference enacted by DIPs on ordinary viruses. But the smaller genome sizes of DIPs immediately afford faster rate of replication compared to full-length viruses, and this size difference alone may be sufficient to explain their competitive advantage (Huang, 1973; Lazzarini et al., 1981; Coburn and Cullen, 2002).

Regardless of the cheating mechanism, if virus co-infection is prevalent, the relative frequencies of DIPs and ordinary viruses are expected to dynamically oscillate through time (Kirkwood and Bangham, 1994). The selective advantage of spontaneously occurring DIP mutants causes them to increase in genotype frequency through time (i.e., invade when initially rare) in the viral population. As DIPs increase in frequency this forces the frequency of ordinary virus genotypes to decline, because DIPs are defined to be fitness advantaged over their full-length virus counterparts during co-infection. But when DIPs reach high frequencies in the population, most cells will be co-infected only by DIP mutants and these dead-end infections

will produce no viral progeny (i.e., DIPs lack one or more essential virus genes, and two fully complementary DIP mutants have low probability of fortuitously entering the same cell). DIPs should then decrease in genotype frequency, allowing ordinary viruses to numerically recover (i.e., invade when initially rare) and the cycle repeats (Huang, 1973). Importantly, this oscillating dynamic (negative frequency-dependent selection) can be revealed by obtaining samples from the virus population through time, and enumerating only the ordinary viruses that are present in the samples. The reason is that estimates of virus densities (titers) rely on assays that place dilutions of population samples onto a substrate (e.g., cell monolayers grown in tissue culture), such that individual particles hit the substrate and give rise to progeny that locally destroy cells to create non-overlapping 'holes' (plaques) visible on the substrate. Necessarily, DIPs lack one or more essential genes and therefore cannot form plaques initiated by an individual particle in such plaque assays. Hence, plaque assays can reliably track densities of the ordinary virus subpopulation through time, but the DIP subpopulation remains invisible in such assays. Nevertheless, enumeration of the ordinary viruses is sufficient to prove that oscillations are occurring, and some *in vitro* experiments have classically demonstrated the expected oscillations when viruses are passaged under frequent co-infection (Huang, 1973; Palma and Huang, 1974; Horiuchi, 1983). However, these studies tended to focus on the molecular virology of DIPs rather than the repeatability of oscillatory dynamics across replicate evolved populations, which is the main goal of the current study.

Here, we employed vesicular stomatitis virus (VSV) as a model to study the repeatability of oscillatory dynamics, across virus populations that were independently passaged under frequent co-infection. VSV is a well-characterized arthropod-borne virus (arbovirus) in the family Rhabdoviridae, which naturally infects various species of insects and mammals, including occasionally humans (Letchworth et al., 1999; Rose and Whitt, 2001). VSV has a ∼11.2 kb negative sense ssRNA genome encoding five proteins: the nucleocapsid (N) protein that encapsidates the genomic RNA, phosphoprotein (P) and large (L) protein which make up the polymerase, glycoprotein (G) involved in cell-surface binding, and matrix (M) protein important both for virion formation and inhibition of host antiviral gene expression (Rose and Whitt, 2001; Lyles and Rupprecht, 2007). As with many ssRNA viruses, VSV has a high mutation rate, rapid generation time, and small genome size, making it a useful and efficient model for examining phenotypic and genotypic evolution (Moya et al., 2004). Although genetic exchange (recombination, reassortment) can be consequential for virus evolution (Perez-Losada et al., 2015), we note that recombination is either extremely rare or non-existent in VSV (Han and Worobey, 2011). VSV is generally not pathogenic to humans, and grows easily in many cell culture systems and tissues owing to its broad tropism (Hastie and Grdzelishvili, 2012). VSV cultured *in vitro* typically reaches very high titers (10^8–10^{10} viruses per mL) within 24 h, on permissive cell types such as baby hamster kidney (BHK-21) cells. Serial passage of VSV under high multiplicity of infection (MOI; ratio of viruses to cells) allows frequent virus co-infection, conditions that promote strong selection for spontaneous DIP mutants in VSV populations (Huang, 1973; Holland, 1991; Thompson and Yin, 2010; Ojosnegros et al., 2011).

We used a single ancestral VSV strain to initiate five replicate virus populations, which were independently passaged for 20 consecutive days on BHK cells at high *MOI*. Results showed that the oscillatory dynamics of ordinary viruses were highly similar across the populations, characterized by high-amplitude oscillations between passages 0 and 10, and followed by dampened oscillations between passages 10 and 20. In addition, full-genome consensus sequencing of mixed population samples showed that parallel molecular evolution occurred across the five populations, and that most of these changes fixed early in the study. Our results suggested that negative frequency-dependent selection experienced by ordinary and DIP genotypes of VSV was highly similar across our study populations, evidenced by ordinary-virus dynamics and molecular substitutions that were repeatable and parallel across independently evolving RNA virus populations.

MATERIALS AND METHODS

Viruses and Culture Conditions

All viruses were derived from a clonal isolate of a low-passage, laboratory-adapted strain of Mudd Summer VSV Indiana serotype that was kindly provided by E. Domingo (University of Madrid). Hereafter, we refer to this clone as wild-type (WT). Viruses and host cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Gibco) with 5% heat-inactivated newborn bovine calf serum (Atlanta Biologicals) and 1% penicillin-streptomycin-L-glutamine (Gibco). Hosts for infection were baby hamster kidney (BHK-21) cells, kindly provided by E. Domingo (University of Madrid) and grown in 25-cm^2 tissue-culture flasks at 37°C, 95% relative humidity, and 5% CO_2 atmosphere to achieve confluent monolayers of $\sim 10^5$ cells/cm^2. Low passage variants of cell lines (i.e., between 5 and 25 laboratory passages) were used for all experiments.

Experimental Evolution of Viral Populations

Wild-type VSV was grown to high titer [$\approx 10^8$ plaque-forming units (PFU) per mL] to create a stock lysate that was used to found five replicate populations (L1 through L5). Confluent monolayers of BHK-21 cells were infected at an initial ratio of 10 viruses per cell ($MOI \approx 10$). This *MOI* created a high probability for multiple virus particles to co-infect individual cells, which would selectively favor spontaneously arising DIPs to gain an intracellular fitness advantage over ordinary viruses (Huang, 1973). At 24 h, an 'undiluted passage' was conducted: from the 3 mL supernatant containing the viral progeny, 0.12 mL was removed and used to infect a new confluent monolayer, with roughly 2.9 mL fresh DMEM. This 24-h propagation cycle was repeated 20 consecutive times for each population. After daily propagation, a sample from each population was stored at –80°C to perform subsequent plaque assays.

Plaque Assays

A serially diluted virus sample was added to a confluent monolayer of BHK-21 cells grown in 6-well plates, and incubated for 45 min at 37°C with rocking every 15 min. Medium containing virus was removed, and replaced with a mixture of agar and 2x DMEM, and incubated for additional 24–30 h at 37°C. Cells were then fixed with 10% formaldehyde, agar and medium was removed, and cells were stained with crystal violet to visualize plaques. Titers (PFU/mL) were estimated as averages of counts from dilutions in two separate wells.

Whole Genome Sequencing

Viral populations were sequenced at three time points in their evolution: the WT virus used to found the replicates (day 0), and the five replicate populations on days 10 and 20. Genomic RNA was extracted from 140 μL of frozen viral stock using Qiagen QiAmp mini viral spin kit. cDNA was generated by reverse transcription with Superscript II (Invitrogen), using random hexamer primers. Virus was polymerase chain reaction (PCR) amplified using GoTaq polymerase (Promega), and VSV specific primers (Supplementary Table S1). Amplicons were purified by enzymatic inactivation: Antarctic phosphatase was used to catalyze the removal of 5′ phosphate from DNA and RNA to prevent self-ligation, and Exonuclease 1 was used to catalyze the removal of nucleotides from single-stranded DNA in the 3′ to 5′ direction. Amplicons were used in Sanger sequencing at the Yale University DNA Analysis Facility on Science Hill. The coverage was between twofold and eightfold from overlapping fragments (range: 300–2100 bp in length) using multiple directional sequencing primers (Supplementary Table S1). Replicate sequences were obtained from at least two independent PCRs, and mutations called only when observed on all reads. Chromatograms were checked for quality and assembled into contigs using CLC Main Workbench 6. Whole genome sequencing constituted the consensus sequence (dominant alleles) of an evolved VSV population, regardless of the genetic background on which these alleles resided. Thus, consensus sequencing of an RNA virus population could be considered analogous to the viral quasispecies, the genetically diverse mixture of genotypes (ordinary viruses, DIPs) within the population at the time of sequencing.

Fast Fourier Transform Analysis

Fast Fourier Transform (FFT) analysis transforms a function or set of data from the time or sample domain to the frequency domain. This means that the Fourier transform can display the frequency components within a time series of data. By inputting the virus titer data into the FFT function in MATLAB (MathWorks, Natick, MA, USA), we obtained an estimated periodicity of the oscillations (number of passages constituting a cycle) in each virus population.

Statistical Analysis

To analyze the effect of evolved VSV population on the estimated titer of the ordinary virus subpopulation, we included the $\log_{10}$ PFU/ml data in a one-way ANOVA that included population as a model effect.

[^{3}H]Uridine Labeling

RNA synthesis was examined by direct metabolic labeling of cells 5 h post infection. Cells were exposed for 6 h to [^{3}H]uridine (33 mCi/ml) in the presence of actinomycin D (10 mg/ml). Cells were harvested, and total cytoplasmic RNA was extracted and analyzed as described previously (Pattnaik et al., 1992; Wertz et al., 1994).

RESULTS

Virus Population Dynamics

In serial passage, a microbial population is cultured in the laboratory for a defined period (typically 24 h) and then subsampled into fresh medium to initiate the next passage. The dilution used to obtain the subsample is termed a population 'bottleneck' because only a portion of the population is transferred (i.e., here, bottleneck is used differently than in population genetics, where bottlenecking indicates that an evolving population is expected to experience effects of genetic drift). To impose frequent co-infection in VSV populations we used so-called 'undiluted' passages, a misnomer in the scientific literature because a dilution actually occurs during this daily serial bottleneck. In our experimental evolution protocol (see the Section "Materials and Methods") the daily dilution was 25-fold. This wide 'undiluted' bottleneck ensured that viruses outnumbered cells in the monolayer, promoting frequent co-infection between viruses as they replicated within host cells. By contrast, most VSV evolution studies employ much narrower population bottlenecks (typically on the order of 10^{-4}–10^{-6} dilution) to specifically prevent frequent co-infection of particles in the same cell (e.g., Turner and Elena, 2000).

Because ordinary viruses have full-length genomes, their densities can be enumerated in plaque assays (see the Section "Materials and Methods") where diluted samples of virus supernatants are plated on host monolayers to count plaques initiated by individual virus particles. In contrast, DIPs lack one or more essential virus genes and therefore cannot form plaques when infecting cells alone; thus, plaque assays can estimate ordinary virus densities, but cannot be used to measure DIP titers. At each passage in the experimental evolution, we obtained diluted samples from each evolving VSV lineage and used plaque assays to estimate the titer (PFU per mL) of ordinary viruses present in the population at each time point. These estimates of ordinary virus titers were then $\log_{10}$-transformed. Results (**Figure 1**) showed that all five experimental VSV populations (L1–L5) presented oscillating (increasing and decreasing) densities in ordinary virus titers through time. These observed population dynamics were similar across the five replicate populations, and in each case the dynamics generally resembled the oscillatory shape of a sine wave with dampened oscillations through time (see further analysis below). Some assays showed that the ordinary virus subpopulation fell to very low densities (i.e., titer $\leq 10^4$ PFU/mL; **Figure 1**); however, in all

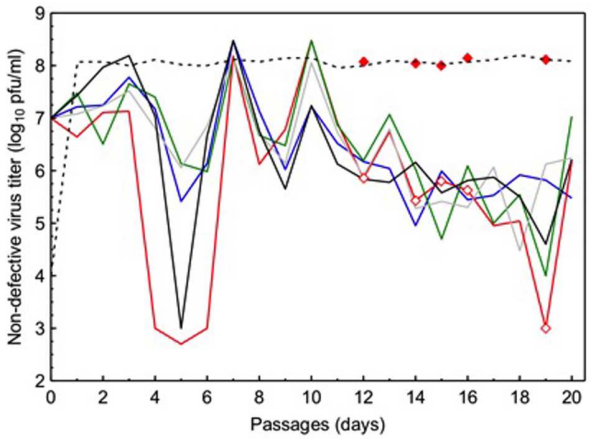

FIGURE 1 | Temporal dynamics of ordinary virus titers in five vesicular stomatitis virus (VSV) populations independently cultured for 20 passages under frequent co-infection of BHK-21 cells. Each solid line depicts titer data from an individual population (purple line: L1; red line: L2; green line: L3; gray line: L4; black line: L5). Open diamonds indicate time points when virus samples were isolated from lineage L2, and assayed for growth on cells at low multiplicity of infection (filled diamonds). Dashed line indicates typical growth ability of a VSV lineage founded by a full-length wild-type (WT) virus genotype, and passaged in the absence of co-infection.

cases the ordinary viruses rebounded in number by the next time point, confirming that ordinary viruses increased in frequency (**Figure 1**). We concluded that the oscillatory dynamics were consistent with expectations from the theoretical and empirical literature on interactions between DIPs and ordinary viruses in an evolving VSV population, because undiluted passages should cause ordinary virus densities to widely fluctuate through time as these full-length viruses and DIPs experience negative frequency-dependent selection.

During the course of the experiment, all five experimental populations showed a trend of decreased titers (PFU/mL) of ordinary viruses as passage number increased. For each lineage, the average size of the full-length virus subpopulation was estimated by calculating the harmonic mean of its observed titer estimates. Hence, the estimated harmonic means equaled: L1: 4.7×10^4 PFU/mL; L2: 3.6×10^3 PFU/mL; L3: 1.1×10^4 PFU/mL; L4: 6.7×10^4 PFU/mL; L5: 1.1×10^4 PFU/mL. These data suggested that the subpopulation sizes of the ordinary viruses were highly similar across our experiment, which was supported by results of a one-way ANOVA that showed the effect of population on ordinary virus titer was not statistically significant [$F_{(2.931,55.68)} = 2.247$; $P = 0.094$].

To further compare population dynamics across replicate lineages, we examined the $\log_{10}$ titer data of ordinary viruses using FFT analysis, which estimated the periodicity of oscillations (number of passages constituting a cycle) in each population (**Figure 1**). Results showed that the number of passages constituting a cycle differed somewhat across the five experimental populations: L1: 2.2 passages/cycle; L2: 2.3 passages/cycle; L3: 3.3 passages/cycle; L4: 3.6 passages/cycle; L5: 4.4 passages/cycle.

Ecological Interactions Dictate Full-length Virus Productivity

The observed low productivity of ordinary viruses could be due solely to ecological interactions with DIPs, which are expected to deleteriously reduce the absolute fitness ($\log_{10}$ titer) of full-length viruses. Alternatively, the inability of ordinary viruses to achieve high titers similar to that of WT VSV ($\approx10^8$ PFU/mL) could have been driven wholly by evolved changes in the full-length viruses. In particular, evolution of ordinary virus resistance to DIPs could have coincided with reduced ordinary virus fecundity; this evolutionary growth trade-off would indicate inability of full-length viruses to simultaneously evolve DIP resistance while maintaining robust growth. If the absolute fitness of an evolved ordinary virus at $MOI = 0.01$ (i.e., in absence of DIPs) was similar to that of the WT ancestor grown at low MOI (i.e., $\approx10^8$ PFU/mL), this outcome would suggest that the observed low productivity of full-length viruses on certain passage days (**Figure 1**) was due to ecological interactions with cheating DIPs. However, if the absolute fitness of an evolved ordinary virus measured at $MOI = 0.01$ was less than the ancestor ($\approx10^8$ PFU/mL), this result would suggest that its decreased fecundity was a trait that evolved during the course of the experiment.

To examine these competing hypotheses, we used plaque assays (see the Section "Materials and Methods") to measure the productivity of representative ordinary viruses drawn from an evolved population, under conditions where virus particles must infect cells on their own ($MOI = 0.01$). We chose to examine ordinary virus samples isolated at five time points during the passage series belonging to population L2. In particular, we isolated samples from passages where the observed titers were relatively low (i.e., passages 12, 14, 15, 16, and 19), because these time points presumably coincided with successful cheating by DIPs that drove down the observed frequencies of ordinary viruses in the population. We used plaque purification to isolate a single clone at random from each of the five time points in population L2's passage history. Each of these clones was used to infect BHK-21 cells at $MOI = 0.01$. After 24-h incubation, we quantified the resulting virus progeny using our standard plaque assay (see the Section "Materials and Methods"). Results (**Figure 1**) showed that all five of the ordinary virus clones had the capacity to grow to high titers ($\approx10^8$ PFU/mL), comparable to results for the WT ancestor grown under identical $MOI = 0.01$ conditions. The data strongly implied that the observed low titers in population L2 were not due to evolved changes in ordinary virus productivity, but were rather due to ecological interactions between the ordinary viruses and the DIPs. However, we do not dismiss the possibility that some evolutionary changes occurred in the full-length viruses in our study, and we present and discuss these data below.

Radiolabeling Analysis Confirming DIP Presence

Although the oscillatory dynamics in **Figure 1** were consistent with expected effects of evolved DIPs on ordinary virus titers, we sought additional direct confirmation of DIP presence in the experimental virus populations. We therefore performed a

preliminary analysis to visualize DIPs that were present at the endpoint (passage 20) of the study, and to estimate whether DIPs were similar across the independently passaged populations. We first obtained a sample of the supernatant from each endpoint population. These samples were then amplified at $MOI = 10$ on BHK-21 cells, and subsequently subjected to RNA extraction followed by labeling with [^{3}H]uridine (see the Section "Materials and Methods"). Results (Supplementary Figure S1) showed the expected mRNA banding pattern for the five VSV genes (N, P, M, G, L; see gene description in the Section "Introduction") when WT VSV infected BHK cells. In contrast, these results showed that DIPs were present in each endpoint population, and that these DIPs presented fewer mRNA bands than WT VSV, consistent with their relatively shortened genomes. Importantly, the observed banding patterns across the four experimental lineages that yielded data suggested that parallel DIP evolution occurred in our study; for unknown reasons one population (L2) failed to yield data in this assay, despite clearly containing viable viruses at passage 20 (**Figure 1**). These preliminary data suggested that the independently passaged VSV lineages had converged in terms of the majority DIPs present at passage 20, at least in the four populations that yielded results.

Genomic Analysis of Evolved Populations

Sanger sequencing can be used to generate a consensus sequence for an evolved virus population, which usefully indicates the alleles that distinguish the evolved population from its ancestor. Although Sanger sequencing cannot determine whether observed mutations exist on a common genetic background (i.e., it cannot identify unique haplotypes), the method can be used to efficiently compare across replicate populations to test whether treatment conditions tended to select for parallel mutations across independently evolved lineages (e.g., Remold et al., 2008; Alto et al., 2013). We employed this approach to test whether parallel changes occurred across the five experimental populations, and whether certain mutations coincided with the early phase of the experiment typified by high amplitude oscillations versus with the later phase exemplified by dampened oscillations. Thus, the genomic analysis further tested for repeatability across the evolved populations, and also tested whether the observed changes in oscillatory dynamics coincided with differing molecular substitutions in the early versus later phases of the study.

Because VSV is a single-stranded RNA virus with a typically high mutational error rate, we expected that 20 passages of experimental evolution would be sufficient to detect polymorphic loci and/or fixed substitutions that separated the evolved lineages from their common ancestor virus, and possibly from one another. Importantly, this expectation assumes that the high *MOI* environment – and presumably DIP presence – exerts strong enough selection for the VSV consensus genome sequence to change in the evolutionary time allowed, especially due to beneficial mutations that spontaneously appear and spread to fixation. Twenty passages in a novel environment, such as a new tissue-culture host or incubation temperature, are sufficient for the VSV consensus sequence to change via fixation of adaptive mutations (e.g., Remold et al., 2008; Alto et al., 2013). In contrast, if VSV is passaged on the typical BHK-21 lab host at 37°C, 20 passages do not typically lead to many detectable changes because the virus is already relatively fit in this environment (e.g., Alto et al., 2013). Finally, we noted that the highly variable population sizes through passage time in our study (**Figure 1**) could prevent natural selection from operating efficiently, thus potentially allowing genetic changes to accrue in ordinary virus and/or DIP subpopulations through genetic drift. In summary, we expected that both adaptive and non-adaptive mutations could increase in frequency during the experiment, making these alleles detectable through consensus population sequencing.

To examine the occurrence of molecular evolution and evidence for parallelism in our study, we focused our attention on the two phases of the experiment that clearly differed in the observed oscillatory dynamics within the evolving lineages: days 0 to 10, and days 10–20 (**Figure 1**). In particular, we defined the ordinary virus-population dynamics occurring between days 0 and 10 as the 'early phase,' where oscillations showed relatively greater amplitude than between days 10 and 20, defined as the 'late phase.' Foremost, we compared the consensus sequence of each lineage across these two phases to test whether (i) observed molecular changes separated the evolved lineage from the common ancestor, indicating that molecular evolution via selection and/or drift occurred in the experiment, and (ii) whether different alleles dominated the population in the early versus late phase. In theory, the differing oscillatory dynamics in the early and late phases could be entirely driven by ecological interactions between full-length and DIP viruses that appeared in the early phase. If true, we would expect that a lineage's consensus sequence at day 10 would be highly similar (or even identical) to its sequence at day 20, perhaps with some early-phase polymorphic loci transitioning to fixed substitutions in the later phase. Alternatively, we might observe highly different consensus sequences in the early versus late phases, suggesting that the differing oscillatory dynamics were driven (at least in part) by both ecological interactions as well as evolutionary interactions, such as extended co-evolutionary changes between ordinary virus and DIP genotypes. Last, overall comparisons in molecular data among lineages (i.e., similarities in consensus sequences) were used to further gage the degree of repeatability in our study, especially occurrence of parallel evolution.

Results showed that the founding VSV ancestor in our study contained 102 mutations separating it from the canonical annotated VSV Mudd-Summers strain Indiana serotype (GenBank #NC_001560.1). In itself, this result is not overly surprising because the two strains have been used in separate laboratories for decades, under unknown but presumably non-identical culture conditions. More importantly for the current study, we identified a total of 93 allele substitutions and/or polymorphisms that separated the consensus sequences of the five evolved lineages from their common ancestor. These data are summarized in **Figure 2**, which shows the locations of fixed, polymorphic, synonymous, and non-synonymous changes, and indicates whether the mutation is observed by day 10 (early phase) versus appears between days 10 and 20 (late phase).

In general, most of the observed mutations occurred in the early phase of the study; across all five lineages, 42 mutations were detected by day 10 and only 14 additional mutations separated the day 20 consensus sequences from their day 10 counterparts (**Figure 2**). Moreover, the vast majority of these additional mutations (11 of 14 changes) were polymorphic at day 20. Overall, these comparisons indicated that most of the molecular evolution in our study had occurred in the early phase when oscillatory dynamics showed relatively high amplitudes (cf. **Figures 1** and **2**).

Our molecular data also indicated that abundant parallel evolution occurred across the five lineages (**Figure 2**). Because VSV is an ssRNA virus, we note that synonymous mutations can affect the secondary structure of the virus and thus potentially impact fitness. One fixed, synonymous mutation occurred in the gene for the VSV phosphoprotein in all five lineages by day 10 (nucleotide position 2151), and was still present at day 20. In the VSV glycoprotein gene, all five lineages showed a non-synonymous mutation at position 3846 (D→N) and at locus 4180 (T→K); one or both of these mutations had either fixed early in an evolved lineage (e.g., L1, L5), late in the experiment (e.g., L2, L4), or remained polymorphic throughout the study. The Large protein showed the largest number of parallel substitutions across the five lineages (**Figure 2**). Here, all lineages showed the same five mutations (changes at loci 5492, 5493, 8176, 8186, and 8736), and most of these substitutions fixed by day 10 (early phase). Interestingly, lineage L2 fixed two of these mutations by day 10, but then reverted back to the ancestral sequence by day 20.

Aside from the large number of parallel mutations across lineages, we observed nucleotide changes specific to a single lineage, or shared among a subset of the replicate lineages (**Figure 2**). However, these changes tended not to bridge the early and late phases of the experiment, and were often observed to be polymorphic. For these reasons, the minority non-parallel substitutions in our study may be considered less impactful, but this tentative conclusion warrants further investigation.

DISCUSSION

Our study examined the repeatability of population dynamics and molecular substitutions across five independently passaged VSV lineages, cultured under frequent virus co-infection that selects for evolution of DIPs (virus cheaters). After 20 days of serial passage that facilitated negative frequency-dependent selection between ordinary viruses and DIPs, all five experimental VSV populations displayed a cycling pattern consistent with theoretically predicted effects of DIPs on full-length virus densities (Palma and Huang, 1974; Depolo et al., 1987; Kirkwood and Bangham, 1994). A molecular assay confirmed that DIPs were present in the endpoint virus populations, and growth of representative full-length viruses at low *MOI* (absence of DIPs) showed that these strains retained robust reproductive ability. Changes in oscillatory dynamics (early versus late phases of the experiment) and patterns of molecular substitutions were highly similar across the five VSV lineages. Altogether, these results suggested that ecological interactions between ordinary viruses and cheaters were extremely repeatable across independently passaged lineages, and that any contribution of virus evolutionary changes to the observed dynamics within lineages was dictated by molecular substitutions occurring relatively early in virus evolution.

Our study was motivated by efforts to better understand fundamental antagonistic virus–virus ecological interactions (Wensel et al., 2003). Only a tiny minority of virus species function in human disease, but the medical importance of viral pathogens causes them to receive undue attention, and defective RNA viruses are sometimes implicated as important (e.g., Gan et al., 2002; Aaskov et al., 2006; Kim et al., 2007). One example is hepatitis delta virus, a medically relevant defective virus often associated with hepatitis B virus in severe cases of chronic active hepatitis (Taylor, 1999). In addition, clinical samples taken from humans suffering from dengue fever show that some mosquito-transmitted dengue virus populations are dominated by defective variants, at least in certain geographical regions (Aaskov et al., 2006). However, we note that DIPs and other defective viruses are necessarily difficult to detect because these variants do not form plaques in standard assays. Thus, the ecological and evolutionary importance of defective viruses in disease systems may be grossly underestimated. For this reason, experiments on the evolutionary ecology of DIPs and other defective viruses remain fertile areas for research. Our work hints that certain aspects of these empirical studies may be more tractable than expected, because virus–virus interactions can be highly conserved within a particular system; of course this assumption must be verified by extending such work beyond the VSV system presented here.

Previous empirical (von Magnus, 1951; Lief and Henle, 1956; Palma and Huang, 1974; Horiuchi, 1983; Depolo et al., 1987) and theoretical (Kirkwood and Bangham, 1994) data predicted the oscillating community dynamics between ordinary and DIP viruses observed in the current study. Such oscillations are generally not observed when VSV is evolved under identical culture conditions and similar time periods as our study, but at strictly low *MOI* conditions that do not foster selection for cheater viruses (e.g., Turner and Elena, 2000). Our FFT analysis confirmed the cyclical nature of these dynamics, but suggested that cycle periodicity differed across the experimental populations, ranging between 2.2 to 4.4 passage days per cycle. It is unknown whether some divergence between the populations helps to explain these observations; e.g., due to differing 'cheating abilities' among DIPs evolved in separate populations. Alternatively, these cycling differences may be purely stochastic, such as due to subtle differences across the populations in the state of host cells on certain passage days (e.g., random differences in host cell physiology or chance fluctuations in available host numbers) and/or due to non-parallel genetic differences among lineages. We note that the FFT analysis is usually used for larger data sets, and that the accuracy of our cycling period estimates may therefore be poor (i.e., weak resolution of cycle measurements). Despite the estimated difference in cycle numbers across test populations, the overall cycling patterns (especially dampened oscillations through time) were remarkably consistent and repeatable.

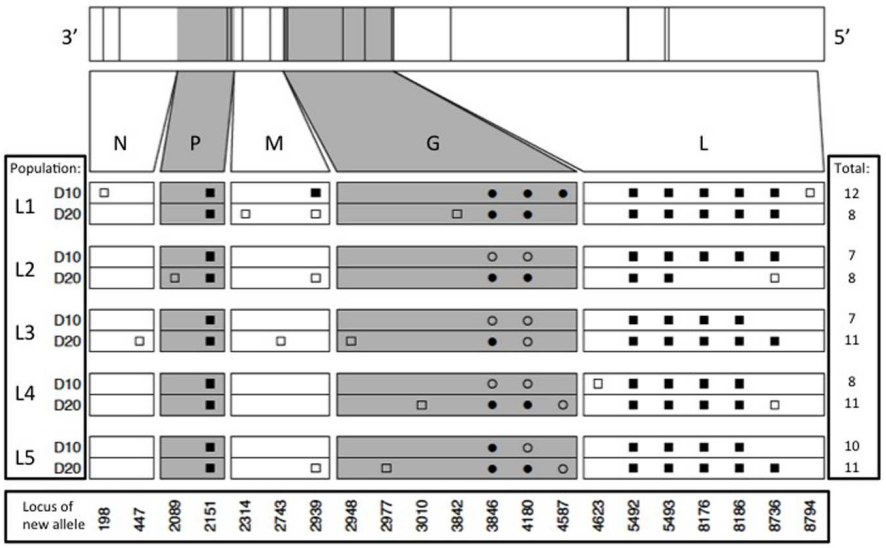

FIGURE 2 | Locations of new mutations relative to the ancestral sequence in the evolved VSV populations, at days 10 and 20 of experimental evolution. Ticks on bars across top indicate physical locations of mutations in the genome. Viral nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and large polymerase protein (L) genes are indicated on both scaled and unscaled bars. Within the 10 lower bars, each symbol represents a new nucleotide. Genome location is shown at bottom. Populations sharing symbols in the same locus share the new allele. Squares and circles represent synonymous and non-synonymous changes, respectively, and filled and open symbols represent fixed and polymorphic changes, respectively.

Although consensus sequencing offers a convenient 'snapshot' of the allele diversity in a virus lineage, these gross changes necessarily omit key details regarding the genetic backgrounds on which mutations arise and accumulate. Nevertheless, our comparison of consensus sequences in the early versus late phases of the experiment, both within and across populations, strongly suggested that molecular evolution occurred and that it was highly repeatable. We concluded that effects of genetic drift did not generally overwhelm those of natural selection, because abundant parallel substitutions were observed and these could not have occurred by chance (Remold et al., 2008). Thus, the available evidence indicated that the most obvious dynamic changes (dampened oscillations that characterized early versus late phases) were probably driven by similar changes across the five lineages, which occurred relatively early during the study. Apparently, frequent virus co-infection exerted strong selection for particular synonymous and non-synonymous substitutions early in the experiment, creating parallel consensus genetic changes across the five virus 'communities' (full-length and DIP genotypes). This parallelism mirrored highly similar population dynamics, especially approaches to roughly the same ecological equilibrium across the five lineages. Additional work beyond the current study, especially VSV reverse genetics, would be necessary to distinguish which of the parallel changes truly constituted adaptive mutations fixed by selection, versus those that rose to high frequencies via other processes (e.g., via genetic hitchhiking).

The population dynamics observed in our experimental populations were reminiscent of those observed in macro-organism predator–prey and host–parasite systems, because predator/prey and parasite/host relative densities can oscillate in a similar fashion (Begon et al., 1996; Gotelli, 1998). In these classic macro-organism examples predators and parasites can sometimes regulate prey and host numbers. For example, removing or minimizing the predator allows the prey to achieve a population size that is much larger, because they are no longer effectively regulated. By analogy, in our system DIPs can be considered the "predator," and ordinary viruses the "prey." By growing ordinary viruses on their own, we determined that presence of DIPs caused the full-length viruses to be regulated at a population size much lower than they could ordinarily achieve. This result suggested that the dampened oscillatory dynamics were driven more by ecological than evolutionary interactions, because representative full-length viruses from lineage L2 had not changed in their ability to produce large population sizes when challenged to grow at low *MOI* on the BHK host cells. Previous work suggested that ordinary viruses could evolve specific resistance to a particular DIP (e.g., Roux et al., 1991), and the possibility existed that coevolution with DIPs could select for resistance mechanisms that were costly for ordinary virus fitness in our study. Rather, we observed that full-length viruses maintained robust growth despite selection favoring DIPs. Moreover, this apparent avoidance of a possible evolutionary growth trade-off indicates that the ordinary viruses should maintain the potential for evolved improvement if they were released from ecological interactions with DIPs. However, further work could examine the intriguing possibility that ordinary viruses suffer evolutionary contingency, if placed in a novel selective environment, such as a new host or incubation temperature. That is, following their ecological history of exposure to cheating by DIPs and any associated genetic-architecture changes in the full-length virus genome, negative

epistasis may limit the evolvability of these ordinary viruses compared to otherwise identical viruses cultured for the same period of time in absence of cheating. Other studies show that various ecological circumstances can prove consequential for performance and evolvability (potential to adapt) of RNA viruses in novel environments, because the selective environment causes changes to viral genetic architecture (Turner et al., 2010, 2012; Alto et al., 2013; Goldhill, 2015). Thus, an intriguing possibility for future work would be to challenge the endpoint full-length virus populations to grow or evolve under new circumstances, to reveal whether historical exposure to cheating affects their performance and/or potential to further adapt.

AUTHOR CONTRIBUTIONS

EW: main execution of genome sequencing and phenotypic analysis of evolved virus populations; writing and editing of manuscript. NM: design and execution of evolution experiment; writing and editing of manuscript. BW: sequence and other data analysis; editing and commenting on manuscript. VB: execution of experiments confirming presence of DIPs. SW: design of experiments confirming presence of DIPs; feedback on manuscript. PT: design and interpretation of experiments; writing and editing of manuscript.

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Development of cell differentiation in the transition to multicellularity: a dynamical modeling approach

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Multicellularity has emerged and continues to emerge in a variety of lineages and under diverse environmental conditions. In order to attain individuality and integration, multicellular organisms must exhibit spatial cell differentiation, which in turn allows cell aggregates to robustly generate traits and behaviors at the multicellular level. Nevertheless, the mechanisms that may lead to the development of cellular differentiation and patterning in emerging multicellular organisms remain unclear. We briefly review two conceptual frameworks that have addressed this issue: the cooperation-defection framework and the dynamical patterning modules (DPMs) framework. Then, situating ourselves in the DPM formalism first put forward by S. A. Newman and collaborators, we state a hypothesis for cell differentiation and arrangement in cellular masses of emerging multicellular organisms. Our hypothesis is based on the role of the generic cell-to-cell communication and adhesion patterning mechanisms, which are two fundamental mechanisms for the evolution of multicellularity, and whose molecules seem to be well-conserved in extant multicellular organisms and their unicellular relatives. We review some fundamental ideas underlying this hypothesis and contrast them with empirical and theoretical evidence currently available. Next, we use a mathematical model to illustrate how the mechanisms and assumptions considered in the hypothesis we postulate may render stereotypical arrangements of differentiated cells in an emerging cellular aggregate and may contribute to the variation and recreation of multicellular phenotypes. Finally, we discuss the potential implications of our approach and compare them to those entailed by the cooperation-defection framework in the study of cell differentiation in the transition to multicellularity.

Keywords: multicellularity, differentiation, multiscale modeling, dynamical patterning modules, cooperation, defection

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1. Introduction

The evolution of multicellular organisms with some degree of cell differentiation and characteristic spatial arrangements has been identified as one of the major transitions in the evolutionary history of life (Maynard-Smith and Szathmary, 2000). Indeed, with the formation of many-celled organisms came a qualitative change in scale and the specialization of coexisting cell types; cell differentiation became spatial and not only temporal (Michod, 2007; Mikhailov et al., 2009).

Multicellular organisms, here defined broadly as an integrated mass of cells with spatial differentiation have evolved at least 25 times (Grosberg and Strathmann, 2007). Nevertheless, the mechanisms that lead or have led to the formation of stable patterns of cell differentiation in emerging multicellular organisms are not yet clear (Maynard-Smith and Szathmary, 2000). There are two main frameworks dealing with the process of cellular differentiation and patterning in emerging multicellular organisms. Here we refer to them as the cooperation-defection framework (Michod and Roze, 2001; Michod and Herron, 2006) and the dynamical patterning modules (DPMs) framework (Newman et al., 2006; Newman and Bhat, 2009). The cooperation-defection framework focuses on the conditions that allow and could produce cell differentiation and patterning in a group of cells that behave as cooperators or defectors (cells that contribute or not to the group fitness, respectively) in emerging multicellular organisms. On the other hand, the DPMs framework focuses on the mechanisms involved in cell differentiation and patterning through cell-to-cell interactions and physicochemical processes. Although both approaches deal with similar questions and can lead to complementary explanations, in some cases they can also imply contrasting assumptions and ideas.

In this article we first provide a description of both frameworks in the context of the transition to multicellularity. Second, we elaborate on the potential role of two specific DPMs in cell differentiation, and present a working hypothesis for the development of cell differentiation in the transition to multicellularity. Third, we present a mathematical model that illustrates this proposal and can be used to perform *in silico* tests. Finally, in light of our results and other available data, we discuss the scope, limitations and predictions of our proposal and its possible impact on the cooperation-defection framework.

2. Two Frameworks for Studying the Origin of Cell Differentiation and Patterning in the Transition to Multicellularity

2.1. The Cooperation–defection Framework

In the cooperation-defection framework (as understood in the context of game theory) it is considered that cell differentiation involves differences in fitness among components of the multicellular organism. For instance, germinal cells, which divide and reproduce, would have a higher fitness at the individual level in comparison to somatic cells, which do not reproduce and instead contribute to the group fitness (Michod and Roze, 2001). Hence, under this framework germinal cells are thought of as defector cells and somatic cells as cooperative cells (Michod and Roze, 2001). Multicellular organisms with different cellular fitnesses at the individual level might always be affected by defector cells that use the resources of cooperative individuals for their own benefit without contributing anything in return (Nowak, 2006), therefore destabilizing the entire organism (Michod and Roze, 2001).

This raises two important questions: (1) how can cooperative behaviors and thus cell differentiation be robustly maintained in emerging multicellular organisms? and (2) how does a cell attain a cooperating or a defecting behavior? The first question is mainly an evolutionary matter, and considerable effort has been invested in answering it. It has been suggested that cell differentiation and patterning cannot appear without mechanisms that enhance and maintain cooperative behaviors in the face of defector cells. For example, when some conditions are met, such as a given spatial structure (Ohtsuki et al., 2006), high relatedness among the individuals of the group (Grosberg and Strathmann, 2007) or the presence of conflict-mediation mechanisms (Travisano and Velicer, 2004), cooperative behaviors can evolve and become fixed in populations, leading to the maintenance of cell differentiation (Michod, 2003; Gilbert et al., 2007; Nowak et al., 2010; Powers et al., 2011). Michod (2007) suggests that once a mass of undifferentiated cells reaches a threshold size, division of labor becomes beneficial for the group even if it implies that some of the cell types will have a relatively low fitness, leading to or maintaining cell differentiation. This is followed by the transformation of the individual cells into essential components of the group fitness, and finally by their spatial organization (Michod, 2007).

To answer the second question, it has been proposed that cooperating and defecting behaviors are intrinsic to individual cells and that genetic differences or specific genotypes could underlie these two types of behavior (Kirk et al., 1987; Michod and Roze, 2001; Travisano and Velicer, 2004; Thompson et al., 2013). It is of course possible that genetic differences could explain changes in cell behavior, but it would be desirable to aim at explanations that account for cellular differentiation and patterning in sets of cells that, much like in most multicellular organisms, do not exhibit critical differences in their genotypes. On the other hand, differences between cell behavior have also been attributed to changes in gene activation profiles within an organism. In fact, in some organisms, differentiation and cell patterning are partially affected by the differential expression of certain genes, such as *regA* in the development of *Volvox carterii* (Michod, 2007). Nevertheless, it is not clear how *regA* or other genes related to cooperation are regulated in a position- and time-dependent manner (Maynard-Smith and Szathmary, 2000), and further investigation of the developmental processes that produce spatial patterns of gene expression is needed (Bonner, 2001).

2.2. The Dynamical Patterning Module Framework

DPMs are sets of well-conserved molecules in interaction with generic physical processes (i.e., those processes common to

living and nonliving chemically and mechanically excitable systems). The DPMs framework postulates that DPMs couple cells and give rise to steady differences among them, as well as to their spatial arrangement. This framework proposes that some molecules already present in single cells may mobilize physicochemical processes that, at the multicellular scale, yield the organization and patterning of multicellular masses. In fact, some authors suggest that the variety of reproducible, yet plastic, patterns generated by DPMs could have been of particular importance in the emergence of specific arrangements at the origin of multicellularity (Newman and Bhat, 2008; Niklas, 2014). Preliminary sets of basic DPMs have been postulated for animal and plant development (Newman and Bhat, 2008, 2009; Hernández-Hernández et al., 2012; Newman, 2012). For example, in both lineages a DPM for position-dependent differentiation has been identified in the interaction between generic patterning mechanisms (e.g., reaction-diffusion processes) and relatively well-conserved molecules that may move among cells (Newman and Bhat, 2009; Hernández-Hernández et al., 2012).

In turn, the physicochemical processes mobilized by these DPMs should be able to interact with intracellular and multistable biochemical networks leading to stable patterns of differentiated cells (Hogeweg, 2000; Ten-Tusscher and Scheres, 2011). The importance of coupling among multistable cells as a patterning mechanism has also been illustrated by diverse modeling approaches (Furusawa and Kaneko, 2002; Benítez et al., 2008; Azpeitia et al., 2010; Salazar-Ciudad, 2010; Inoue and Kaneko, 2013).

The problem of cell differentiation and patterning evolution has been recognized by both the cooperation-defection and DPMs frameworks, each with very different assumptions. Here, we explore the application of the DPMs framework to the origin of cell differentiation and patterning in the transition to multicellularity.

3. DPMs Framework for the Development of Patterns of Differentiated cells in the Transition to Multicellularity

It has been argued that some of the key ingredients for the transition to multicellularity are cell adhesion and cell-to-cell communication (Grosberg and Strathmann, 2007; Rokas, 2008b; Niklas, 2014). Therefore, in this work, we postulate that the fundamental DPMs for the appearance of multicellular organisms are those involved in communication and adhesion. However, for these DPMs to be involved in the evolution of cell differentiation and patterning during the emergence of multicellular organisms, they (i) should be capable of generating cell patterning among multistable cells and (ii) the molecules involved in these DPMs should precede or evolve during the transition to multicellularity. In the following sections we elaborate on these two matters and then postulate our main hypothesis.

3.1. Adhesion and Communication DPMs are Capable of Generating Cell Patterning among Multistable Cells

It has been shown that DPMs are capable of generating patterns by coupling cells' properties (e.g., Furusawa and Kaneko, 2002; Newman and Bhat, 2008, 2009; Zhu et al., 2010a). For example, some authors have pointed out that mechanical forces exerted on an extracellular matrix can couple cells in a tissue and regulate cellular differentiation and behavior (Ingber, 2002). This is also the case for the adhesion and the communication DPMs. It has been experimentally and theoretically demonstrated that cells with different adhesive properties can generate organized cell patterns (Armstrong and Armstrong, 1984; Swat et al., 2012), and that communication-related DPMs like those involving diffusion and reaction of molecules play an important role in coupling cells and producing patterns at the multicellular or tissue scale. Some of these mechanisms were explored by Turing (1952) with the reaction-diffusion model and by Meinhardt and Gierer (1974) with the lateral-inhibition model. In addition, there is now vast evidence confirming the existence and relevance of these mechanisms in living organisms (e.g., Sternberg, 1988; Affolter and Basler, 2007; Kondo and Miura, 2010; Lander, 2011; Rogers and Schier, 2011). For instance the lateral inhibition and the diffusion DPMs can couple neighboring cells and have proven to be critical for the generation of gradients and patterns of molecules involved in the development of plant, animal and microbial systems. These DPMs have been found to render periodic arrangements in leaves, animal skin and developing bones, and cellular filaments (Newman and Bhat, 2008, 2009; Zhu et al., 2010a,b; Benítez et al., 2011; Hernández-Hernández et al., 2012; Watanabe and Kondo, 2015).

DPMs generate dynamic patterns that provide position-dependent information during development. Moreover, cells in developing organisms have multistable biochemical networks whose steady states have been interpreted as specific cell types (Kauffman, 1969; Thomas, 1973). Thus, position-dependent information might affect the dynamics of intracellular biochemical networks and bias the cells to a particular differentiated state. This interaction between multistable networks and DPMs has been illustrated by modeling in diverse study systems. For example, work in *Arabidopsis thaliana* shows that the spatial organization of the cellular types identified in the root stem cell niche emerges from the coupling of multistable gene networks via molecules that can move among neighboring cells (Azpeitia et al., 2010). Similarly, a model employed to explain the segmentation process in *Drosophila melanogaster* shows the importance and generality of the coupling of multistable networks for pattern formation during development (von Dassow and Odell, 2002; Jaeger and Reinitz, 2006). In another theoretical work, Furusawa and Kaneko (2002) showed that when an aggregate of virtual multistable and coupled cells grows, new steady states (virtual cell types) arise as a consequence of the emergence of microenvironments inside the aggregate.

Experimental studies focusing on microbial organisms that can form multicellular masses show that DPMs coupling can give rise to spatial structuring and cellular patterning. For

instance, the cellular arrangement within fruiting bodies of the bacterium *Myxococcus xanthus* is partially determined by cell-to-cell communication via a contact signal that is also involved in cellular adhesion (C-signal) and that enables patterning mechanisms. This membrane-associated protein is localized at the cell poles; at low intensity it triggers the aggregation phase, while at high levels it triggers sporulation, leading to a concentric pattern that determines the final cellular arrangement in fruiting bodies (Julien et al., 2000). In the development of another model organism, *Dictyostelium discoideum*, prespores and prestalk cells are predetermined in a distinctive centripetal pattern with prespore cells confined to the center and the base and prestalk cells located in the periphery. This pattern is generated prior to the formation of the fruiting body by the enhanced differentiation in prestalk cells in the presence of the higher oxygen concentration at the periphery, and is reinforced by internal cellular coupling via ammonia and cAMP (Bonner et al., 1995, 1998; see review in Bonner, 2001).

In all of the above examples, initially indistinguishable cells are eventually specified as particular cell types or acquire a particular cellular behavior. The determination and organization of these cell types emerge as a result of the dynamic coupling of cells via direct or indirect cell-to-cell communication, suggesting that differentiation and patterning in early multicellular systems does not need to be traced back to differences among single cells, but that patterning may result from the collective dynamics of an initially homogeneous (or at least statistically homogeneous) group of coupled cells.

3.2. Multistable Cellular Networks and the Molecules Involved in the Fundamental Adhesion and Communication DPMs are Well-Conserved and could had Preceded the Transitions to Multicellularity

The key components of the adhesion and communication DPMs, critical for the emergence of multicellularity, appear to be present in a variety of multicellular plant and animal lineages and their closest unicellular relatives.

In the case of adhesion, the proteome of the unicellular choanoflagellates has many adhesion proteins, including cadherins, inmunoglobulins and alfa-integrins (Rokas, 2008b). In the choanoflagellates, some of these proteins seem to be used as a food-catching device. Similarly, some unicellular fungi have collagen (King et al., 2008; Rokas, 2008a) and the algae *Chlamydomonas reinhardtii* has a cell wall with the same components as the extracellular matrix of multicellular organisms, like the one observed in *Volvox* (Kirk, 2005). Similarly, communication molecules like Notch, Hedgehog, and MAPK, well known for their role in cellular communication, have been identified in unicellular organisms (Rokas, 2008a). Another example is the autoinducer molecules that serve as signals already present in bacterial populations (Shapiro, 1998). It is worth noting that in addition to the conservation of some critical molecules for adhesion and communication, simple cellular communication and adhesion could have emerged relatively simply via certain genetic or environmental changes leading to, for example, incomplete division and the formation of intercellular channels.

Finally, multistable gene expression and differential cellular states have been identified in many unicellular organisms (Sanford et al., 2002; Lopez et al., 2009; Maisonneuve et al., 2013). One example is in *C. reinhardtii*, where the presence of light activates the movement of the organisms toward the source of light, while the absence of light induces cell division, clearly showing two different cell states (Kaiser, 2001). In the same organism, nitrogen deficiency induces the differentiation of vegetative cells to pregametes, which are converted to gametes after a light impulse (Beck and Acker, 1992).

Multistable networks, cellular communication and adhesion molecules seem to be well-conserved among multicellular organisms and their closest unicellular relatives. Therefore, it is plausible to assume that these elements were already present before the transitions to multicellularity (Miller and Bassler, 2001; Abedin and King, 2008; Davidson and Surette, 2008; Newman and Bhat, 2009; Hernández-Hernández et al., 2012; Suga et al., 2013).

3.3. DPMs-based Hypothesis for the Development of Patterns of Differentiated Cells in the Transition to Multicellularity

If, as argued above in Sections 3.1 and 3.2: (i) DPMs (specifically adhesion and communication DPMs) are capable of generating cell patterning among multistable cells and (ii) the molecules involved in the fundamental adhesion and communication DPMs are well-conserved among multicellular and their unicellular relatives (being likely then that these molecules were present before the transitions to multicellularity), then one can hypothesize that:

> *In the transition to multicellularity, robust patterns of differentiated cells can result from the adhesion and communication DPMs that couple multistable cells and, thus, the origin of such patterns does not need to assume any pre-established or individual cell behaviors (cooperation or defection) nor fitness differences among cells.*

In the following section we present a dynamical model that incorporates the conditions (i) and (ii) reviewed above and illustrates the possible cell differentiation patterns generated by adhesion and communication DPMs in a mass of multistable cells. We also use this model to explore how changes in the parameters of the DPMs generate variation in the observed multicellular patterns. Importantly, the cells in the model are identical. Hence, they do not have any pre-established cell behavior or fitness difference. As other previous models (e.g., Furusawa and Kaneko, 2002; Zhu et al., 2010a), our model supports that patterns can result from the interactions of DPMs, but it aims to explore this process in the transition to multicellularity, so it only considers components associated to cell adhesion and communication, present in the unicellular and multistable virtual elements.

4. A dynamic and *in silico* Exploration of our DPMs-based Hypothesis

In the previous sections we provided empirical and theoretical evidence that supports the conditions (i) and (ii) underlying our hypothesis. The following model illustrates how the dynamical coupling of cells by DPMs can, in principle, give rise to complex and steady patterns, even from cells that are initially indistinguishable from each other.

4.1. Model Description

We model a population of identical cells, each with a simple intracellular regulatory network. The network has the structure of the so-called activator-inhibitor system (**Figure 1**) (Meinhardt and Gierer, 2000). This network has two nodes, A and I, that can correspond to metabolites, proteins or other molecules, and reaches two steady states. We define each steady state as different cell types, identified here as activated ($A \geq I$; blue) and not activated ($I > A$; red). (For a detailed explanation see **Box 1**).

The virtual cells are set to interact under different scenarios of cell adhesiveness and communication. Cell adhesiveness could vary in strength (none, low, high) and as adhesiveness values increase, cell movement within the group decreases regardless of the specific nature of the molecules and processes involved. In the high adhesion scenario we fix the cells, avoiding membrane fluctuations. In the other scenarios, cell shape is variable over time (**Box 1**). The coupling of cells in a mass can be considered as a consequence of intercellular communication, which can be defined as direct or indirect. In direct intercellular communication, cells communicate only with adjacent cells they are in contact with, for example via membrane proteins or by intercellular channels. In indirect communication, cells secrete a signal that once in the medium can be detected by surrounding cells. In our model, communication is (i) direct, (ii) indirect or (iii) mixed, when both direct and indirect communication are present. Our model thus contains multistable cells and the key elements of a DPM: molecules present in single cells associated with adhesion and communication mobilizing a generic patterning mechanism (here, activator-inhibitor) as cells transition to the multicellular scale. We performed a set of simulations using CompuCell3D (CC3D), a software that allows modeling of multiscale biological systems using the Potts formalism (Swat et al., 2012).

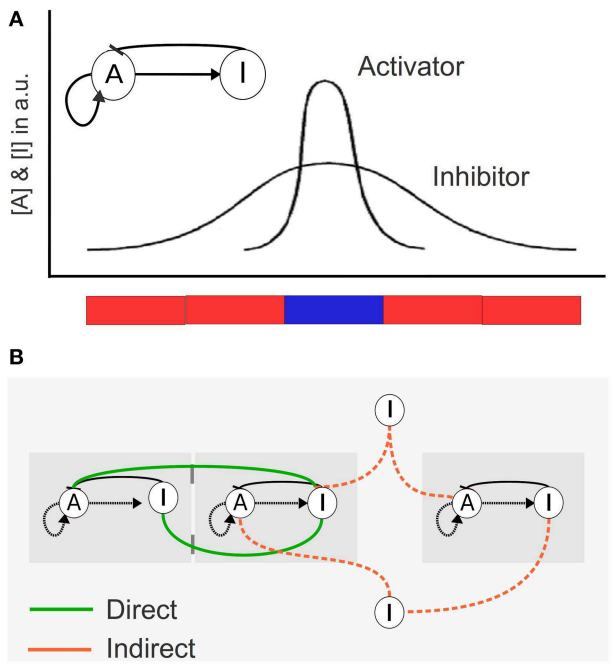

FIGURE 1 | Schematic description of the mathematical model for cellular coupling via different modes of communication. (A) Internal network composed by an Activator (*A*) and an Inhibitor (*I*) in each cell. The *A*/*I* ratio determines the color of the cell (if $A \geq I$: blue cell, if $I > A$: red cell). In this figure we represent the concentration of *A* and *I* in arbitrary units (a.u.) of five cells in a filament, where each cell is blue or red depending on the *A*/*I* relation. **(B)** Different types of cellular communication. Cells can communicate in three ways: (i) direct communication mediated by the diffusion of a signal to adjacent contacting cells (green), (ii) indirect communication in which the signal can be diffused to and sensed from the medium (orange), and (iii) a mixed scenario in which both direct and indirect communication are allowed.

4.2. Simulations and Results

We carried out simulations first for a single cell and then for a cell population. In a single cell, the network dynamic always leads to the blue cell type ($A \geq I$), irrespective of the kind of communication being simulated (**Figure 2**). Similarly, in a cell population (non adhesive cells) without any kind of communication ($s = 0$, $d = 0$ in Equations 5 and 6 in **Box 1**), the network dynamic always reaches the blue cell attractor. Nevertheless, when indirect communication is allowed, the population can become heterogeneous (**Figure 2**). Then, when we include cell adhesion, diverse cellular arrangements with the coexistence of different cell types emerge. As suggested by Furusawa and Kaneko (2002) for a model of cellular masses, when there is both intercellular adhesion and communication, cell patterns with differential gene expression states arise. In our simulations, the nature of the particular arrangements depends on the kind of communication (*s* and *d* in **Box 1**).

As shown in the final states depicted in **Figure 2**, direct communication gives rise to a pattern of spaced blue cells surrounded by red cells. Indirect communication renders radial patterns in which the red cell type arises preferentially on the border of the aggregate, surrounding the blue cell type. Finally, mixed communication results in an interesting pattern of blue dots tending to locate in the center of the aggregate.

When adhesion decreases, there are differential effects on the final pattern, depending on the kind of communication. The decrease in cell-to-cell adhesion provokes an increment in cellular movement and, as a consequence, a continuous change in the relative position of cells in the aggregate. In the direct communication scenario, this continuous change does not affect the qualitative pattern obtained. Nevertheless, in the case of indirect and mixed communication scenarios, where the position in relation to the external medium is critical, the final pattern is highly modified by the continuous movement of cells in the aggregate. Finally, our simulations indicate that such

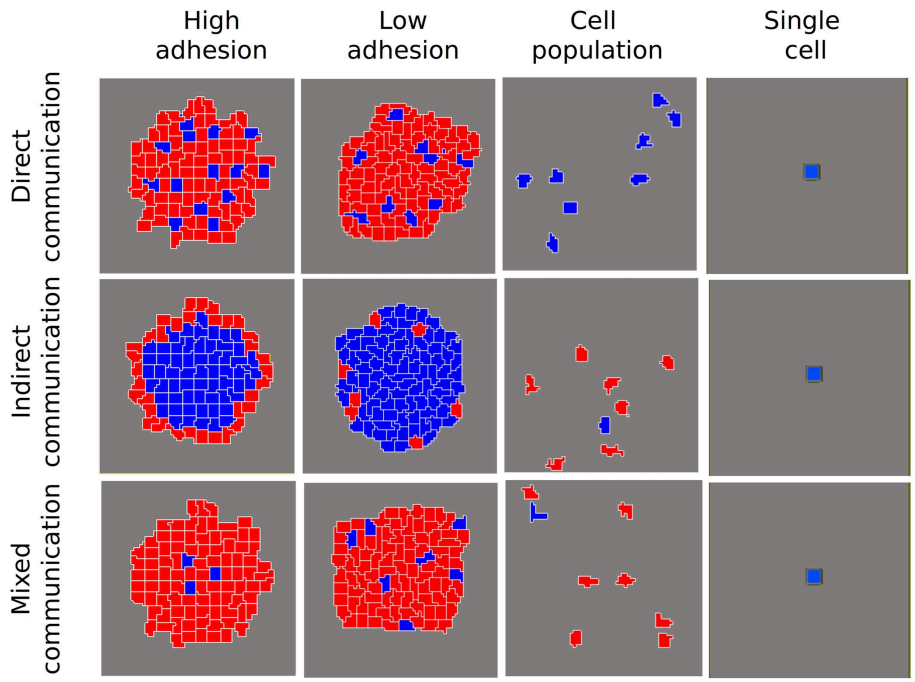

FIGURE 2 | Patterns of differentiated cells emerging in different scenarios of cell-to-cell communication and for different degrees of adhesion among cells. We show a control scenario of individual cells where, independently of the communication type, cells always reached a steady state where $A \geq I$ (blue cells). Another control scenario shows the simulation of a population of cells without adhesion. In this case, when indirect communication is allowed, different cell states are reached (blue cells: $A \geq I$; red cells: $I > A$), without any patterning. When cellular adhesion is introduced and is high, three different patterns of differentiated cells are attained. If adhesion is low (i.e., cellular movement allowed) these patterns are qualitatively similar, but are not stable.

arrangements are robust to changes in the aggregate size and transitory perturbations (data not shown).

In accordance with previous work (e.g., Salazar-Ciudad et al., 2001; Furusawa and Kaneko, 2002; Salazar-Ciudad, 2010; Zhu et al., 2010a), our results suggest that initially identical cells can give rise to a variety of patterns as a consequence of their coupling (in this case via adhesion and communication), which produces differences in local and internal properties. While DPMs render robust patterns, these arrangements can be modified by intracellular or environmental factors, providing a source of variation. In particular, our simulations suggest that the specific mode of communication has an effect on the emerging cellular arrangements and should be further investigated.

Interestingly, some of the patterns we observe (**Figure 2**) resemble the arrangement of some of the classical model systems in the study of multicellularity, even if the particular coupling mechanisms are different from the ones we modeled. A typical example of an emerging multicellular arrangement is that of spore cores surrounded by accompanying cells in *M. xanthus* (Sager and Kaiser, 1993; Julien et al., 2000; Holmes et al., 2010). This arrangement is similar to that generated in the mixed communication scenario, though our model used different specific coupling mechanisms than occur in *M. xanthus*. However, our simulations support the hypothesis that this particular arrangement could be, in principle, originated from similar cells coupled by different types of communication. Another interesting example comes from the predetermination of preespore and prestalk cells in *D. discoideum* before the formation of the fruiting body. This arrangement, where peripheral cells become prestalk cells and internal cells become prespore cells, is determined by the effect of oxygen in the medium, reinforced by internal loops of ammonia and cAMP (Bonner et al., 1995, 1998; Bonner, 2001). This emphasizes the role of the position of the cells with respect to the medium in their determination. The pattern is similar to our indirect communication scenario where the contact with the medium is decisive (**Figure 2**).

5. Impact of Our Hypothesis on the Cooperation–defection Framework

According to our hypothesis and simulations, the development of cell specialization and patterning in emerging multicellular organisms could be a mechanistic consequence of cell interactions and physicochemical properties. In all our modeling scenarios, stable and non-trivial patterns of cellular states emerged without assuming that individual cells had an intrinsic initial cooperative-defective strategy. Hence, the differentiation between cell types (for example, between somatic and germinal cells) and the spatial arrangement in emerging multicellular

BOX 1 | GGH Simulation and Description of the Model

1. Virtual Cells

In Glazier–Graner–Hogeweg (GGH) simulations, cells are defined as a set of pixels in an *ixj* lattice (**Figure 3**). Each cell has its own identifier (τ) and belongs to a particular cell type (σ). In our model, there are two cell types (blue or red) and an external medium. The cell type depends on the ratio of two internal molecules; activator A and inhibitor I (if $A \geq I$: blue, if $I > A$: red).

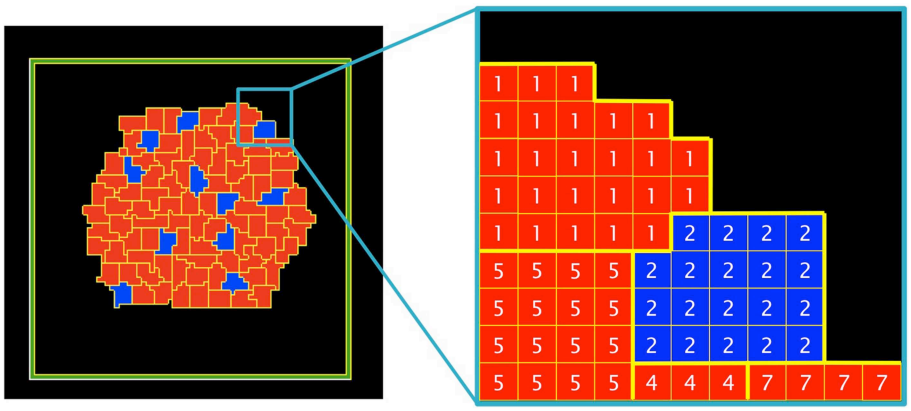

FIGURE 3 | Detail of the cells and the external medium in a bi-dimensional lattice. Five different cells are depicted (τ = 1, 2, 4, 5, 7) belonging to two different cell types (σ = blue, red), a non-flux frontier (in green) and the external medium (in black).

2. Effective Energy: Adhesion, Volume

The core of GGH simulations is the *Effective Energy* H_{GGH} which describes some cell behaviors and interactions. The objective of the GGH simulations is to find the configuration of cells and cells types with the minimum H_{GGH}. As such, characteristics of cells are described by different instances of H:

$$H_{GGH} = H_{boundary} + H_{constraints} \tag{1}$$

To begin with, we define a boundary energy $H_{boundary}$ to simulate cell adhesion between cells of different types:

$$H_{boundary} = \sum_{\vec{i},\vec{j}\ neighbors} J\left(\left(\tau\left(\sigma\left(\vec{i}\right)\right)\right)\left(\tau\left(\sigma\left(\vec{j}\right)\right)\right)\right)\left(1-\delta\left(\sigma\left(\vec{i}\right),\sigma\left(\vec{j}\right)\right)\right) \tag{2}$$

Where $J((\tau(\sigma(\vec{i}))),(\tau(\sigma(\vec{j}))))$ is the boundary energy per unit area between two cells (σ_1, σ_2) of given types $(\tau(\sigma(\vec{i}))),(\tau(\sigma(\vec{j})))$ at a contact point. The term $(1-\delta(\sigma(\vec{i}),\sigma(\vec{j})))$ avoids taking into account pixels belonging to the same cell. If J between cells is high compared to the contact between the cells and the medium, adhesion between both cells will be low and *viceversa*. In our model, we perform three different adhesion scenarios (high, low and no-adhesion).

Volume, surface and other characteristics are defined through an elastic form, where each of the variables tends toward an objective value. For example in the case of volume (v):

$$H_{constraint=v} = \sum_{\sigma} \lambda(v(\sigma) - v_{tg}(\sigma))^2 \tag{3}$$

Where $v(\sigma)$ is the actual volume of the cell and $v_{tg}(\sigma)$ is the target volume. λ is the spring constant which determines the constraint strength.

3. Dynamics and shape

The configuration of the lattice changes over time through random index-copying attempts between pixels (for example pixel $\vec{i}$ tries to copy its own identifier (τ) to $\vec{j}$) which are accepted or rejected according to the Boltzmann probability:

$$P(\sigma(\vec{i}) \rightarrow \sigma(\vec{j})) = \{1 : \Delta H \leq 0, e^{-\Delta H/T} : \Delta H > 0\} \tag{4}$$

Where T is the *membrane fluctuation* parameter. These dynamics enable a cell to move, grow and change shape during the simulation. Since cells' membranes are in constant fluctuation, their shape is only determined by the optimization of the target volume and target surface. In the high adhesion scenario, we established that cells do not move, so we fixed the cells in order to eliminate the unnecessary fluctuations. When a cell is fixed, there are no attempts for index copies (as we will see below). As a result, cells keep the same shape all over the simulation.

4. Cells internal network and cell communication.

Each of the cells σ in the simulation has an identical Activator-Inhibitor network (**Figure 1**), defined in CC3D through Ordinary Differential Equations (ODEs) and Partial Differential Equations (PDEs). This network has two nodes or genes, an Activator (A) and an Inhibitor (I). A promotes the production

(Continued)

BOX 1 | Continued

of itself and that of I. On the other hand, I inhibits the production of A. This is summarized by Equations (5) and (6) (without the communication terms).

We considered three types of communication: direct, indirect and mixed. This model assumed the existence of a signal (specifically, the signal was the node I) that moves between cells through diffusion without any receptors. All scenarios are included in Equation (6). When communication is direct, I can only move through cells that are in direct contact with each other (s is set to 0; and only the second term is considered in Equation 6). When communication is indirect, cells can only communicate with the outside medium (d is set to 0; and only the first term is considered in Equation 6). When communication is mixed, cells can communicate both with their direct neighbors and with the external medium (both terms are considered).

$$\frac{dA_\sigma}{dt} = \frac{k_1 A_\sigma^2}{I_\sigma} - k_2 A_\sigma \tag{5}$$

$$\frac{dI_\sigma}{dt} = k_3 A_\sigma^2 - k_4 I_\sigma + \overbrace{s(I_{ext_\sigma} - I_\sigma)}^{\text{Indirect Comunication}} + \overbrace{d(\sum I_n - \#(N_\sigma) I_\sigma),\, n \in N_\sigma}^{\text{Direct comunication}} \tag{6}$$

Where A_σ and I_σ are the concentration of A and I in cell σ. I_{ext_σ} is the concentration of I outside the cell σ – in its boundary. N_σ is the set of neighbors of cell σ. n is a cell identifier that belongs to N_σ. $\sum I_n$ is the sum of I in the neighbors of cell σ. $\#(N_\sigma)$ is the number of neighbors of cell σ. k_1, k_2, k_3, k_4, s and d are parameters that could account for bistability. Neighbors are continually changing in time and are calculated in each step.

When I is present in the external medium, it only diffuses passively through the medium (Equation 7). In the boundary in contact with the cell σ, there is an exchange of I with the cell σ (Equation 8).

$$\frac{\partial I_{ext}}{\partial t} = D\nabla^2 I_{ext} - k_5 I_{ext} \tag{7}$$

$$\frac{\partial I_{ext_\sigma}}{\partial t} = D\nabla^2 I_{ext_\sigma} - k_5 I_{ext_\sigma} + s(I_\sigma - I_{ext_\sigma}) \tag{8}$$

Where I_{ext_σ} is the concentration of I in the boundary with cell σ. D corresponds to a diffusion rate and k_5 stands for the degradation rate of I in the medium.

Finally, it is important to emphasize that we employed these Equations (5) and (6) due to their bistability that could account for the different cell types.

5. CC3D global dynamics.

Swat et al. (2012) contains a useful and detailed explanation of the CC3D method. In brief, CC3D performs simulation in discrete time steps, the so-called MonteCarlo Steps (Swat et al., 2012). In each of the steps there are as many random index copy attempts (accepted or not according to the Boltzmann equation probability) as there are pixels in the lattice. Then the ODE and PDE are calculated through the Forward Euler Method and cell types are updated depending on their A/I ratio. The cycle then repeats, for approximately 30,000 steps.

organisms using a DPMs approach do not need to be interpreted under a cooperation-defection framework. Consequently, we argue not only that the DPMs approach relaxes some of the cooperation and defection assumptions often invoked when explaining the development of cellular differentiation and patterning in emerging multicellular organisms, but even that some of these assumptions could be incompatible with the DPMs-based hypothesis.

To begin with, under the cooperation-defection framework, individual cell behaviors (cooperator or defector) are usually pre-established independently of their context (e.g., internal chemical networks or coupling mechanisms). Assigning a particular behavior and fitness to individual cells in an aggregate implies conceptualizing them as independent agents and averaging a non-additive property among single cells. However, once cell coupling mechanisms are considered, a cell's behavior, and thus its fitness, is dependent on the internal properties of the cell, the cellular context and the interactions with other cells. Since under this vision cellular states are not individually acquired features but inseparable parts of a whole, an important consequence is that under the DPMs framework, defector cells would not give rise to defector cell populations. Instead, the identity and behavior of the cells, as well as their relative proportions, in an integrated multicellular organism would depend again on the cell properties and the cellular context and interactions. In addition, the cooperation-defection framework invokes paired relationships between cells (Nowak, 2006; Michod, 2007), while the non-linear nature of the processes that characterize developing organisms makes it seem unlikely that collective or systemic features are the result of paired interactions (Axelrod, 2006; Jaeger and Reinitz, 2006).

Finally, we argue that the DPMs approach can help to avoid or relax assumptions such as the presence of initial strategies due to genetic differences or the capacity of cells to establish beneficial relationships with those cells that are more genetically similar. In principle, cell-to-cell coupling can arise and render patterns of cell differentiation in cells that are initially identical as in our simulations, or in cells that are heterogeneous. The fact that in both empirical (e.g., *Volvox* or filamentous cyanobacteria) and theoretical (e.g., our simulations) systems patterns are generated from initially homogeneous groups of cells suggests that initial genetic strategies are not necessary for pattern formation.

In our opinion, the consequences of our hypothesis on the cooperation–defection framework open other questions that could be explored in future research. Hence, in the last section, after a brief recapitulation of our ideas, we discuss some of these possible questions.

6. Discussion

The cooperation-defection and DPMs frameworks are the two main frameworks for explaining the origins of cell differentiation and patterning in the transition to multicellularity. While the cooperation-defection framework focuses mainly on evolutionary questions, the DPMs one is more interested in the developmental mechanisms that could be involved in cell differentiation and patterning (although it may also have evolutionary implications). In this work we postulate and test the hypothesis that cell differentiation and patterning in emerging multicellular organisms are the consequence of the coupling of multistable cells via communication and adhesion DPMs. These DPMs, along with intracellular networks, are fundamental ingredients for multicellularity to arise. Our hypothesis is supported by demonstrations of cell patterning as a result of adhesion and communication DPMs and their coupling with multistable cells, as well as on evidence suggesting that the coupling molecules and multistable behaviors pre-date multicellular organisms and could be coopted in the transitions to multicellularity. Once the cells are coupled, cell behaviors are dependent on the interactions between cells and hence, it is unnecessary to assign inherent behaviors or fitnesses to individual cells. We implemented a model considering the assumptions entailed by our hypothesis and further explored *in silico* its outcomes and some possible sources of phenotypic variation (communication types and adhesion strength). Our simulation results, along with the current available evidence, support our hypothesis and enable us to further discuss the possible implications of our proposal on the cooperation-defection framework.

Our simulations show that particular cellular arrangements could be originated from initially identical cells that can both directly and indirectly communicate, spontaneously reaching different identities in the aggregate (**Figure 2**). Moreover, if the external and internal conditions are maintained, this arrangement could be in principle re-created in a population of interacting cells with certain local and shared properties (e.g., adhesiveness and a type of response to communication). The variability in the cellular patterns can be generated by changes in particular DPMs (e.g., type of communication), which can in turn be due to changes in the single cells of a population, or to environmental changes that favor different types of coupling (Newman and Müller, 2000; Newman and Bhat, 2008, 2009). Further theoretical and empirical explorations might help to clarify how different types of communication and sources of variation affect the generated patterns of cell differentiation, as well as to identify robust cellular patterns and the cellular and environmental processes that are necessary and sufficient to reproduce them.

The DPMs framework emphasizes the role of cellular interactions and physicochemical processes between cells and the external environment in producing phenotypic variation in the first multicellular organisms (Newman et al., 2006). Indeed, Newman et al. (2006) do not deny the role of genetic changes in the organism variation, but they hypothesize that genes might act as regulators (rather than creators) of change, through the processes of stabilizing selection (Schmalhausen, 1949), canalization and genetic assimilation (Waddington, 1953; West-Eberhard, 2003). They explain that if individuals with certain patterns of cell types, developed through the action of DPMs, have some advantage (or no disadvantage) they will act as a template that permits genetic changes that further regulate and make those pattern more robust. For example, the internal genetic network could be modified and make the pattern more reliable and robust against internal mutations (Salazar-Ciudad, 2010). This would in principle stabilize the process of re-creation with DPMs through generations.

Considering fitness is certainly important for the evolution of multicellularity in populations, as natural selection will modify these populations selecting for the fittest individuals. Yet our proposal does not rely on fitness as the sole explanation for the development of phenotypic variation in emerging multicellular organisms. The evolutionary scenarios discussed above, as well as those considering competition, nutrient availability, populations of reproducing aggregates, etc., could be explored in future versions of this and other dynamical models and would shed light on the evolutionary implications of our proposal.

Another important issue that could be addressed in future modeling efforts refers to the role of stochastic fluctuations among genetically homogeneous cells. This has indeed been reported as a relevant factor in the determination of cell types in some multicellular organisms formed by aggregation (Nanjundiah and Sathe, 2013).

Our results imply that it is possible to reach and re-create cell differentiation and cell patterning, in a variety of spatial arrangements, without appealing to cooperative or defective behaviors. To our knowledge, this is the first time this proposal is discussed in the context of the transitions to multicellularity and contrasted with the cooperation-defection framework. Nevertheless, the role of conflict-mediating mechanisms in systems of coupled cells should still be carefully studied and discussed, for example in a set of independent, non-coupled individuals.

If there is some initial heterogeneity between largely independent cells due for example to mutations (Travisano and Velicer, 2004), cell behavior could be associated to such initial cell heterogeneity, and the cooperation-defection framework could apply. Nevertheless, this framework is better suited to explain the persistence of cooperative populations, in spite of the presence of cheaters (Foster et al., 2004; Travisano and Velicer, 2004; Santorelli et al., 2008) and not the mechanisms that lead, or have led, to the evolution of spatial cell differentiation. For example, mutant cheating strains have been identified in *M. xanthus* and in *D. discoideum*. When mixed with wild type strains, these cheating strains are overrepresented as spores and underrepresented in the stalk (Strassmann et al., 2000; Velicer et al., 2000). This imbalance

might lead the population either to collapse (due to its inability to form fruiting bodies for dispersion) or to a complete switch from the wild type to the mutant strain where there would be, again, two types of cells: stalk and spore cells in normal proportions (e.g., facultative cheaters, Santorelli et al., 2008). However, in neither of both cases, the presence of cheaters affects nor explain the patterns of cell differentiation themselves.

The cooperation-defection framework has also been used to explain differences in the degree of complexity attained by different organisms and lineages. For instance, there are two ways by which groups of cells can arise, namely, aggregation of initially isolated cells (e.g., *M. xanthus* or *D. discoideum*) and incomplete division (e.g., animals and plants, which develop from a single cell). Since the multicellular organisms that are often identified as the most complex in terms of size, number of cell-types and body plans are generated by incomplete division, it has been speculated that the minimization of genetic conflict caused by genetic homogeneity allows them to generate more cell types and spatial structures (Grosberg and Strathmann, 2007). Aggregates formed from cells that can descend from different lineages are thought to have more genetic conflict and thus reach less complexity. There are complementary ways to tackle this problem. Some sources suggest that genetic homogeneity is not a necessary condition for cell differentiation (for example in the case of *D. discoideum*, Bonner, 2001) nor a sufficient condition (*Eudorina elegans*, a 32 cell homogeneous aggregate, does not have any spatial cell differentiation, Kirk, 2005). From the DPMs framework we could speculate than some differences between aggregates and organisms arising from incomplete division might be due to the dynamic differences between clusters that result from cells that become coupled in a single and relatively fast event (aggregation) vs. clusters resulting from cells that are gradually incorporated and coupled into the cluster (incomplete division).

Similarly, the relationship between size of the aggregate and cell differentiation has been well documented (Bonner, 2004). In the context of the cooperation-defection framework, it has been suggested that once a mass of undifferentiated cells reaches a threshold size, division of labor becomes beneficial for the group even if it implies that some of the cell types will have relatively low fitness, leading to or maintaining cell differentiation (Michod, 2007). However, another explanation based on the dynamical properties of coupled cells is also possible; Kaneko has suggested that in larger aggregates of coupled cells, more microenvironments of nutrient concentration or signals can emerge from cell-to-cell and cell-medium interactions, which in turn bias the cellular fates and yield more cell types (Furusawa and Kaneko, 2002).

Finally, it is worth noting that our work and discussion focuses on the process of cellular differentiation and patterning in emerging multicellular organisms and that any extrapolation to other biological or social scales are beyond the scope of our model (though it would be interesting to address how the coupling of different dynamic mechanisms could change our understanding of collective organization at other scales).

We have pursued a modeling approach based on the DPM framework to address one of the questions we consider central in evolutionary developmental biology: the origin of cell differentiation and patterning in the transition to multicellularity. This approach relies on different assumptions than the cooperation-defection framework on the problem of cell differentiation and provides new working hypotheses, complemented with dynamical mathematical modeling. This approach is specially interesting for the transition to multicellularity, as Bonner (2001) put it: *"In trying to reconstruct the beginning, our most effective tool is mathematical modeling, which allows us to ask: What is the minimum signaling needed to produce a pattern?"* We believe that it would be interesting to evaluate these ideas with oriented experiments in order to validate the possible predictions. In fact, we consider that joint theoretical and experimental approaches (e.g., experimental evolution) will be key to uncovering some fundamental principles behind the development and evolution of multicellularity.

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Extracellular enzyme production and cheating in *Pseudomonas fluorescens* depend on diffusion rates

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Bacteria produce extracellular enzymes to obtain resources from complex chemical substrates, but this strategy is vulnerable to cheating by cells that take up reaction products without paying the cost of enzyme production. We hypothesized that cheating would suppress enzyme production in co-cultures of cheater and producer bacteria, particularly under well-mixed conditions. To test this hypothesis, we monitored protease expression and frequencies of *Pseudomonas fluorescens* producer and cheater genotypes over time in mixed liquid cultures and on agar plates. In mixed culture inoculated with equal frequencies of cheaters and producers, enzyme concentration declined to zero after 20 days, consistent with our hypothesis. We observed a similar decline in cultures inoculated with producers only, suggesting that cheater mutants arose *de novo* and swept the population. DNA sequencing showed that genetic changes most likely occurred outside the protease operon. In one experimental replicate, the population regained the ability to produce protease, likely due to further genetic changes or population dynamics. Under spatially structured conditions on agar plates, cheaters did not sweep the population. Instead, we observed a significant increase in the variation of enzyme activity levels expressed by clones isolated from the population. Together these results suggest that restricted diffusion favors a diversity of enzyme production strategies. In contrast, well-mixed conditions favor population sweeps by cheater strains, consistent with theoretical predictions. Cheater and producer strategies likely coexist in natural environments with the frequency of cheating increasing with diffusion rate.

Keywords: cheating, diffusion, extracellular enzyme, protease, protein, *Pseudomonas fluorescens*, social evolution, spatial structure

INTRODUCTION

The breakdown of complex organic matter is a fundamental biogeochemical process that is largely controlled by microbes. Living biomass contains a wide range of polymeric macromolecules, including proteins, nucleic acids, and structural carbohydrates (Stevenson, 1994; Kögel-Knabner, 2002; Lee et al., 2004). Microbes produce extracellular enzymes to degrade these compounds into smaller molecules that can be taken up across the cell membrane and metabolized (Burns, 1982; Sinsabaugh, 1994). Thus carbon and nutrient cycles in ecosystems depend on microbial extracellular enzymes.

Microbes can benefit from enzyme production by accessing energy and nutrients in complex compounds, but this strategy also requires a substantial resource investment in enzyme synthesis and excretion (Frankena et al., 1988). Enzyme production may consume ∼1–5% of bacterial productivity (Giuseppin et al., 1993; Christiansen and Nielsen, 2002), and also requires large amounts of nitrogen because enzymes have C:N ratios of ∼3:1 (Sterner and Elser, 2002).

Extracellular enzymes belong to a class of chemical compounds known as "public goods" because they are costly to individual microbes but increase resource availability for other organisms. Public goods include compounds like signaling molecules, antibiotics, siderophores, and secreted proteins (West et al., 2007). The production of public goods represents an evolutionary conundrum because natural selection should favor "cheater" variants that exploit public goods without paying the cost of production (Velicer, 2003; Travisano and Velicer, 2004). By avoiding this cost, cheaters can gain a competitive advantage and increase their individual fitness and population size relative to enzyme producers.

Theoretical studies suggest that cheaters can suppress enzyme producers and reduce substrate degradation rates in model systems (Allison, 2005). Theory also shows that cheating and cooperation depend on spatial structure (Nowak and May, 1992; Wakano et al., 2009). Under well-mixed conditions, cheaters have equal access to the benefits of enzyme production and readily out-compete enzyme producers. In contrast, restricted diffusion causes the products of the enzymatic reaction to remain spatially close to enzyme producers, giving producers a local competitive advantage against cheaters. Thus limited diffusion and resulting spatial structure promote coexistence of cheater and producer strategies.

Similar patterns have been observed with other public goods. For instance, colicin-producing, colicin-resistant, and colicin-susceptible strains of *E. coli* can coexist in spatially structured but not well-mixed environments (Chao and Levin, 1981;

Durrett and Levin, 1997; Kerr et al., 2002). Similarly, multiple strains of *Pseudomonas fluorescens* can evolve and coexist in spatially heterogeneous microcosms (Rainey and Travisano, 1998), and siderophore production by *P. aeruginosa* is less susceptible to cheating when dispersal is limited (Kümmerli et al., 2009a). Christensen et al. (2002) showed that when growing on benzyl alcohol, *P. putida* and *Acinetobacter* coexist in a biofilm but not in a well-mixed environment.

Although some empirical studies are consistent with cheating in extracellular enzyme systems, few studies have confirmed that reductions in enzymatic function are due to cheaters. For example, Worm et al. (2000) found that a protease-negative *P. fluorescens* strain survived on complex protein as a primary N source when grown with a wild-type strain that was protease-positive. However, it was not clear if the putative cheater in this system had a negative impact on enzyme production. Another study by Romaní et al. (2006) found that bacteria had a negative impact on enzyme-producing fungi that were decomposing plant litter in aquatic mesocosms. In this case, negative interactions could have resulted from cheating or some other mechanism, such as toxin production (Mille-Lindblom et al., 2006).

The predicted negative impact of cheaters on enzyme producers has been demonstrated via controlled competition experiments with genetically manipulated microbes. Gore et al. (2009) used yeast strains lacking the invertase gene as an extracellular enzyme cheater and defined a range of conditions under which cheater and producer strains could coexist. Cheaters were demonstrated to have a negative impact on producer growth, but mainly at high population densities. At low population densities, invertase producers were able to maintain a growth advantage through a small (∼1%) positive differential in access to hydrolysis products.

The objective of our study was to analyze the effects of diffusion, cheating, and evolution in extracellular enzyme systems. Although spatial structure is known to promote diversity in many microbial systems, theoretical predictions about the effect of diffusion on enzyme cheating have not been confirmed. Based on findings with similar public goods (Kümmerli et al., 2009b), we hypothesized that restricted diffusion should allow enzyme producers to preferentially access reaction products and thereby coexist with cheaters. In contrast, we expected cheaters to outcompete enzyme producers under well-mixed conditions in liquid cultures. We tested these hypotheses using a protease-producing strain of *P. fluorescens* and an isogenic knockout strain that was protease-negative (the cheater). We tracked cheater-producer dynamics over time in liquid culture or on agar plates with transfers to new medium every few days. This design, commonly used in experimental evolution studies (Elena and Lenski, 2003), also allowed us to observe the *de novo* emergence of new enzyme production phenotypes.

MATERIALS AND METHODS

STRAINS

We obtained strains of *P. fluorescens* ON2 from Dr. Ole Nybroe's laboratory at the University of Copenhagen, Denmark. The original strain ON2 was isolated from a freshwater sediment and subjected to genetic manipulation via electroporation with plasmid pJBA28. This plasmid contains a pUTmini-Tn5-*gfp* gene cassette with a kanamycin (kan) resistance marker (Christoffersen et al., 1997; Worm et al., 2000). The wild-type "producer" strain ON2 secretes a single extracellular metalloprotease, whereas strain ON2-pd5 (the "cheater") contains the Tn5 gene cassette and does not secrete protease. Protease phenotype was screened by selective plating on kan agar containing skim milk; the cheater strain was unable to metabolize skim milk. Subsequent sequencing of the cheater strain showed that the transposon was inserted downstream of the protease structural gene and likely interfered with expression of an ABC transporter required for protease secretion (Worm et al., 2000).

MEDIA

Strains were grown on either M63 medium or casein medium. The base M63 medium (pH 7.0) contained 2.0 g/l $(NH_4)_2SO_4$, 13.6 g/l KH_2PO_4, 0.5 mg/l $FeSO_4[H_2O]_7$, and 0.2% (w/v) glucose. Casein medium contained 0.4% (w/v) casein in diluted M63 medium (0.25 × in deionized water). Base and casein media were supplemented with 1 mM $MgSO_4$, 0.35 mM $CaCl_2$, and 50 μg/ml ampicillin. Solid media for plate experiments contained 1.5% agar.

EXPERIMENTAL DESIGN

For experiments in shaking flasks, cells were grown overnight in M63 medium to an OD_{600} of ∼1 and inoculated into triplicate 50 ml flasks containing 10 ml casein medium. One set of triplicates was inoculated with overnight culture containing only cheater cells. We added 5 mg/l proteinase K to these flasks to hydrolyze casein and allow for cheater growth. This concentration of proteinase was equivalent to the protease present in producer cultures at stationary phase. These flasks served as a control for cheater growth and allowed us to confirm that the Tn5 insertion was maintained in the absence of kan for the duration of the experiment. Another set of triplicate flasks contained casein medium and was inoculated with a mixture of 50% cheater cells and 50% producer cells to examine competition between the strains. A final set of triplicate flasks also contained casein medium but was inoculated with only producer cells. This treatment was designed to test whether protease function was maintained in the absence of inoculated cheaters. For all three experiments, starting OD_{600} in each of the triplicate flasks was 0.01.

All flasks were maintained at 28°C in a dark incubator with orbital shaking at 200 rpm. After the cultures reached stationary phase, 100 μl culture fluid was transferred to a new flask containing 10 ml fresh medium. For trial 1 of this experiment, transfers were done every 1 or 2 days for 23 days depending on the availability of laboratory staff. For the second trial, transfers were standardized to occur every 48 hours for 30 days. Optical densities at stationary phase were ∼1.0, which corresponds to $\sim 3 \times 10^9$ cells/ml (Worm et al., 2000). Given that the culture was diluted 100-fold at each transfer, growth to stationary phase represents approximately 6.64 bacterial generations. Relative abundances of ON2-pd5 were determined at several time points by diluting the culture fluid, plating out on LB agar plates (all with 50 μg/ml ampicillin), and counting the number of colonies on plates with and without kan (25 μg/ml).

For the agar plate experiments, triplicate plates were inoculated with ∼6.6 × 10^6 producer cells or a 50–50 mixture of cheaters and producers, as in the flask experiment. Because growth was slower on agar, transfers occurred every 3 days for 81 days. Cells were transferred to new agar medium by contact plating. Our shaking flask experiment showed that after a period of time, protease phenotype did not necessarily correspond to kan resistance phenotype. Therefore, we assayed protease expression directly on clones isolated from agar plates to determine the frequencies of cheaters and producers in this experiment.

PROTEASE ASSAYS

For the shaking flask experiment, we used the colorimetric azocasein method (Tomarelli et al., 1949) to assay protease activities in the culture fluid at 2–7 day intervals during each trial. Protease activities were expressed as enzyme concentrations (proteinase K equivalents) based on a standard curve of absorbance versus proteinase K concentration (440 nm after 60 min incubation at 37°C). We also measured the frequency of cheater and producer phenotypes on days 12 and 24 of the second trial in flasks inoculated with producers only. The culture fluid was diluted and plated out on agar, and 22 individual colonies were picked and assayed for protease activity with the azocasein method after 48 h growth on casein medium. For the agar plate experiment, we used a high-throughput fluorimetric assay (Pierce® Fluorescent Protease Assay, Thermo Scientific) to assay protease expression in 100s of randomly isolated clones.

GENETIC ANALYSES

To identify possible genetic variation underlying changes in protease phenotype, we extracted and sequenced genomic DNA from all replicates of trial 2 (day 30) in the shaking flask experiment. We also extracted and sequenced the genomic DNA of the ancestral strain *P. fluorescens* ON2 for a total of 10 libraries. Genomic DNA was extracted with the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions, sheared to ∼300 bp with the Covaris S2 System, and barcoded with the TruSeq DNA sample prep kit (Illumina). Fragments were sequenced on the Illumina platform at the UC Irvine Genomics High-Throughput Facility. The resulting reads from each library were assembled into contigs using PRICE (Ruby et al., 2013), Velvet (Zerbino and Birney, 2008), and Minimus (Sommer et al., 2007). A 94 kbp contig (GenBank accession KJ540107) from the ON2 assembly was used as a reference sequence for mapping Illumina reads. This contig contained the extracellular protease operon as confirmed by BLASTn alignment to a 434 bp sequence upstream of the Tn5 insertion point provided by Dr. Nybroe (GenBank accession KJ540106). Prior to mapping, all 100 bp reads from ON2 and each of the shaking flask libraries were trimmed to 40 bp with the FAST-X Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and aligned to the reference contig using BLASTn with a >90% sequence identity cutoff and hit length >30 bp. BLAST matches were mapped to the reference contig using Geneious 7.0.6 (Biomatters Ltd., www.geneious.com). Geneious software was used to search for genetic differences between the reference contig and the mapped sequences from each experimental library. Mean coverage after mapping ranged from 120 to 190× within libraries with an overall average of ∼160×.

STATISTICAL ANALYSES

Optical densities were compared across inoculation treatments using analysis of covariance with time as the covariate. We used Tukey tests to determine if changes in optical densities over time were significantly different across treatments. For the agar plate experiment, we calculated the frequencies of cheaters versus producers based on relative enzyme expression of isolated clones. Background fluorescence of the substrate and medium was subtracted from the fluorescence of each clone to calculate net fluorescence. Relative expression values were calculated by dividing the net fluorescence of each clone by the maximum net fluorescence observed at a given transfer. For the agar plate experiment, clones with relative expression values >0.56 were considered producers whereas clones below this value were classified as cheaters. We also calculated the coefficient of variation in relative protease expression across all clones at each time point and used linear regression to determine if there was a significant increase in variation over time.

RESULTS

SHAKING FLASKS

In trial 1 with shaking flasks, we observed significant ($P < 0.001$) declines in optical density from >1.0 to <0.3 over a 23 day period in mixed and producer cultures growing on casein medium (**Figure 1A**). We also observed a slight but significant decline in the optical density of cheater cultures growing on hydrolyzed casein ($P < 0.01$). However, the declines in the mixed and producer cultures were significantly greater ($P < 0.001$) than in the cheater cultures, which had optical densities near 0.9 for most of the trial. Producer optical densities were higher than mixed cultures until day 21 when they converged on similar values, but the rate of decline was not significantly different between the two treatments.

Concurrent with the decline in optical density, protease concentration and kan sensitivity also declined during trial 1 in cultures growing on casein medium. Protease was initially higher in producer cultures than in mixed cultures, but neither culture showed detectable protease by day 20 of the trial (**Figure 2A**). The fraction of kan sensitive clones indicative of the producer genotype also declined from 50% to nearly 0% during this time period (**Figure 2C**).

The second mixed flask trial was similar to the first in overall patterns of optical density (**Figure 1B**) and protease concentration (**Figure 2B**). However, the rate of decline in optical density of the producer culture was not as large as in the mixed culture ($P < 0.05$). This difference is largely due to the recovery of protease concentration in one of the three replicate producer flasks. In this flask, optical density and protease concentration recovered to levels of 0.9 and 14.4, respectively, leading to increased variability in these parameters after 28 days (**Figures 1B** and **2B**). Also in contrast to the first trial, kan sensitivity indicative of the producer genotype increased to nearly 100% after 28 days in the mixed cultures (**Figure 2D**). However, assays on individual clones isolated from producer cultures revealed that ∼76 and ∼86% of the cells

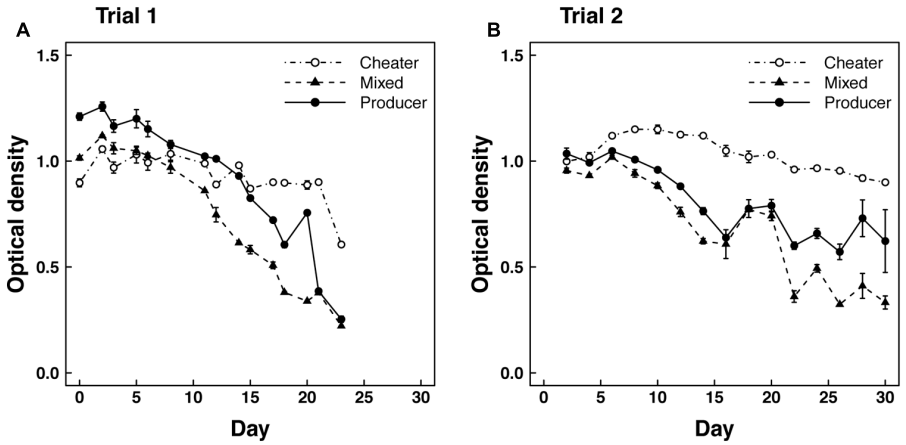

FIGURE 1 | Optical density over time in (A) Trial 1 and (B) Trial 2 for cheaters grown alone on hydrolyzed casein and producers grown alone or mixed (50–50%) with cheaters on casein medium.

were negative for protease expression on days 12 and 24 of trial 2, respectively.

GENETIC ANALYSES

We did not find evidence for genetic changes in the protease operon or surrounding regions that could account for observed differences in protease phenotype. Although we detected multiple genetic variants with frequencies >10% in each of the mapped genomic libraries from trial 2, these variants were also present in the ancestral ON2 strain. Our genetic analyses were clearly capable of detecting genetic variation because sequences containing Tn5 genetic material were found in all libraries that contained the ON2-pd5 cheater strain.

AGAR PLATES

To assess the effect of spatial structure and restricted diffusion on enzyme production, we monitored the frequency of cheater and producer phenotypes in cultures maintained on agar plates. When starting with 50% cheater and 50% producer genotypes, cultures on plates were initially dominated by producer phenotypes (>80%), but cheater phenotypes became dominant after 30 days and remained dominant through the end of the experiment (**Figure 3A**). Similarly, cultures started with 100% producers were dominated by producer phenotypes through the first few transfers, but cheater phenotypes became more abundant over time (**Figure 3B**). After 50 days, roughly equal frequencies of cheater and producer phenotypes were maintained on agar plates. Consistent with these results, we observed a significant ($P < 0.001$) increase over time in the coefficient of variation for protease production among clones isolated from the agar plates initiated with producers only (**Figure 3C**). These results indicate an increasing diversity of enzyme expression levels over the course of the experiment.

DISCUSSION

Data from both trials under shaking conditions support the hypothesis that cheater phenotypes have a selective advantage. In the first trial, a loss of protease function over 23 days was accompanied by a loss of the kan sensitive genotype characteristic of our producer strain. Under shaking conditions, protease-negative mutants likely have a selective advantage because they do not pay the costs of enzyme production, yet they can access casein hydrolysis products (Allison, 2005).

Even if no cheater knockouts were inoculated into the initial culture, we still observed loss of protease function over time (**Figures 2A,B**). This pattern could have arisen from *de novo* genetic mutations that conferred a protease-negative phenotype. Our genetic analyses suggest that any such mutations must have occurred outside the protease operon. We did not observe genetic variation in or near the operon that could account for loss of protease function. Still, this result does not rule out a genetic basis for the change in phenotype. Mutations in many enzyme and regulatory genes can have pleiotropic effects that increase fitness in bacteria (Hottes et al., 2013). One or more of these distant mutations could have reduced protease expression in our study.

Our second trial under shaking conditions suggests that cheaters may arise by multiple evolutionary pathways with different fitness consequences. In this trial, we observed a decline in protease expression in the 50–50 mixed culture, but there was no associated loss of kan sensitivity. This pattern suggests that *de novo* mutations in the wild-type strain may have generated cheater strains with higher fitness than our original knockout strain. Since our knockout was generated by random transposon insertion, it is not surprising that other genetic changes affecting protease production would result in higher fitness. The transposon in our knockout was inserted downstream of the protease gene but upstream of genes involved with protease secretion, meaning that our ON2-pd5 cheater strain may still incur the costs of transcription and translation for other genes in the protease operon. *De novo* mutations that reduce or eliminate these costs would be expected to confer higher fitness relative to our original cheater knockout.

It is somewhat surprising that we observed distinct evolutionary outcomes in trial 1 versus trial 2. Cheaters derived

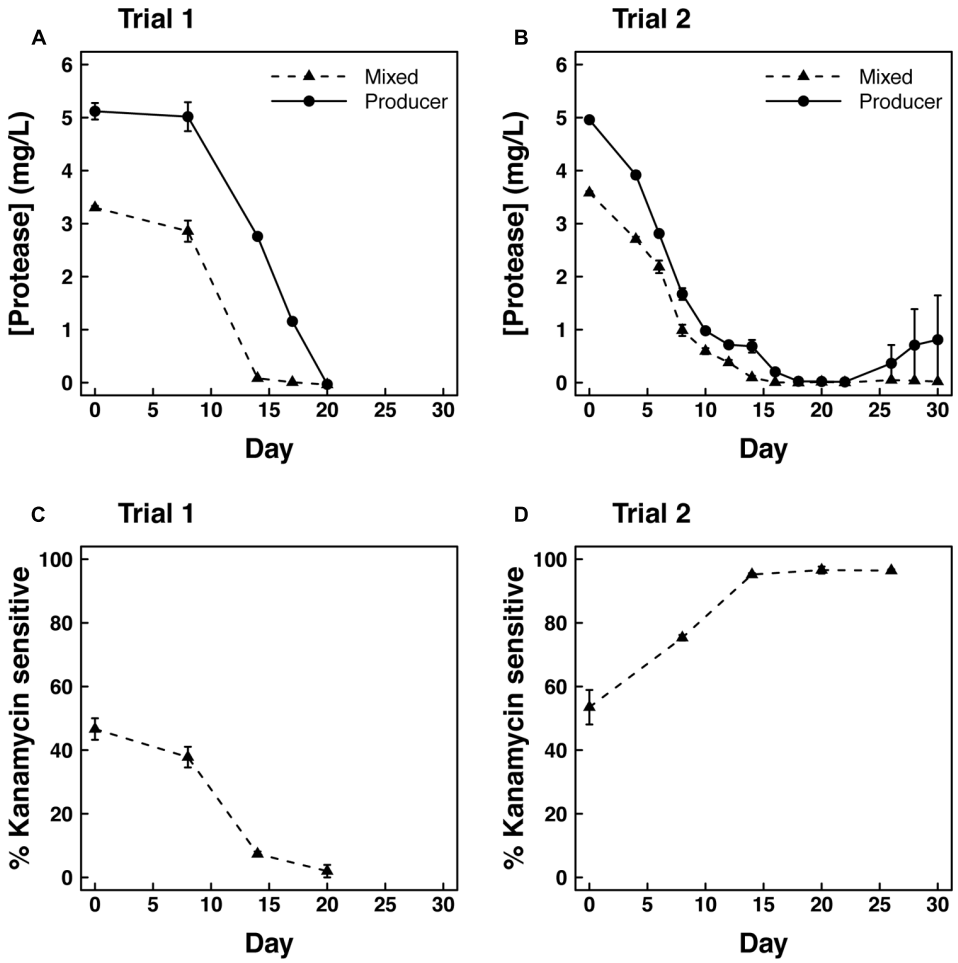

FIGURE 2 | Protease concentration (mg/l proteinase K equivalents) over time in (A) Trial 1 and (B) Trial 2 for producers grown alone or mixed (50–50%) with cheaters. Kanamycin sensitive fraction of isolated clones over time in **(C)** Trial 1 and **(D)** Trial 2 in the mixed culture.

from the wild-type swept all three replicate flasks in the 50–50 mixture of Trial 2, but the original cheater knockout (or its descendants) swept the populations in Trial 1. With only three replicates, we cannot rule out that the different outcomes were due to chance ($P = 0.1$; Fischer's exact test). Alternatively, it is possible that the selective environment differed between the two trials. In particular, the transfers were not regularly spaced for the first trial (every 1–2 days) as they were for the second trial (every 2 days). On average, the cells in trial 1 spent a larger fraction of time growing on fresh medium and less time in stationary phase, which would alter the chemical environment and selective pressures in the flasks.

The results from the liquid producer culture in trial 2 suggest that the loss of protease function is reversible. Protease loss during the first 25 days of the trial reduced the availability of casein hydrolysis products. Under these conditions, protease-producing variants are favored because proteolysis increases resource availability for growth while cheater densities are relatively low. The protease-positive phenotype that emerged after 25 days could have resulted from compensatory mutations or positive selection on rare producer genotypes that survived the population bottleneck on day 22.

Alternatively, loss and recovery of protease function could have resulted from changes in gene expression. However, this mechanism is unlikely given that most clones isolated on days 12 and 24 from producer flasks in trial 2 did not express protease even after plating on agar and regrowth on casein medium. Furthermore, the complete loss of protease function and crash of producer populations probably would not have occurred if gene expression were flexible.

The recovery of protease expression is indicative of frequency-dependent selection, whereby producers are favored at low population densities and cheaters are favored at high densities (Greig and Travisano, 2004). Theoretical studies show that frequency-dependent selection may be driven by spatial structure that allows rare producers to gain preferential access to reaction products (Durrett and Levin, 1994; Wakano et al., 2009). Spatial structure is important because rare producers under perfectly mixed conditions should be out-competed by cheaters since both variants would have equal access to (dilute) reaction products. There may

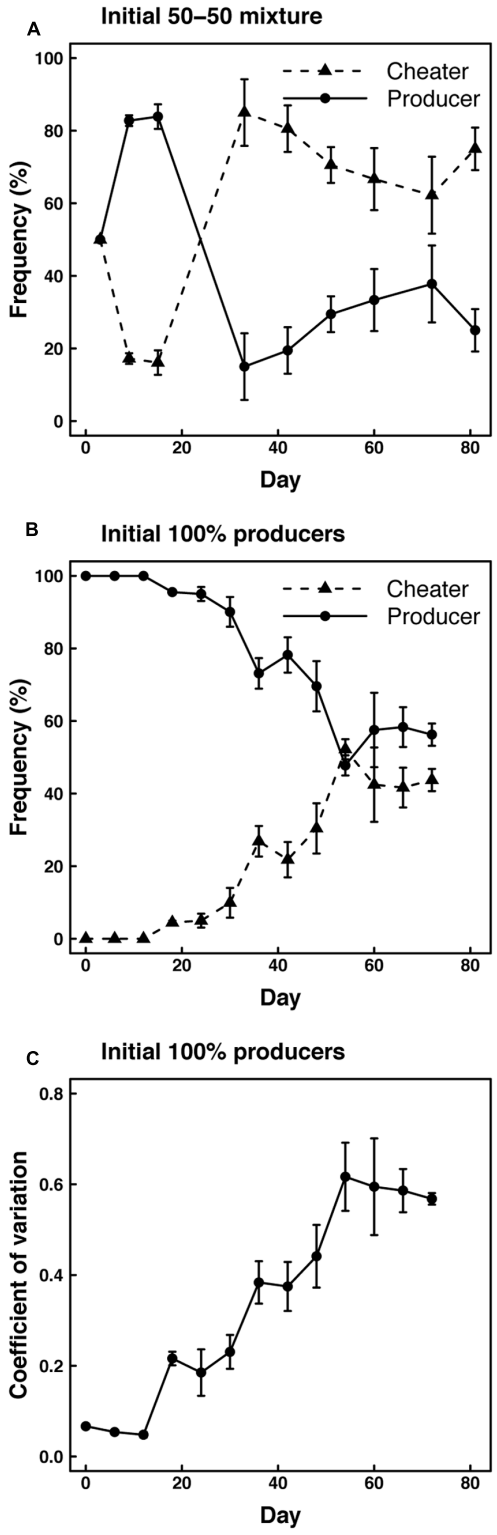

FIGURE 3 | Frequencies of cheaters and producers based on protease expression in experiments with (A) cheaters and producers (50–50%) or only producers (B) initially inoculated onto agar plates. (C) Coefficient of variation in protease expression level based on clones isolated from the agar plates inoculated with producers only.

have been some undetected spatial structure in our flasks that facilitated frequency-dependent selection, such as biofilm formation. This spatial structure was probably limited since we only observed a recovery of protease expression in one flask of the six included in our trials. Clearly defining the role of spatial structure and frequency-dependent selection in shaking flasks would require a larger number of replicates and experiments lasting more than 30 days.

Results from maintaining cultures on agar plates support the hypothesis that limitations on diffusion enhance the coexistence of cheater and producer strains. Frequency variations from the 50–50 mixture are suggestive of oscillations predicted by theoretical models under intermediate diffusion levels (Allison, 2005). Although additional experiments would be required to confirm the consistency and duration of these cycles, they are consistent with the theoretical effect of spatial structure on frequency-dependent selection (Durrett and Levin, 1994).

As in the shaken flask experiment, the agar plate experiment showed evidence for evolution of phenotypes with reduced protease expression. However, the shaking flasks were subject to sweeps by cheaters with undetectable levels of protease expression, whereas the agar plates hosted strains with a continuum of enzyme production levels that we arbitrarily classified into cheaters versus producers. This diversity is evident from the significant increase in the coefficient of variation in protease expression across randomly selected isolates (**Figure 3C**).

Reduced diffusion and increased spatial structure likely both contributed to increased diversity on agar plates. Limits on diffusion allow cheaters and cooperators to coexist because cooperators gain preferential access to the benefits of public goods production (Kümmerli et al., 2009b). In addition, the formation of colonies on agar generates spatial gradients in enzyme, substrate, product, and by-product concentrations. This spatial structure likely leads to differentiation in enzyme production strategies by increasing the number of chemical resource niches. Such a relationship between growth, spatial structure, and strain diversity has been observed with *P. fluorescens* growing in unshaken flasks (Rainey and Travisano, 1998).

Our results have implications for the maintenance of microbial diversity and ecological function in environmental settings. Few natural environments are completely well-mixed, and therefore the selective sweeps that may have occurred in our shaking flasks are probably uncommon. Even in aquatic and marine ecosystems, particulate organic matter generates spatial heterogeneity that could select for a range of enzyme production strategies (Smith et al., 1992). Biofilm formation is common on particles and surfaces in aqueous environments, further increasing chemical and spatial heterogeneity that can facilitate coexistence (Hansen et al., 2007; Cordero et al., 2012). Multi-phase environments such as soils and sediments are likely to have even lower diffusion rates and greater spatial heterogeneity than our agar plate system (Moldrup et al., 2001). Therefore it is likely that evolutionary processes and spatially mediated coexistence are constantly maintaining microbial diversity in these environments.

Although we identified important mechanisms for maintaining diversity and function despite the potential for cheating, our

results also suggest that cheaters play a role in many microbial systems. At least in aquatic and saturated systems most similar to our experimental conditions, cheaters are probably always present to some degree. Broadly defined, cheating need not involve a complete loss of enzyme expression or other function. A reduction in the amount of enzyme production relative to neighboring cells is also a form of cheating if the enzyme benefits are distributed equally but the costs are unequal.

Regardless of its form and magnitude, cheating can impact ecological processes, such as hydrolysis rates for biomolecules. In the case of enzyme production strategies, greater diversity and overall microbial growth may not translate into maximal rates of hydrolysis (Folse and Allison, 2012). Gore et al. (2009) showed that adding glucose (a reaction product of invertase) actually reduces the equilibrium fraction of invertase producers in the population and therefore the hydrolysis rate of sucrose, the invertase substrate. Although our experiments only examined proteolysis under controlled conditions, our results raise the possibility that enzyme-driven decomposition may not increase with diversity as has been observed with other ecosystem functions (Tilman et al., 2006). The evolutionary and ecological processes that generate diversity need not maximize biogeochemical rates because of the constant selective pressure for cheating.

AUTHOR CONTRIBUTIONS

Steven D. Allison conceived the project, designed experiments, analyzed data, and wrote the manuscript. Lucy Lu conducted the experiments, analyzed data, and helped write the manuscript. Alyssa G. Kent and Adam C. Martiny conducted the genetic analyses and helped write the manuscript.

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Impact of spatial distribution on the development of mutualism in microbes

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The evolution of mutualism is one of the long-standing puzzles in evolutionary biology. Why would an individual contribute to the group at the expense of its own fitness? Individual bacterial cells cooperate by secreting products that are beneficial for the community, but costly to produce. It has been shown that cooperation is critical for microbial communities, most notably in biofilms, however, the degree of cooperation strongly depends on the culturing conditions. Spatial community structure provides a solution how cooperation might develop and remain stable. This perspective paper discusses recent progresses on experiments that use microbes to understand the role of spatial distribution on the stability of intraspecific cooperation from an evolutionary point of view and also highlights the effect of mutualism on spatial segregation. Recent publications in this area will be highlighted, which suggest that while mechanisms that allow assortment help to maintain cooperative traits, strong mutualism actually promotes population intermixing. Microbes provide simple and suitable systems to examine the features that define population organization and mutualism.

Keywords: cooperation, non-producer, assortment, microbial population, surfaces

Evolutionary biologists have had a long-standing interest in elucidating the mechanisms sustaining the persistence of cooperation. Why would an individual restrict its own fitness to benefit the whole population? The evolution of cooperation has extensively been studied in animals, while the advantages of research on microbes have recently been exploited to address this puzzle. Microbial cells cooperate by secreting and sharing products that benefit the community, but are costly to produce. Numerous examples have been described that exemplify cooperation in microbes and the dynamics among producing and non-producing members of the population (reviewed in West et al., 2006; Nadell et al., 2008). Importantly, the establishment of cooperation simultaneously results in strategies that ensure the stability of cooperative traits by actively or indirectly reducing the presence of cheaters (Travisano and Velicer, 2004). Microbial colonization of surfaces provides one of the solutions to maintain the stability of cooperation. As microorganisms settle and inhabit biotic or abiotic surfaces, spatial variances in nutrient composition produce subtle environmental differences that allow for a variety of ecological interactions. Cooperation is critical in certain microbial communities, most notably in biofilms, where the degree of cooperation strongly depends on the culturing conditions. In nature, most microbes persist in surface-attached sedentary communities known as biofilms. These communities provide a simple illustration of microbial complexity: distinct cells residing in a biofilm deliver cooperative traits, like the secretion of extracellular polysaccharides (EPS), and these traits are advantageous at the biofilm level, because they improve its resilience and ability to grow, while the costs arise at the single cell level within the population. Here, the focus is given to experimental systems that study intraspecific interactions.

Theoretical studies reveal the importance of microbial spatial distribution. Local environmental differences, e.g., in nutrient concentration, diversify the cell growth and alter the spatial distribution of cells within the population (Xavier and Foster, 2007). Moreover, cooperation is favored when certain parameters are present in a structured environment according to simulation (Allen et al., 2013). Small diffusion rates (low diffusion retains secreted compounds and enzymes close to producers), low colony dimensionality (flat versus complex three-dimensional structures), and small rates of decay of the commonly used and accessible metabolites (public goods) all support cooperation according to mathematical simulations (Allen et al., 2013). Local spatial differences in the microbial population composition might originate from a stochastic distribution of founder cells. Theoretical simulations suggest that EPS producers in biofilms outcompete non-producers in the presence of solute gradient (e.g., oxygen or resource gradient; Xavier and Foster, 2007). Under non-realistic conditions, when no gradient is present, the competition is purely driven by growth rate, and since non-producers are not paying the costs, their growth rate is superior. The competitive advantage of EPS producers originates from the benefit of EPS used only locally. Simulations suggest that when cells in a biofilm population start to grow, EPS production results in lower growth rates (Xavier and Foster, 2007). In later stages, EPS secretion actually helps the producer cells to push the progeny out from the focal cell layer. This process presents a primitive form of kin selection, where cells provide benefits to their descendants, as EPS is most likely shared among cells that are close to each other. While at the population level a variety of microbes are present with low relatedness, locally, highly related clonal population might exist, affecting mathematical simulations,

and evolutionary outcome (Xavier and Foster, 2007). Secretion of EPS by a cell allows it to altruistically push its descendants into a more nutrient-rich environment. There is a fascinating analogy to plants here where vertical growth and leaf coverage increase access to light at the expense of competitors (Xavier and Foster, 2007). Simulations suggest that negative frequency-dependent selection, i.e., the fitness of a phenotype increases as it becomes rarer, is also relevant in microbial populations, where both EPS producers and non-producers are able to invade the population primarily made of cells following the other strategy; however, cooperation will be stabilized long-term at the population level (Xavier and Foster, 2007). Thus, EPS production is an altruistic behavior, as secreting cells have lower growth rates and division, but aid other cells (mostly their descendants) to reach nutrient rich sectors. EPS production in these cases provides a selective advantage by allowing the producer population to rise up and over other cells, therefore reaching more nutrients or oxygen and suffocating others that are left behind.

The consequence of EPS production on cooperation has been further studied in various experimental approaches. In *Vibrio cholerae* chitinase production is exploited by non-producers in mixed environments, while production of thick biofilms or increased flow rate could solve the public good problem, i.e., how cooperative traits are stably maintained in microbes (Drescher et al., 2014). Specifically, thick biofilm allows cooperators to sequester all liberated sugars (public goods) to themselves, while flow removes the majority of liberated sugars so the exploitative non-producer cells cannot use them. Various initial ratios of producers versus non-producers were examined and it was suggested that under mixed conditions (shaked culture) a higher ratio of producers helps the population as a whole resulting in higher growth rate, while the non-producers grow more rapidly by not paying the metabolic cost of chitinase production. Growing on a surface itself does not solve the public good dilemma, as deliberated sugars distribute within the chamber, even at low initial founder cell densities. A solution is provided when cells form thick biofilms. In such a situation, cells far away from the chitin source or outside of the biofilm experience low concentrations of liberated sugars while those cells that inhabit the thick biofilm of producers will benefit from the available resources. Further, biofilm matrix production also increases spatial segregation. Flow of the surrounding medium clarifies the public good dilemma in a different way: in the presence of a current in the medium, all cells experience a reduced concentration of public goods (Drescher et al., 2014). This is selectively disadvantageous for the non chitinase producers because these cells do not benefit from chitin degraded by chitinase in their intimate vicinity, while producers still harvest enough resources close to the enzyme production site.

Excretion of EPS, although benefitting producers, comes with a fitness trade-off. The experiments of Nadell and Bassler (2011) using *V. cholerae* biofilms showed that while EPS production locally benefits clonal cells and gain dramatic advantage, it has an ecological cost in the form of restricted dispersal. EPS producers are impaired in their ability to escape from the biofilm and colonize new niches, presenting a trade-off on matrix production. Their experiments comprised a flagellum and quorum-sensing (QS) deficient strain that constitutively produces EPS (EPS+) and an isogenic but *vpsL* variant that is not producing the biofilm matrix any more (EPS−). While QS mutant *hapR* produces EPS in a constitutive manner, the *vpsL* mutant is highly motile. To examine the sole effect of EPS producing ability, a flagellin A (*flaA*) mutation was introduced into both strains and they were labeled with a fluorescent dye that had no fitness effect on the growth. While in liquid culture, the EPS− cells have a benefit when co-cultured with the EPS+ strain, however, under biofilm conditions, EPS+ cells increase more rapidly in number depending on their initial fraction. One of the advantages could be that the production of the matrix helps the cells to stick to the substratum and resist shared stress (Nadell and Bassler, 2011). EPS+ cells benefit themselves and their daughter cells similarly to what was suggested by the simulation described above. Therefore each tower like biofilm structure predominantly contains cells of one linage. This is also true if genetically identical EPS+ strains are used to initiate flow cell biofilms of *V. cholera*, labeled with different fluorescent reporter proteins. This might explain why EPS production could develop in nature and how exploitation by non-producers might be avoided. However, local competition is not the only factor contributing to long term evolutionary dynamics. The liquid effluent was also monitored during the experiments. While EPS producers were major part of the biofilm obtained in a microfluidic device, the effluent mainly contained EPS− cells at weak but also under strong disturbance. Therefore the competition between EPS+ and EPS− cells also depends on how often empty spots are available that are colonized by dispersing clones (Nadell and Bassler, 2011). Therefore the regulation of both biofilm formation and dispersal is important from an evolutionary point of view. *Staphylococcus aureus* is an important example where after establishing dense community, biofilm gene expression is decreased in a QS [i.e., accessory gene regulator (Agr) system] dependent manner and dispersal is activated (Boles and Horswill, 2008).

Spatial population expansion was recently suggested to also facilitate the evolution of cooperation (**Figure 1**). The studies of van Dyken et al. (2013) and van Gestel et al. (2014) used colonies of *Saccharomyces cerevisiae* and *Bacillus subtilis*, respectively, to demonstrate the impact of assortment within the population on public good exploitation by non-producers. The experiments on *Saccharomyces cerevisiae* employed a simple system where microbial genetic drift can be followed. In this colony expansion system, described first by Hallatschek et al. (2007), radial growth from a founder area depends on cell division mediated expansion (i.e., daughter cells push neighboring cells). As cells deplete the nutrients at the colony's edge, only few cells contribute to propagation leading to a series of genetic bottlenecks that causes high local fixation probability of clonal linages. Theory predicts that cooperation is favored at high genetic relatedness of the microbial cells (van Dyken et al., 2013). However, non-producers might arise rapidly within a population stochastically fixed at the front and increase in number during expansion. The sucrose invertase secretion ability of *Saccharomyces cerevisiae* was employed to examine the dynamics between fluorescently marked strains of public good producers

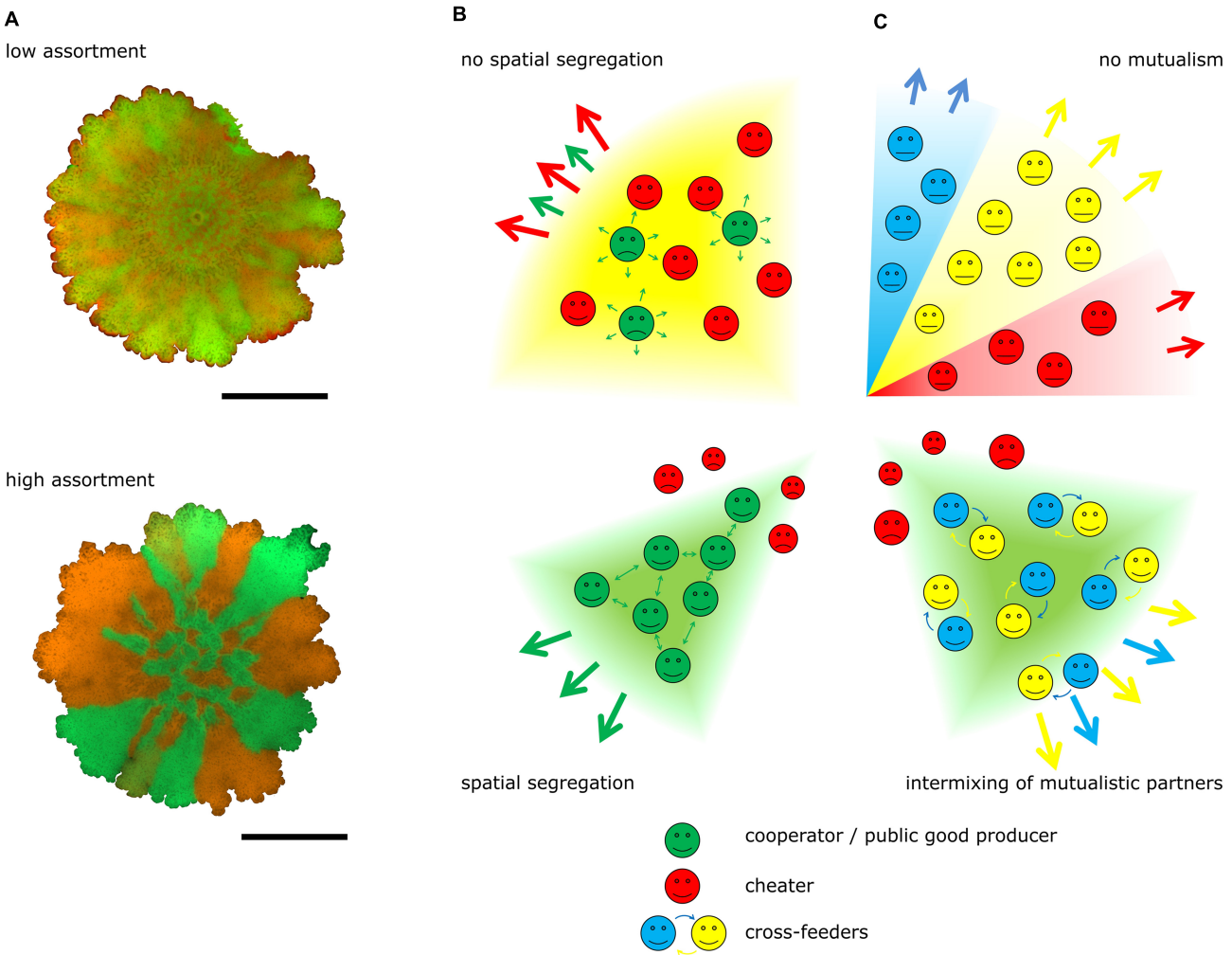

FIGURE 1 | Cooperation and spatial distribution. (A) Experimental examples of *Bacillus subtilis* colony biofilms are shown with low (above) and high (below) spatial assortment of genetically identical strains with different fluorescent labels. Strains producing green- or red-fluorescence proteins were mixed and used to initiate colony biofilms as described by van Gestel et al. (2014). Pictures were obtained with AxioZoom V16 microscope (Zeiss). Scale bars indicate 5 mm. **(B)** Schematic figures depict the role of spatial segregation on the stability of cooperation: non-producers exploit the public good produced by cooperators if no spatial segregation exists in the expanding population (above), while cooperative traits are sustained under high spatial segregation (below). **(C)** Spatial segregation during competition (above) is suppressed when microbial strains or species are exchanged in a mutualistic interaction (below), which results intermixing of partners. Green and red symbols indicate public good producers and non-producers, respectively, while blue and yellow symbols denote mutualistic cross-feeders. Thick arrows show colony expansion, whereas thin arrows designate public **(B)** or exchanged **(C)** goods.

(cooperators) and non-producers in expanding colonies (van Dyken et al., 2013). The exoenzyme sucrose invertase catalyzes the digestion of sucrose to monosaccharides, which can be imported by the cells and metabolized. Under well-mixed unstructured conditions, cooperators decline at all initial frequencies due to the cost of cooperative trait production, in spite of the growth advantage in pure cultures comprising cooperators only. In contrast, during radial colony growth on the surface (i.e., structured environment), cooperators increase in frequency as expansion proceeds. Cooperators also invade non-cooperator populations when initiated at low frequency, and resist public good exploitation by non-producers in later stages due to a faster spreading ability of the producer strains. Thus, under these conditions, high assortment reduces the direct local competition between cooperators and non-producers, and therefore diminishes the potential benefit of defection (van Dyken et al., 2013). As cooperators establish themselves locally, their productivity is higher resulting in a fitness advantage outweighing the costs of cooperation. This leads to a further increase in cooperator frequency.

Spatial pattern formation in *B. subtilis* biofilms was examined using a distinct system (van Gestel et al., 2014). Colony expansion of *B. subtilis* depends on the production of EPS, a biofilm component with fitness costs associated with the production. Genetic drift observed for *Saccharomyces cerevisiae* was not observed in colony biofilms of *B. subtilis*. However, spatial patterns can be modulated on a continuous scale by altering the

founder cell density. Mathematical modeling and experimental approaches both showed that higher dilution of initiating cell numbers increases the degree of assortment that occurs during colony biofilm development, while low dilution, i.e., high founder cell density, results in reduced spatial segregation (van Gestel et al., 2014). Competition experiments that exploit the possibility to alter the level of assortment were employed to show that EPS-producing cells have a selective advantage over non-cooperative mutants when colonization occurs at high spatial segregation, while they have a disadvantage when assortment is low. In addition, adjusting EPS production to diverse levels indicated that the level of EPS production in the wild type cells facilitates surface colonization at an optimal level and secretion of surplus matrix does not aid to further the expansion properties (van Gestel et al., 2014). These experiments therefore showed that colonization dynamics of complex microbial communities could determine the persistence of cooperation.

As discussed above, structured environments define spatial distribution of microbes, which, in turn, impacts the stability of cooperation. From a different perspective, ecological interactions might also define pattern formation and, therefore, the degree of mixing (**Figure 1**). Several recent studies showed that strong mutualistic interactions can stimulate partner intermixing where spatial segregation is suppressed (Momeni et al., 2013a,b; Müller et al., 2014). Using the yeast colony expansion system described above, Müller et al. (2014) genetically engineered two cross-feeding yeast strains and followed the degree of genetic drift in expanding colonies. In these experiments, growth of auxotroph mutants depends on the metabolites excreted by a producer strain, and *vice versa.* They found that nutrient rich medium allows spatial segregation (i.e., distinctive sectors are observed corresponding to the different genotypes). However, when a nutrient poor medium was employed where cell growth and therefore colony expansion depends on cross-feeding, intertwinned patches of the strains could be observed during spatial expansion as mutualism requires physical proximity of the interacting partners. The degree of intertwinement was suggested to be related to the diffusion properties of the mutualism compounds. Genetic drift and mutualism are opposing forces, as one separates, while the other mixes the members of the microbial community, respectively (Müller et al., 2014). Mutualism is the driving force only until a certain nutrient concentration is reached. From then on, genetic drift has the higher impact on colony expansion and community structure. By varying the concentration of different cross-feeding metabolites, the degree of mixing could be examined. At concentrations where mutualism would be still beneficial (i.e., level of nutrient that still enhances growth of the auxotroph strain in pure culture), genetic drift already outcompetes mutualism (i.e., in these experiments, 25% of the minimal nutrient concentration that supports maximal growth rate). Thus mutualistic microorganisms can expand only if the benefit of mutualism is sufficiently strong and if dispersal of the partners is sustained (Müller et al., 2014). It is plausible to assume that the degree of assortment and mutualism driven intermixing also fluctuates depending on the local environmental conditions.

Mutualism driven partner intermixing is also maintained in surface-grown communities where microbes settle at distinct spatially distributed spots and expand to colonize the available niche. Momeni et al. (2013a) utilized metabolite-exchanging yeast strains with different degrees of interactions (i.e., from commensalism to cooperation), and showed that robust mutualism leads to partner intermixing, when ecological interactions are the major patterning factor. Such a stable mutualistic community can return to a stable population composition after it is disturbed. Conversely, competing populations with no metabolic interdependence tend to segregate, described as competitive exclusion (Momeni et al., 2013a). Simulations and analytical calculations based on three-dimensional fitness models with different interaction scenarios showed that initial partner ratios can converge over time if the interaction benefits at least one of the partners, but not for competitive communities. Also, these simulations predict partner intermixing for strong cooperation. Interestingly, further experiments revealed that mixing of mutualistic populations results in a layered pattern where the intermixing index increased proportionally as a function of community height. As observed for colony expansion, layering of the yeast communities, and local patch sizes were suggested to be determined by the localized nutrient supply and consumption, i.e., the distance a secreted nutrient can diffuse within the community. Curiously, while initial partner ratio did not significantly affect the level of intermixing, at very high initial cell densities, intermixing was also observed in the absence of cooperation (Momeni et al., 2013a).

Community patterning is also influenced via partner fidelity feedback, where non-producers are unable to evade cooperator populations. Again, a combination of experiments on yeast and mathematical modeling was applied by Momeni et al. (2013b) to inspect partner fidelity feedback in surface-attached structured environments. Two cross-feeding strains were mixed with a non-producer strain that consumes one of the metabolites produced by the mutualistic cooperators, but not releasing any public good. Non-producers had increased fitness in an unstructured, well-mixed environment. On the contrary, in a structured environment, the cooperators self-organized into mixed clusters, as above, while the cheating strain was excluded from these clusters of mutualistic cells. Partner choice could be ignored in this phenomenon as yeast cells are incapable of partner recognition (Momeni et al., 2013a). Additionally, simulations suggested that self-organization of the mutualistic partners and exclusion of non-producers are driven by asymmetric fitness effects, i.e., unequal spatially localized benefits that the partners supply to the heterotrophic partner during cell growth and expansion. Isolation of non-producers therefore enabled cooperators to rise in frequency regardless of the intrinsic advantage of non-producers over cooperators (Momeni et al., 2013a).

The experiments above all point to the eligibility of microbial systems to demonstrate basic evolutionary theories. These experiments clearly support the idea that genetic drift maintains cooperation within clonal linages; intermixing supports close mutualism in structured environment and in some cases, intermixing with preferred partners might even promote exclusion of non-producers. All these experiments demonstrate that spatial self-organization provides a solution for stability of intraspecific cooperation without the need for specific molecular mechanism

for partner recognition. Experimental microbial systems greatly help us to understand and emphasize the importance of ecology and significance of spatial structures for the evolution of cooperation.

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Ecological perspectives on synthetic biology: insights from microbial population biology

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The metabolic capabilities of microbes are the basis for many major biotechnological advances, exploiting microbial diversity by selection or engineering of single strains. However, there are limits to the advances that can be achieved with single strains, and attention has turned toward the metabolic potential of consortia and the field of synthetic ecology. The main challenge for the synthetic ecology is that consortia are frequently unstable, largely because evolution by constituent members affects their interactions, which are the basis of collective metabolic functionality. Current practices in modeling consortia largely consider interactions as fixed circuits of chemical reactions, which greatly increases their tractability. This simplification comes at the cost of essential biological realism, stripping out the ecological context in which the metabolic actions occur and the potential for evolutionary change. In other words, evolutionary stability is not engineered into the system. This realization highlights the necessity to better identify the key components that influence the stable coexistence of microorganisms. Inclusion of ecological and evolutionary principles, in addition to biophysical variables and stoichiometric modeling of metabolism, is critical for microbial consortia design. This review aims to bring ecological and evolutionary concepts to the discussion on the stability of microbial consortia. In particular, we focus on the combined effect of spatial structure (connectivity of molecules and cells within the system) and ecological interactions (reciprocal and non-reciprocal) on the persistence of microbial consortia. We discuss exemplary cases to illustrate these ideas from published studies in evolutionary biology and biotechnology. We conclude by making clear the relevance of incorporating evolutionary and ecological principles to the design of microbial consortia, as a way of achieving evolutionarily stable and sustainable systems.

Keywords: spatial structure, mass action environment, cooperation, cheating, microbial consortia, synthetic ecology

INTRODUCTION

Microbes have a long biotechnological history, since the first use of yeast and bacteria for fermentation. Modern innovation in biotechnology has harnessed the diversity of microbial metabolic capabilities, almost exclusively by selection or engineering of single strains. The single strain approach has a long record of success, such as the engineering of insulin producing *Escherichia coli* (Williams et al., 1982). Even so, in many cases single selected or engineered strains are not capable of producing the desired product. For example in engineered strains, genetic modification is limited by metabolic load and the number of exogenous elements that can be cloned and optimized in a single cell, among other problems (Glick, 1995; Brenner et al., 2008; Shong et al., 2012). A main goal of synthetic biology is to realize the potential of microbial consortia, to develop systems that can usefully generate products beyond that of single strains. Natural or engineered microbial consortia show great promise in overcoming the limitations of single strain systems, because of their capability for complex metabolic interactions (Verduzco-Luque et al., 2003; Raghoebarsing et al., 2006), and their inherent compartmentalization which prevents undesired cross-reactions and side products (Shong et al., 2012).

Microbial interactions are the primary advantage of consortia and provide its structure and function. Most microbial interactions in consortia are mediated by the use and excretion of metabolites in the form of small molecules (e.g., nutrients, chemical cues, etc.). Extraordinary efforts have been made in modeling metabolic networks to predict the type of interactions among species within microbial consortia to inform engineers in their design (Freilich et al., 2011; Harcombe et al., 2014). These modeling approaches include biophysical parameters (e.g., metabolite concentration, diffusion rates, viscosity of the media, etc.) that necessarily influence the strength of the interactions and the cellular growth of the microbial populations. Using these

models provides the metabolic basis for product generation and frequently can be directly used to predict product output rates.

The main challenge for synthetic ecology is that consortia are frequently unstable, largely because evolution by constituent members affects their interactions. This leads to reduced yield and lower productivity (Field et al., 1995; Hamer, 1997; Kato et al., 2005; Kim et al., 2008), and thus far there are few examples of successful microbial consortia (recently reviewed in Sabra et al., 2010). Current practices in modeling consortia largely consider interactions as fixed circuits of chemical reactions, which greatly increases their tractability. This simplification comes at the cost of essential biological realism, stripping out the ecological context in which the metabolic actions occur and the potential for evolutionary change in biological systems. Consideration of metabolic, biophysical and trophic interactions from an ecological and evolutionary perspective are scant (Momeni et al., 2013). Because ecological and evolutionary principles are frequently not included in design, consortia *evolutionary* stability is not engineered into the system.

This realization highlights the necessity to better identify the key components that influence the stable coexistence of microorganisms, not only in terms of the biophysical variables and stoichiometric modeling of metabolism (Freilich et al., 2011), but also by inclusion of ecological principles as well as organismic and evolutionary understanding of the system into microbial consortia design (Freilich et al., 2009; Kuhn et al., 2010). Fortunately, there is a large literature on community stability from population and evolutionary biology, which includes experimental studies of microbial systems (Stewart and Levin, 1973; Lenski and Hattingh, 1986; Turner et al., 1996). In contrast to biotechnologists, evolutionary biologists have focused on identifying ecological mechanisms and selective regimes that affect community stability. Integrating results from microbial population biology is a powerful addition to engineering of microbial-mediated processes, as has been previously recognized (Goldman and Brown, 2009). Such integration has been hampered by the different technical terms, goals and perspectives of biotechnologists and evolutionary biologists.

Competition and cooperation are two of the most important ecological processes, and are frequently mediated by resources. Resource-based competition involves two or more species that consume the same resources, thereby potentially limiting one another's growth. In contrast, resource-based cooperation promotes growth and persistence, and typically involves consumption of metabolic products. It has two general forms, cascade or reciprocal. Cascade interactions involve one-way consumption, in which the metabolic products of one species are consumed by others. Reciprocal cooperation involve consumption of metabolic products by all species involved in the cooperative interaction (Freilich et al., 2011; Momeni et al., 2013; Grosskopf and Soyer, 2014). In natural communities, it is likely to find species as part of cooperative cycles (Freilich et al., 2011), and these type of interactions, we believe, may be the more stable in evolutionary terms for coexistence when designing microbial consortia.

Access to resources is directly affected by *connectivity*, the propensity for molecules and cells to flow across the system, or as some have referred to as the viscosity of the media (Momeni et al., 2013). Depending on the type of interaction among microbes, competition and the type of cooperation, we argue that spatial structure plays a key role in the stability of the consortia, affecting simultaneously cellular location (Momeni et al., 2013) and resource availability. For communities involving reciprocal cooperative interactions, spatial structure limits the potential for non-cooperative individuals (cheaters) to evolve and spread, and is therefore essential for stable coexistence. In contrast for non-reciprocal interactions, access to resources is imperative and restricting the flow of resources does not contribute to coexistence stability.

This review aims to bring ecological and evolutionary concepts to the discussion on the stability of selected or engineered microbial consortia. In particular, we present a critical review to frame our argument on the relevance that the combined effect of spatial structure and ecological interactions has on the persistence of these consortia. We discuss exemplary cases to illustrate these ideas from the evolutionary biology and biotech-engineering perspectives. We conclude the review by making clear the relevance of incorporating evolutionary and ecologically principles to the engineering approach taken to date in the "design" of microbial consortia as a way of achieving evolutionarily stable, sustainable, and productive biological systems.

ECOLOGICAL PRINCIPLES OF COEXISTENCE DERIVED FROM MICROBIAL POPULATION BIOLOGY STUDIES

Ecological and evolutionary research has demonstrated that biodiversity can emerge and be maintained when there is ecological opportunity and competitive trade-offs that allow the existence of different ecological types (Tilman, 1977; Rainey and Travisano, 1998). Microbial coexistence can be promoted and maintained, by simple changes that affect either resource availability or physical structure (Rainey et al., 2000). The physical structure of environments can promote stable coexistence of genetically distinct individuals by localizing connectivity and interactions (Amarasekare, 2003). Spatial structure can lead to locally depleted resources than can limit growth of competitors and result in a patchy environment with multiple niches and ecological opportunity (Chao and Levin, 1981; Rainey and Travisano, 1998; Greig and Travisano, 2004). However, spatial structure can also limit diversification by reducing connectivity, for example by decreasing resource availability in cases where resources are made available through facilitation (Saxer et al., 2009). We refer to the physical structure of an environment as either *mass action* or *structured*, the key difference between these two categories is their differences in connectivity, respectively high and low, which greatly affects the flow and potential for interactions of cells and molecules within the system (**Box 1**).

For a single species, biological functions tend to persist when the selective benefit of the function or trait is greater than its cost, and are lost if the reverse is true. In multispecies communities, coexistence criteria have a similar structure, building up from component species. For a multispecies community to persist, the biological functions associated with the interspecies interaction must be concomitantly more beneficial to the component species than their respective costs. Therefore when evaluating the effects of ecological interactions and spatial structure, it is necessary to

Box 1 | Key concepts and definitions

Cascade interaction—This is a unidirectional interaction characterized by consumption of another species waste product. In this sense, is a non-reciprocal interaction. A→B.

Cheater—An individual obtaining benefits from a public good produced by other(s) that are disproportionately large relative to its own contribution to such good.

Connectivity—The flow of molecules and cells across the system.

Cooperation—Provision of a benefit available to others at a cost to self.

Ecological interaction—Refers to the interactions between producers and consumers of metabolic products in an ecosystem.

Fitness—Measure of evolutionary success of an individual in terms of survival and reproduction.

Frequency dependence—It refers to evolutionary processes where the fitness of an organism is determined by its relative abundance in the population. It could be positive if fitness increases with frequency or negative when fitness decreases as the organism becomes common in the population.

Interaction—Refers to any interaction between the members of an ecosystem, either biotic or abiotic.

Mass action environment—We refer to mass action environment as a well-mixed culture where resources are available to all members of the population or community.

Polymorphism—Coexistence of two or more clearly different phenotypes, which are in principle the result of genetic differences. It can be understood as biodiversity.

Reciprocal interaction—A form of cooperative two-way interaction, or feedback cooperation, where one organism (A) produces resources for others (B) to the detriment of its own fitness and *vice versa* A↔B, the reciprocal benefits should exceed the cost of the production for the interaction to be maintained.

Selection regime—Is an experimental design that provides the necessary conditions to allow the survival of only those individuals from a population expressing a particular phenotype or performing a specific function. Only individuals selected by the experimental conditions can reproduce.

Structured environment (spatial structure)—This type of environment is best exemplified by either non-mixed cultures, plates or biofilms where resources are localized to the immediate environment of consumers, restricting availability to other members of the population or community. In this environment interactions occur in a localized manner.

simultaneously consider both the costs and benefits of the traits of interest and what affects this ratio. We evaluate two main categories of interactions, cascade (non-reciprocal) and reciprocal. The first is a strategy dependent mainly on large-scale community connectivity with broad availability of resources and other molecules, whereas reciprocal interactions involve cooperative feedback and local connectivity.

CASCADE INTERACTIONS

Cascade type interactions are non-reciprocal, in which metabolites produced by one species or genotype affect the growth of other species. These interactions are characterized by niche partitioning and unidirectional cross-feeding, also called incidental cross-feeding (*sensu* Bull and Harcombe, 2009), where one species uses another's waste as a resource. This is because resource specialist genotypes are generally competitively superior to generalist genotypes when there is an abundance of resources. Surprisingly, this is true even for resources produced by the microbes themselves (Friesen et al., 2004) and adaptation to growth on an exogenously supplied primary nutrient causes reductions in the ability to grow on metabolites. Waste metabolites are excreted to the environment, which are then available for use by other strains that subsequently specialize for growth using these secondary metabolites (Rosenzweig et al., 1994). The waste metabolite resources are inherently associated spatially with the producing bacteria, and as a consequence the biological significance of cascade interactions crucially depends upon the movement of nutrients away from the producing microbes.

Cascade interactions are well known in bacteria (Helling et al., 1987; Rosenzweig et al., 1994; Turner et al., 1996; Treves et al., 1998; Rozen and Lenski, 2000). Probably the best-studied example involves the appearance of polymorphisms during evolution of *E. coli* in glucose minimal medium, which was first reported by Helling et al. in 1987. The work by Helling describes the emergence of stable genetic variants in *E. coli* populations during evolution in a chemostat with a single carbon resource (glucose). *E. coli* variants were first identified on Tryptone Agar (TA) plates, where different colony sizes were observed (Helling et al., 1987). Based on chemostat model of coexistence, only a single strain can persist when there is a single limiting resource in a temporally constant environment, the strain that can replace itself at the chemostat washout rate at the lowest resource concentration (Hansen and Hubbell, 1980; Tilman, 1982, 1988). Thus, it was not immediately clear how an *E. coli* multiple genetic variants could arise and be maintained. In later experiments Rosenzweig et al. (1994) demonstrated that these genetic polymorphisms were maintained by cross-feeding interactions, where a glucose specialist consumes only glucose and produces, as byproducts, glycerol and acetate, which in turn, are consumed by two other genotypes (acetate and glycerol specialists) (**Figure 1A**).

Insights on the origin and maintenance of these polymorphisms have been made by working with polymorphic *E. coli* types from a long-term evolution experiment (Rozen and Lenski, 2000). The populations were derived from replicate cultures, starting from single identical clones in liquid glucose-limited medium, grown under identical conditions of temperature and

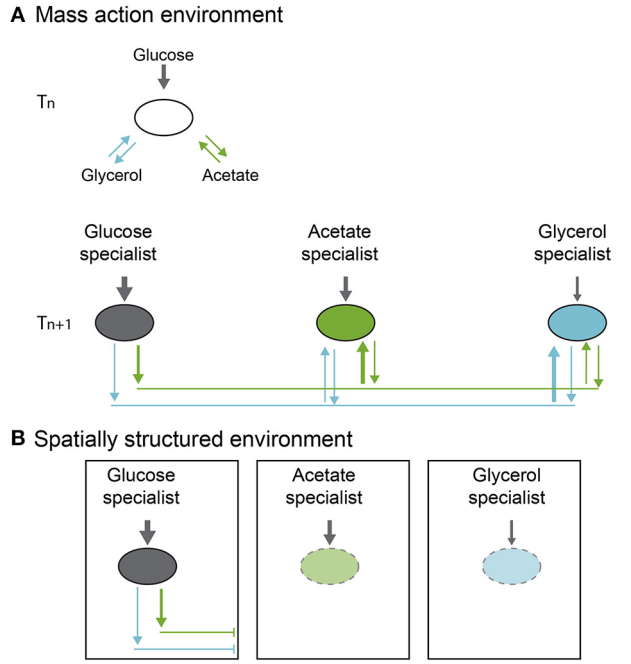


FIGURE 1 | Polymorphism evolution in a cross-feeding cascade system. (A) In a mass action environment, polymorphism is maintained through free flow of metabolites (arrows). **(B)** On a spatially structured environment, connectivity is limited and consequently flow of metabolites or resources is reduced affecting polymorphism stability (figure based on the results of Rosenzweig et al., 1994; Saxer et al., 2009).

shaking, and transferred each day to fresh media. In one of the replicate populations of this evolution experiment, a polymorphism evolved after 20,000 generations of selection. Two morphotypes, one with small colonies (*S*) and one with large ones (*L*) were identified. After isolation of the colony types, competition experiments were performed and showed that each type grew better when rare, and coexistence was possible after frequency fluctuations toward an equilibrium point. This polymorphism was maintained even though the *L* type had much higher maximum growth rate in the culture medium and was, therefore, expected to exclude *S* by competition. However, the *S* clone had two advantages that allowed it to invade and coexist with the *L* morphotype. The first advantage was an increasing death rate of *L* when *S* is more abundant, and second, both, *L* and *S* excrete to the medium metabolites that promote *S* growth (metabolite cascade). Coexistence is therefore maintained through frequency dependence and cascade type interactions (**Figure 1A**).

Following up on the ecological causes for the persistence of polymorphisms, Saxer et al. (2009) showed that when there is no nutrient limitation and spatial structure is added to these polymorphic *E. coli* populations, diversity is lost (**Figure 1B**). They first selected populations under culture conditions similar to those in Rozen and Lenski's work (2000), but at higher nutrient concentrations to promote production of metabolites and cross-feeding. After obtaining different specialists with differing colony morphotypes (*L* and *S*), the culture conditions were modified to a spatially structured environment, the same culture media with added solidifying agent (non-nutritive agar). Propagation of the cultures was performed by extracting a plug from the agar, dispersing the bacteria in saline solution and transferring them onto fresh medium. Environmental structure in the agar plates impeded dispersal of metabolic waste products, disadvantaging the non-glucose specialists and disrupting coexistence by breaking the stabilizing ecological interaction. Diversity plummeted 50% over 7 days (**Figure 1B**).

Cheating is not possible in cascade interactions, since there is no requirement for cooperation, the only requirement is resource availability to the specialists. Thus, as long as there is diffusion of resources, coexistence will be maintained. It should be noted, however, that different trade-offs in resource utilization must also exist among populations, since competition for the same resource represents a severe constraint for stability or long-term coexistence (see simulation in **Box 2**).

RECIPROCAL INTERACTIONS

Reciprocal interactions involve codependency. Individual organisms produce resources that facilitate the growth of others, and these other organisms provide 'reciprocal' resources to the first group of individuals. Frequently, both sets of resources are produced at a short time scale fitness cost to the organisms producing them (i.e., cooperative cross-feeding; Bull and Harcombe, 2009), but with a gain at intermediate time scale (by reciprocation). This type of interaction can readily breakdown, due to the evolution of "cheater" individuals that receive the benefits of the facilitation without contributing (Nowak, 2006). Typically cheaters do not produce resources, but still exploit the resources produced by partner organisms. Most studies that have investigated cooperative behavior invariably consider the evolution of cheater individuals in the populations, and the consequent destabilization and eventual crash of the cooperative system. Depending on the type of cooperative system and environmental spatial structure, this potential meltdown can be overcome or delayed by one or more of three cheater control strategies (Travisano and Velicer, 2004). There can be mechanisms enabling individuals to differentially reward cooperative instead of non-cooperative partners via targeted benefit or targeted punishment limits (Travisano and Velicer, 2004; Momeni et al., 2013), The physical structure of the environment can limit the spread of cheating genotypes, such as spatial structure. A third strategy are physiological and developmental mechanisms that essentially structure the environment temporally, again limiting the spread of cheater genotypes that would disrupt cooperation (Furusawa and Kaneko, 2002; Winther, 2005; Hammerschmidt et al., 2014).

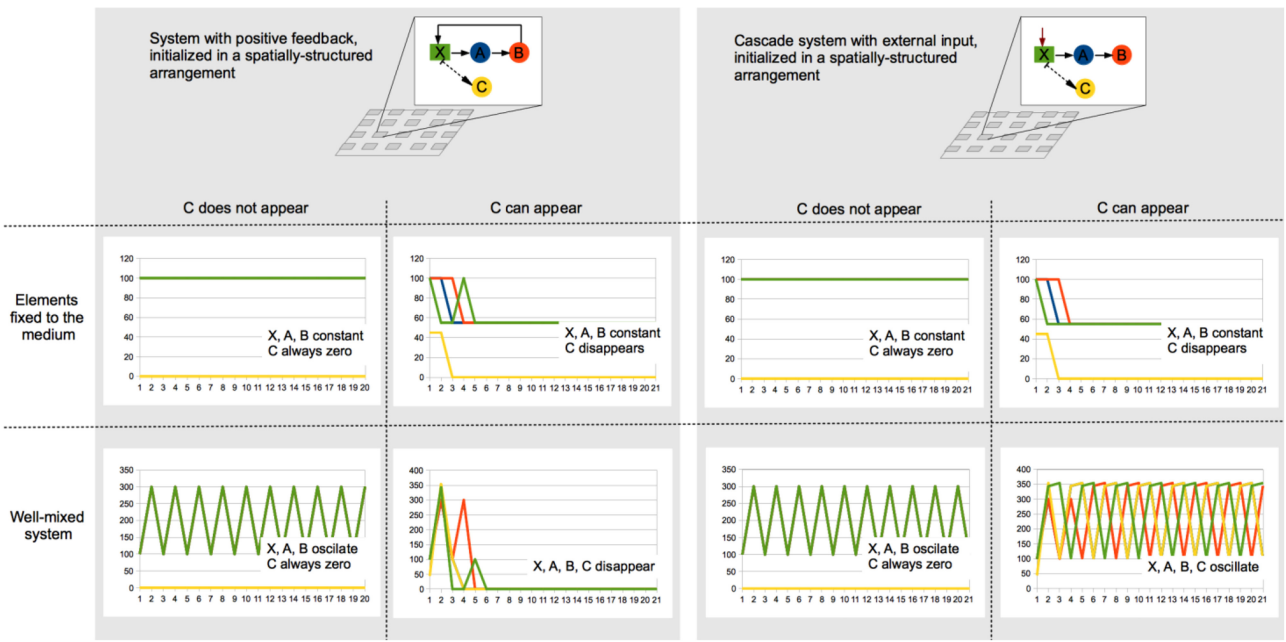
Cancer is an example of cheating in a multicellular system that provides some insight in understanding how cheaters can be overcome in microbial systems. If cheating (cancer) cells remain localized forming a benign tumor, cheating has only modest effects, as the effects are localized. However, if the cheating cells spread (metastasize), then the individual typically dies, as the deleterious effects of cheaters are global. Similarly, in communities, diversity can be maintained if cheaters cannot spread through so that beneficial reciprocal interactions persist. Community spatial structure provides a route for sustained reciprocity as the benefits of resource production are localized to the individuals bearing the

Box 2 | Dynamic comparison between two types of consortia in different conditions.

This review postulates that spatial structure and positive ecological feedbacks promote stability in microbial consortia, especially while looking for cooperative interactions and cheater control. To illustrate this, a couple of toy models corresponding to systems with and without positive feedback (cooperative and cascade-like, respectively) were mathematically specified, which allowed us to analyze their dynamics in different scenarios. In these models, X represents a substrate that is consumed by A, which in turn produces food for B. In the cooperative model (left-hand side), B is able to produce X, thus closing the positive feedback. In the cascade model (right-hand side), X is always available, which could correspond to an external input along the simulations. Note that in the case of the feedback system, C represents a cheater that feeds on X but does not contribute to the persistence of A and B. In the cascade model, C would correspond to a competitor that consumes the same substrate that A consumes.

Both models were initialized exactly with the same amount of A, B, and X. Also, in both cases the consortia were assumed to be on a 2D medium with modular spatial structure, as shown in the figure. These basic models were simulated in scenarios that combined the following conditions: (i) the components of the system remain at the place where they were initially set, (ii) the components of the system can move or are moved in a diffusive manner, therefore homogenizing the spatial arrangement, (iii) the elements denoted as C in the graphs are never present, and (iv) the elements denoted as C arise in each spatial module with a 0.5 probability.

Although these simulations do not explore the possible conditions and parameters exhaustively, they illustrate the dynamics that each of the consortia could exhibit under different conditions. The plots in the figure show how the absolute population sizes changes in time in each of tested scenarios. In summary, these toy-model simulations show that in cooperative systems cheaters can be controlled and the whole population can persist if the spatial structure remains modular, this is, if the members of the community remain where they started or if the environment is not being mixed. A similar situation is observed for the cascade system, but with competitors arising in the community instead of cheaters. However, in the cascade system, the well-mixed condition does not lead to the collapse of the system in the presence of competitors, but to an oscillatory state with even higher average population sizes. It is worth noting that while the cascade system does not collapse in any of the scenarios, its persistence depends on the external X input, while the cooperative system is in principle able to self-sustain once it is "set on."



cost of resource production. Spatially structured environments provide a mechanism that directs benefits to cooperating individuals (Griffin et al., 2004; Sachs et al., 2004) facilitating the direction of such benefits by localizing interactions (Harcombe, 2009). Indeed, it has been shown that for certain models of cooperation, the organization of communities in subsets of closely interacting individuals can lead to the stabilization of cooperation. Such an organization may certainly correspond to spatial structure, but also to temporal isolation of subsets of individuals or to the non-random architecture of ecological networks (Nowak, 2006).

Recently, Harcombe et al. (Bull and Harcombe, 2009; Harcombe, 2009, 2010) have shown that cooperation and the associated diversity can evolve and be maintained in laboratory conditions if there is preexisting reciprocal feedback for cooperation, and if reciprocal interactions are selectively superior to non-reciprocal (cascade) interactions for individual microbes. The experiments demonstrate that it is possible to create and maintain cooperation, if there is initially a low cost production of a resource that a second party can utilize and reciprocally benefit the producer to have more of the waste product. However, this stability is only sustainable in a spatially structured environment

where the competitive benefits to cheaters are severely limited. If the environment loses spatial structure, the benefits are available to all, even non-cooperators, making possible the rise of cheaters and the breakdown of cooperation. Harcombe's experiments are direct evidence that spatial structure of the environment is a determinant in directing the benefits to cooperating individuals and localizing interactions (**Figure 2**), which facilitates stability of a system where ecological reciprocation exists.

These examples of reciprocal interactions show the primary importance of cheating control when looking for stable and long-term coexistence of microbial populations. The examples also illustrate that engineering a spatially structured selective environment can assist in maintaining reciprocal interactions. Both resources (communication signals, nutrients, etc.) and interactions are locally contained and community meltdowns due to invasions by cheaters only occurs at a local scale (see toy-model simulation in **Box 2**).

ECOLOGICAL PERSPECTIVES OF COEXISTENCE OF ENGINEERED CONSORTIA

In the previous section we used key examples from experimental evolution of microbial populations to present evidence demonstrating the importance of ecological interactions and spatial structure on long-term system stability in terms of polymorphism maintenance or genetic variants coexistence. In this section we give an ecological and evolutionary perspective for some of the challenges in the field of synthetic biology. Such challenges frequently occur because microbial communities or engineered consortia are visualized as fixed circuits, rarely considering the importance of ecological interactions and adaptation.

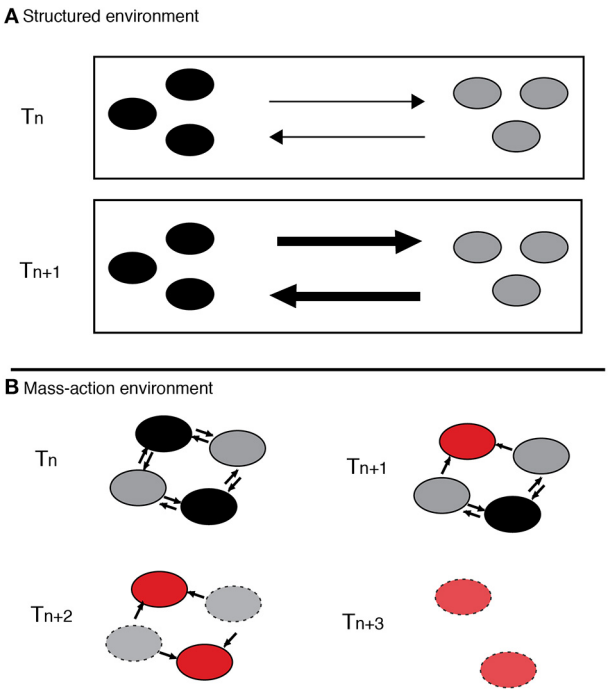


FIGURE 2 | Polymorphism evolution in a reciprocal interactions system. (A) On a spatially structured environment diversity is maintained through cooperative interaction. With time this interaction is selectively reinforced, and as a result each species' cooperativeness is increased. Arrows represent metabolites flux. **(B)** On a mass-action environment, however, there is more connectivity, all cells obtain the benefits, even if they do not pay the cost. Therefore, over time, cheaters arise and spread destabilizing the system.

We review recent publications on engineered microbial consortia to show that ecological and evolutionary complexity of these systems can easily reach levels where their evolutionary fate becomes hard to predict, thus control of the functionality of the system in the long run can be problematic. Kwok (2010) has attributed the general uncertainty on the behavior of engineered consortia to their complexity in terms of the number of components, potential incompatibilities among them and the impossibility of maintaining all parameters, components and conditions without variation. We argue that, at least in part, design of microbial communities is limited by the lack of an ecological and evolutionary perspective. Adding insights from ecology to the current approach will not overcome all the challenges, however it can help improving performance, stability and predictability of the systems. We focus our discussion of the reviewed examples in three aspects of their design: type of ecological interaction (reciprocal or non-reciprocal), physical structure (mass-action or spatially structured) and observed or predicted outcomes in terms of evolutionary stability of microbial consortia.

Work by Shou et al. (2007) is a good example of how engineered consortia can be better understood and designed, if simple ecological and evolutionary principles are taken into consideration. The system was designed taking into account ecological feedbacks (reciprocal interaction), consisting of two engineered yeast genotypes that were cultured with no explicit reference to any spatial structure in liquid media. Each genotype was auxotrophic for a specific amino acid that was overproduced by the other genotype. This strict dependence on the production of essential amino acids generated frequency-dependent selection that stabilized the community. In this system, a genotype increases in frequency when it is rare due to the abundance of its growth limiting essential amino acid. In contrast, the more common genotype decreases in frequency because of the scarcity of its growth limiting amino acid, which is produced by the rare genotype (**Figure 3A**).

The stabilizing ecological interactions could be lost, however, by the evolution of "cheaters" that do not contribute amino acids to the culture media and gain a growth advantage by using the resources for their own growth and reproduction. Waite and Shou (2012) subsequently showed that the engineered system could be maintained, despite the appearance of cheaters, "…if during adaptation to an environment, the fitness gain of cooperators exceeds that of cheaters by at least the fitness cost of cooperation." Moreover, a recent follow up on the Shou group system (Momeni et al., 2013) explicitly investigates the role of spatial structure in the successful coexistence of cooperative consortia. What the authors found experimentally, and with computer simulations, is that given certain viscosity of the media (structured environment) and the genetically engineered cooperative behavior, a spatial self-organization favors cooperation over cheating since, the difference in fitness between cooperators and cheaters

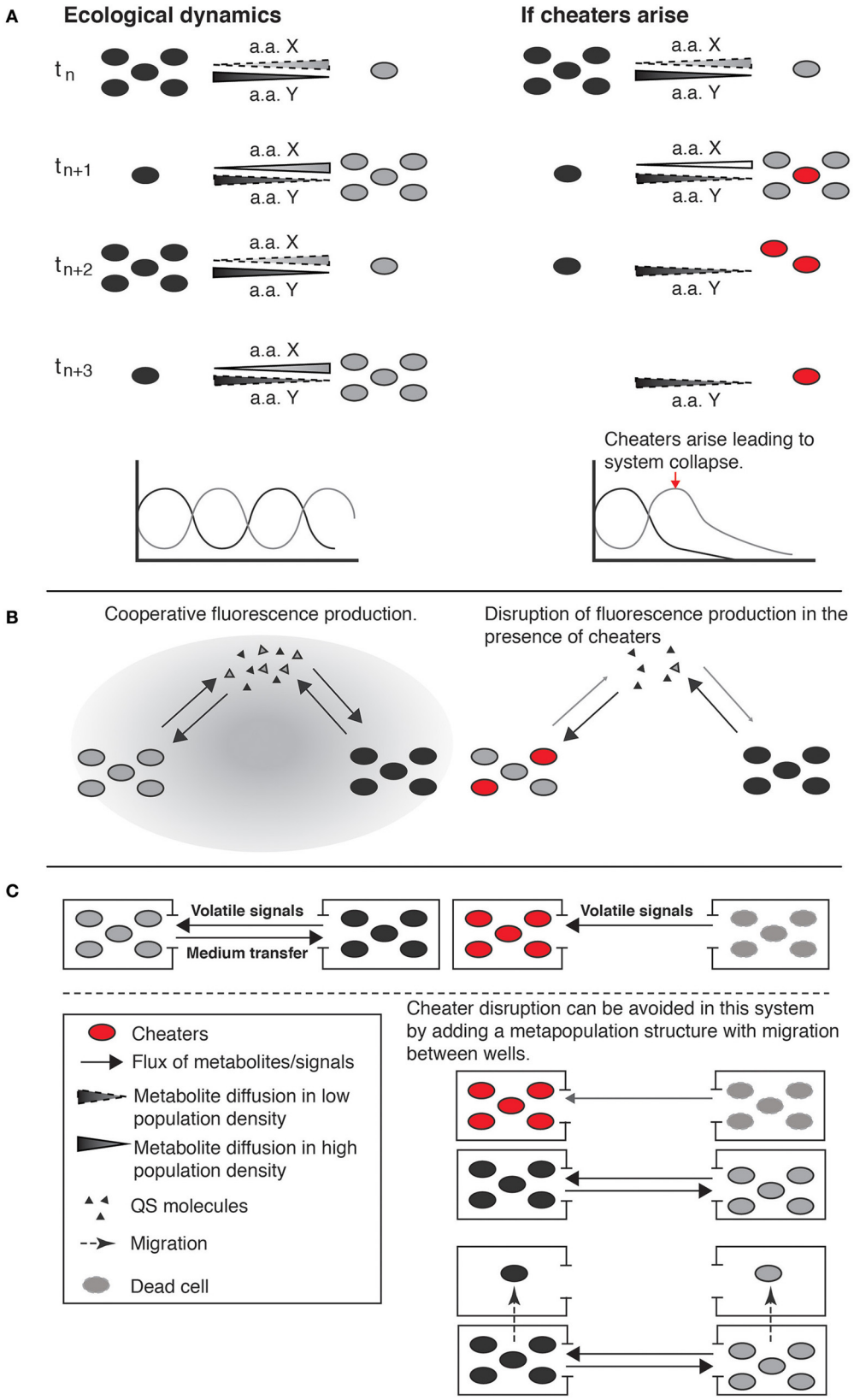


FIGURE 3 | Ecological dynamics and potential evolutionary outcomes of engineered cooperative consortia. In the left column are stable short-term dynamics; the right column, illustrates long-term outcomes if cheaters arose by mutation. **(A)** Two populations with strict interdependence by the production of essential aminoacids (one strain produces the aminoacid that

(Continued)

FIGURE 3 | Continued
the other cannot and *vice versa*). The release of the specific metabolic product is associated with near cell death of the producer and the decline of population density, producing population oscillatory dynamics. Over longer periods of time, cheaters can appear in the population, obtaining the benefits without paying the associated costs, and in the absence of spatial structure cheaters can spread leading to the collapse of the system (example from Shou et al., 2007). **(B)** A microbial consortium produces fluorescence as a cooperative trait regulated by a mechanism of consensus quorum sensing (QS). The signal molecules of this QS mechanism act as positive feedback between the two populations of cells for the production of both fluorescence and more signal molecules. The ecological role of each population is not described but cheaters can arise and disrupt the system, impeding fluorescence production (example from Brenner et al., 2007). **(C)** Reciprocal interaction circuit where two populations of cells are maintained in culture. One of the populations provides essential components to the other through volatile signals or direct transfer of medium, this system is thus open to the evolution of cheaters and collapse of the system. System collapse could potentially be prevented by including a redundancy component and local extinction and migration (metapopulation structure) via the replacement of populations in which cheaters appear with populations in which cheaters are absent (example from Weber et al., 2007).

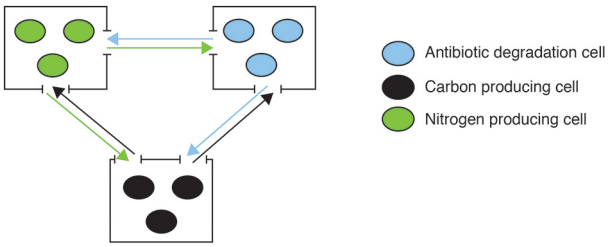


FIGURE 4 | An engineered consortia with three different soil bacterial populations each contributing with essential resources to the system. Each of the populations produces different resources (N, C, and antibiotic degradation enzymes) that are necessary and not produced by the others. All populations are kept in independent culture wells, imposing structure with constant connectivity through resources flow (example from Kim et al., 2008).

on the local partners during colony growth into available space drives assortment and automatically grants cooperators instead of cheaters more access to cooperative partners, thus disfavoring cheaters and ensuring partner fidelity (Momeni et al., 2013).

Brenner et al. (2007) similarly engineered a microbial consortium, but with more limited stability. The system involved positive feedbacks, in this case producing a fluorescence compound with a consensus quorum sensing (QS) control mechanism, and tested the cultures in liquid and solid phase (mass-action and spatially structured) (**Figure 3B**). While the QS mechanism acted as positive feedback for the production of QS molecules and thereby of fluorescence, there was no ecological feedback (cost/benefit of production) maintaining stability as with Shou et al. (2007). Thus this system could potentially be destabilized by loss of function mutants, involving reduced expression of QS molecules, reduced sensitivity to QS control, or inability to produce the fluorescing compounds. These examples illustrate how consideration of ecological and evolutionary dynamics could counteract problems in understanding and engineering consortia that involve long-term instability and unpredictability.

In contrast, Kim et al. (2008) present the case of an engineered consortium that initially involved spatial structure to maintain stability. It consisted of three different bacteria each contributing with essential resources to others, establishing reciprocal interactions (**Figure 4**). Each population was grown in individual culture wells, imposing structure with connectivity maintained by chemical communication flow. The system was stably maintained, in part because spatial structure provides the means for cheating control by allowing asymmetric fitness effects of cooperators and cheaters on coexisting populations during colony growth (Momeni et al., 2013), and maintaining disruptive effects of cheaters local while favoring intraspecific (cooperative versus cheating types within wells) over interspecific competition (between wells) (Amarasekare, 2003). When grown in co-culture (mass action) interspecific competition increases and, due to differences in growth rates, one strain becomes dominant, displacing the others. In this case the community was engineered not only as a circuit but also as an open system highly influenced by external interactions with both the environment and other organisms.

Weber et al. (2007) also involved spatial structure in their engineered biological circuit connecting different cell populations, but with less satisfactory results. They show various types of ecological interactions, but two are particularly relevant for our purposes: first, mutualistic or reciprocal, and second commensal or cascade. In the first case (reciprocal), two populations of cells are maintained in culture conditions (spatially segregated) where one population provides essential components to the other through volatile signals or direct transfer of medium from one population to the other (**Figure 3C**). If the essential resources produced by one population fail to reach the other, both populations die and the system collapses, in a manner similar to that in **Figure 3A**, even though the system different components (populations) are spatially segregated. Including our ecological and evolutionary perspective, system collapse could, potentially, be prevented by including component redundancy, replacing populations in which cheaters appear with populations in which cheaters are absent (Kerr et al., 2006). Because the evolution of cheaters is stochastic, depending on mutations, the likelihood of all populations containing cheaters depends upon the number of individuals within a population and the number of populations, both of which are under control of the experimenter.

In the second case (non-reciprocal) in Weber et al. (2007), commensal or cascade interactions also involve two populations spatially segregated, but one of them does not require resources from the other, completely changing the ecological dynamics of the system. In this new arrangement of the system, the main risk for the stability is access to resources, not cheating control, thus spatial structure is counterproductive. However, the original design of the circuit (airborne transport of signals) does not compromise access to resources despite culture of the populations in independent vessels, making the system spatially unstructured in this respect (**Figure 3C**). This example illustrates the ease with

which interactions (and therefore the ecological dynamics of the system) are modified while engineering a system, implying a pressing need to consider ecological aspects to assemble stable and productive microbial consortia.

The examples in this section highlight the importance of interactions and spatial structure on the establishment and maintenance of a stable community or consortium, in other words the incorporation of the ecological context whilst designing the setting to maintain the biological circuit. In summary, if reciprocal cooperation is involved in the design, cheating control strategies should also be included in the design, otherwise cheaters are likely to overtake the system and cause it to collapse. In cascade interactions, cheating will not be a problem, but then the connectivity of the system becomes crucial to allow diffusion of interaction's relevant molecules that may be critical for efficient product output.

PERSPECTIVE

The use of microbial consortia to carry out processes in industrial and domestic applications (e.g., pharmaceutical, food, materials, effective microorganisms in agroecosystems) is long established. Mixed microbial consortia can perform complex processes that would prove inefficient or impossible for single species systems. The merits of these systems are realized by the avoidance of trade-offs associated with different steps in a process. Despite their utility and common use, the vast majority of microbial consortia were developed on an *ad hoc* basis, and frequently contain a variety of poorly known genotypes and partially understood processes. Because of this, genetic engineering of microbes and consortia has drawn much attention with the promise of higher control over the microbial systems. However, ecological and evolutionary instability has arisen as a pervasive problem.

Successful cases of synthetic microbial communities have shown the feasibility of engineering genetic circuitries to construct efficient cellular machines through the manipulation of genetic parts. Nonetheless, there have been major difficulties in developing microbial consortia. These systems are typically very complex (Kwok, 2010) and while there has been substantial engineering effort in their development, there has been insufficient inclusion of the necessary biological realism for system analysis and design (Kuhn et al., 2010). With the current engineering approach, the whole organism and ecosystem perspective is frequently missed, efficiency problems are commonly encountered (e.g., difficult to control production due to changes in community composition resulting in low yields and economic losses; (Shong et al., 2012), and the potential for system collapses is always present. Very recently, there have been impressive efforts to incorporate more biology into the circuit design through co-occurrence analyses (Berry and Widder, 2014), and stoichiometry and metabolic network modeling of specific microbial strains (Freilich et al., 2009, 2011; Harcombe et al., 2014). These efforts provide substantial information on the type of interactions that can be established within microbial consortia. We argue that better predictability of consortia behavior will only come from evaluations that take into account the evolutionary dynamics of ecological systems in which cheating, connectivity and costs can be controlled through appropriate selection regimes.

Knowledge achieved through microbial population biology experiments is key for considerable improvements and long-term stability of genetically modified communities. Given this, it is not surprising that some of the great successes in the appropriate use of ecological and evolutionary concepts meet the desired goals of productivity, but also stability, resilience and adaptability. Such successes are frequently accompanied by the emergence and maintenance of cooperative behavior. We foresee fulfillment of the promise of microbial consortia coming from metabolic modeling and engineering approaches, by predicting "successful" interactions between two or more microbial strains through their metabolic capacities (Freilich et al., 2011). To achieve this, we believe that a further step is needed in the design and "engineering" of microbial consortia: explicit application of ecological and evolutionary design principles, involving the specifics of the interactions between microbes (direction, feedbacks, non-reciprocity) and the evolutionary consequences that physical structure of the environment. This ecological and evolutionary view, going beyond gene activity, will be crucial in the assessment of new applications and practices involving microbial consortia.

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Enrichment experiment changes microbial interactions in an ultra-oligotrophic environment

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The increase of nutrients in water bodies, in particular nitrogen (N) and phosphorus (P) due to the recent expansion of agricultural and other human activities is accelerating environmental degradation of these water bodies, elevating the risk of eutrophication and reducing biodiversity. To evaluate the ecological effects of the influx of nutrients in an oligotrophic and stoichiometrically imbalanced environment, we performed a replicated *in situ* mesocosm experiment. We analyzed the effects of a N- and P-enrichment on the bacterial interspecific interactions in an experiment conducted in the Cuatro Cienegas Basin (CCB) in Mexico. This is a desert ecosystem comprised of several aquatic systems with a large number of microbial endemic species. The abundance of key nutrients in this basin exhibits strong stoichiometric imbalance (high N:P ratios), suggesting that species diversity is maintained mostly by competition for resources. We focused on the biofilm formation and antibiotic resistance of 960 strains of cultivated bacteria in two habitats, water and sediment, before and after 3 weeks of fertilization. The water habitat was dominated by *Pseudomonas*, while *Halomonas* dominated the sediment. Strong antibiotic resistance was found among the isolates at time zero in the nutrient-poor bacterial communities, but resistance declined in the bacteria isolated in the nutrient-rich environments, suggesting that in the nutrient-poor original environment, negative inter-specific interactions were important, while in the nutrient-rich environments, competitive interactions are not so important. In water, a significant increase in the percentage of biofilm-forming strains was observed for all treatments involving nutrient addition.

Keywords: mesocosm, nutrient enrichment, Cuatro Cienegas Basin, proteobacteria interactions, community structure

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Introduction

A central goal of ecology is the understanding of the driving principles underpinning biodiversity (Gaston, 2000). Several lines of research have tried to explain the differences in diversity among local communities, focusing on approaches ranging from network interactions in food webs to random assemblages resulting from the dispersion capacity of organisms (Hutchinson, 1959; Dykhuizen, 1998; Kassen et al., 2000; Bennie et al., 2006; Marini et al., 2007). These

theories, as well as other more complex explanations of biodiversity (such as chaotic interactions between competing strains; Huisman and Weissing, 1999) are partially successful, depending on the system studied and are frequently complementary. The patterns of biodiversity are determined by the combined impacts of interactions of several biotic and abiotic environmental factors, and cost-benefit strategies followed by each species can change according to the complexity of the community and its nutrient availability (Marini et al., 2007; Werner et al., 2014).

For instance, it is known that the abundances and proportions of nitrogen and phosphorus in the environment have a major influence in the composition of species at macro and microscopic scales (Makino and Cotner, 2005; Jansson et al., 2006; Marini et al., 2007; Christofoli et al., 2010; Nelson and Carlson, 2011). Classic trade-off competition theory places resource availability at the center of the processes that influence community structure (Tilman et al., 1981; Smith, 1993; Brauer et al., 2012). MacArthur and Wilson (1967) coined the terms "r-selection" and "K-selection." This theory takes into account biotic and abiotic factors such as climate, mortality, survivorship, population size, intra and interspecific competition, relative abundance and length of life. Under this theory, r-strategists are adapted to abundant nutrients, which are rapidly exploited. On the other hand, K-strategists are adapted to a long-term survival on limited resources (Pianka, 1970; Fuchs et al., 2000; Singer et al., 2011). Experimental evidence from different microcosm experiments shows that nitrogen and phosphorus enrichments result in significant changes in community structure in terms of uniformity and species richness (Schäfer et al., 2001; Nelson and Carlson, 2011).

The changes in community structure are probably driven mainly by the effects of nutrients on the growth rates of individual strains (Smith, 1993), but other factors could also play a role such as seasonal changes (Rodríguez-Verdugo et al., 2012; Bevivino et al., 2014), as well as changes in mutualistic interactions due to fluctuations in the supply and demand of "public goods" by cause of the entrance of nutrients to the ecosystem (Morris et al., 2012; Sachs and Hollowell, 2012; Werner et al., 2014).

The release of chemical compounds into the environment, which are toxic or inhibitory to the competitors, is one of the commonly observed antagonistic interactions (Riley and Gordon, 1999; Lenski and Riley, 2002; Riley and Wertz, 2002; Kirkup and Riley, 2004; Hibbing et al., 2010; Kohanski et al., 2010; Majeed et al., 2011, 2013; Pérez-Gutiérrez et al., 2013; Aguirre-von-Wobeser et al., 2014). These secretions highly influence community structure and maintain cohesion of bacterial populations by leading to the extinction of sensitive strains in liquid media, but to coexistence in a structured media (Validov et al., 2005; Greig and Travisano, 2008; Rypien et al., 2010; Cordero et al., 2012). In the non-transitive model rock-paper-scissors (RPS), one antagonist, one sensitive and one resistant strain coexist in structured media (Czarán et al., 2002; Kerr et al., 2002; Kirkup and Riley, 2004). In a natural environment, this non-transitive relation may occur if toxic production is costly, both sensitive and resistant strains exist and costs associated with resistance are less than those of toxin production. However, the relative magnitude of each of these features is critical for coexistence (Kerr et al., 2002).

The effects of nutrient addition in microbial community structure can also depend on the extant biodiversity prior to the increase of nutrient availability, as shown by an experiment performed on the bacterial community of Owasso Lake (Minnesota, USA), which has been reported as one of the least diverse bacterial communities known (Makino and Cotner, 2005). When this lake was enriched with nitrogen, phosphorus and carbon, the community showed a response that would be expected from a single strain, rather than from a community (Jürgens and Güde, 1990), suggesting that if bacterial diversity in a given environment is low, this diversity will remain low, because of its low potential to respond at a community level to changes in its environment. In contrast, in more diverse environments, nutrient enrichment usually homogenizes the composition of the community assemblage (Donohue et al., 2009).

The Cuatro Cienegas Basin (CCB) located at the Chihuahuan desert of north central Mexico is a good system to study the effects of the addition of nutrients on bacterial communities. CCB harbors a number of highly oligotrophic aquatic ecosystems that have very low available phosphorus levels, as well as a stoichiometric disequilibrium with nitrogen and thus are strongly limited by phosphorus (Elser et al., 2005, 2006). We have suggested that the high microbial species diversity in CCB is strongly shaped by the stress of low nutrient supplies and the impacts of interspecific competition (Souza et al., 2008). Previous studies indicate that CCB bacterial communities display strategies to cope with this lack of nutrients, including a high representation of genes involved in phosphorus assimilation (e.g., *pho* and *pst* in Mesquites river; Breitbart et al., 2009) and the presence of a large number of genes related to the production and resistance to antibiotics (in Pozas Rojas; Bonilla-Rosso et al., 2012; Peimbert et al., 2012).

Pseudomonas and other cultivable proteobacteria are abundant in different aquatic systems in CCB, including the Churince system (Escalante et al., 2009) and in the Los Hundidos region (Bonilla-Rosso et al., 2012; Peimbert et al., 2012). In a different study in the flow system Churince, it was found that the *Pseudomonas* genus exhibited a seasonal variation across summer and winter (Rodríguez-Verdugo et al., 2012).

In this paper, we analyze the seasonal response of bacteria cultivable in *Pseudomonas* Isolation Agar (PIA; Difco[1], Detroit, MI) in response to the nutrient amendment. Our study seeks to understand the effects of nitrogen and phosphorus fertilization on the interaction potential of the cultivable gammaproteobacteria community in two different habitats, water and sediment, by determining how nutrient enrichment affected various features related to microbial species interactions, such as the tendency to form biofilms and resistance to antibiotic compounds. We are clearly aware of the limitations of culture media in order to capture the diversity of a site, as we have described elsewhere (see Souza et al., 2006, 2012), nevertheless, only in culture we can study the physiology and sociology of particular strains, which is the particular aim of this study. Our findings shed light on how the supplies of key nutrients, such as N

[1]DifcoTM and BBLTM Manual, 2nd Edition. Pseudomonas Isolation Agar.

and P modulate community structure, as well as the nature and intensity of interspecific interactions.

Materials and Methods

Study Site

The nutrient enrichment experiment was conducted *in situ* in a small shallow evaporitic pond, Lagunita (26.84810° N, 102.14160° W), lateral to the main Churince flow system. The Churince flow system, located at the western region of CCB is dominated by gypsum sediments and has a strong longitudinal gradient of salinity, temperature, pH and dissolved oxygen (Cerritos et al., 2011). Lagunita is characterized by low phosphorus concentrations (PO_4 as low as 0.1 μM and often below detection) (Elser et al., 2005), but relatively high concentrations of inorganic N and thus high N:P ratios (>200:1 for total nutrients; Lee et al., in press). Lagunita is subjected to strong evaporation, the greatest water depth at the beginning of the experiment was 32 cm and decreased to as low as 12 cm by the end of the experiment.

Experimental Design of Mesocosm

The mesocosm experiment was conducted from May to June 2011. Each mesocosm consisted of a round clear plastic tube with a diameter of 40 cm. The tube had a depth of 20 cm into the sediment and approximately 20 cm above the water surface. The mesocosms were arranged in a randomized complete block with a total of 5 blocks, separated about 2-4 m from each other. Each block consisted of four treatments. A non enriched control treatment, a phosphorus enrichment treatment (P), amended with KH_2PO_4 and maintained at 2 day intervals at a final concentration of 1 μM; a nitrogen and phosphorus enrichment (NP), amended as above with KH_2PO_4 to 1 μM but also with NH_4NO_3, to achieve N:P of 16:1; and a nitrogen and phosphorus enrichment with extra nitrogen (NNP), amended as above with 1 μM KH_2PO_4 but also with NH_4NO_3 at an N:P ratio = 75:1.

Methodology for Chemical Analyses

For the chemical analyses, water was collected in acid-washed 2 L cubitainers. Water samples were filtered through pre-combusted (24 h at 450°C) GF/F filters (Whatman, Piscataway, NJ) for seston elementary analysis and stored at −20°C. To measure total dissolved nutrients, water samples were filtered through 0.2 μm polyethersulfone membrane filters. Samples for dissolved organic carbon (DOC), and total dissolved nitrogen (TDN) were acidified with 12 N HCL to pH < 2 and stored in the dark at room temperature, while the remaining filtrate was frozen for total dissolved phosphorus (TDP) and soluble reactive phosphorus (SRP) analyses.

GF/F with seston were thawed, dried at 60°C and packed into tin disks (Elemental Microanalysis, U.K.) for N analyses with a Perkin Elmer™ PE 2400 CHN Analyzer at the Arizona State University Goldwater Environmental Laboratory (ASU GEL). Another set of dried GF/F filters from the same water samples was used in order to estimate seston P content. These filters were digested in persulfate followed by a colorimetric analysis to determine PO_4^{3-} (APHA, 2005). TDP concentrations were determined using the colorimetric assay after persulfate digestion as previously described; SRP was measured without the persulfate digestion. DOC and TDN were analyzed using the Shimadzu TOC-VC/TN analyzer at the ASU GEL. Total Phosphorus (TP) concentration was calculated as the sum of the seston and total dissolved pools.

Sample Collection and Processing

Water and sediment samples were taken for each one of the four treatments for each experimental block (20 samples total). Initial samples were obtained from surface water and top of sediment prior to the application of the treatments (T0; 14 May 2011) and after 21 days of enrichment (4 June 2011) using sterile BD Falcon vials (BD Biosciences, San Jose, CA). Cultivable bacteria were obtained by plating 100 μl of each water sample or 100 μl of a 1:10 sediment dilution, prepared with 0.9% NaCl solution. Strains were isolated from water and sediment using PIA medium. Strains were incubated on agar plates at room temperature for 2 days at the field, and then were kept at 4°C until isolation in the laboratory. Individual colonies were transferred to new PIA plates and then were grown at 30°C. A total of 960 isolates were obtained.

DNA Extraction and PCR Amplification

Phylogenetic identification of the strains was performed using the 16S rRNA gene. For the isolates, DNA extraction was very complicated, and thus several methods were tested (Chen and Kuo, 1993; Aljanabi and Martinez, 1997; Reischl et al., 2000, and DNeasy Blood and Tissue kits, Qiagen, Hilden, Germany). Ultimately, the DNeasy Blood and Tissue kit was the method used, and from the 960 isolates, good quality DNA was obtained for 152 strains. 16S rRNA genes were amplified using universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′) (Lane, 1991) and high fidelity *Phusion* hot start DNA polymerase (Finnzymes, Espoo, Finland). All reactions were carried out in a Techne TC-3000 thermal cycler (Barloworld Scientific, Staffordshire, UK) with the following program: 94°C for 5 min, followed by 30 cycles consisting of 94°C for 1 min, 50°C for 30 s, 72°C for 1 min and 72°C for 5 min. Polymerase chain reaction (PCR) amplification products were electrophoresed on 1% agarose gels. Sanger sequencing was performed at the University of Washington High Throughput Genomics Center. The sequences have been uploaded to GenBank with accession numbers (KF317734-KF317770, KM352505-KM352636).

Phylogenetic Analysis

The 16S rRNA sequences were aligned with CLUSTALW (Larkin et al., 2007) and MUSCLE (Edgar, 2004), and the alignments were manually revised. For the reconstruction of the phylogenetic tree, a maximum likelihood analysis was done with PhyML version 3.0 (Guindon et al., 2010) with the TrN+G model. The substitution model was calculated with jModelTest 2.1.3 (Darriba et al., 2012). The degree of statistical support for the branches was determined with 1000 bootstrap replicates. Genera level identification of the strains was made using the classifier tool (Wang et al., 2007) from the Ribosomal Database Project (RDP) Release 10, update 30 (Cole et al., 2009; **Table 1**). We performed a local

TABLE 1 | Summary of nutrient-induced shifts in relative abundance of dominant bacterial lineages.

Class	Consensus clade	Total Count	T0		Control		NP		NNP		P	
			H_20	Sed	H_20	Sed	H_20	Sed	H_20	Sed	H_20	Sed
α-Proteobacteria	*Rhizobium*	**8**	0	**6**	0	0	0	0	0	0	0	**2**
γ-Proteobacteria	*Stenotrophomonas*	**9**	0	**9**	0	0	0	0	0	0	0	0
γ-Proteobacteria	*Pseudomonas*	**54**	**20**	0	**9**	0	0	0	**4**	0	**21**	0
γ-Proteobacteria	*Shewanella*	**1**	**1**	0	0	0	0	0	0	0	0	0
γ-Proteobacteria	*Aeromonas*	**20**	**5**	0	0	**3**	**10**	0	**2**	0	0	0
γ-Proteobacteria	*Citrobacter*	**1**	**1**	0	0	0	0	0	0	0	0	0
γ-Proteobacteria	*Rheinheimera*	**5**	**5**	0	0	0	0	0	0	0	0	0
γ-Proteobacteria	*Halomonas*	**47**	**7**	0	0	0	0	**18**	0	**22**	0	0
Actinobacteria	*Kocuria*	**4**	0	0	0	0	0	**1**	0	0	0	**3**
Actinobacteria	*Brachybacterium*	**3**	0	**3**	0	0	0	0	0	0	0	0

BLAST search (Altschul et al., 1990) to find the nearest neighbors using the 16S ribosomal RNA. These analyses were performed with 700 bp from the 5′ end of all the sequences.

Antibiotic Resistance Assays

The 960 isolates were grown on LB plates supplemented with the following antibiotic concentrations: Carbenicillin 500 μg/ml, Kanamycin, 200 μg/ml, Tetracycline, 150 μg/ml, Streptomycin, 200 μg/ml and Gentamicin 150 μg/ml. Isolates were labeled as resistant to an antibiotic if we observed bacterial growth; otherwise, they were considered as nonresistant.

For statistical inference, the antibiotics data were organized as contingency tables, and Barnard's exact test (Barnard, 1945) was performed. Independent tests were conducted for each treatment against the initial time, for water and sediment data. Since several treatments were compared to the initial condition, a Bonferroni correction was applied for multiple tests.

Biofilm Formation Assay

A microtiter dish assay was performed for the same 960 isolates as described by O'Toole (2011) with the following modifications. The overnight culture was grown in LB medium. The microtiter plates were incubated for 18 h at 30°C. Each strain was analyzed by triplicate and we used the strains *P. aeruginosa* PAO1 and *E. coli* MC4100 as positive and negative controls, respectively. After incubation, plates were gently washed with water, and subsequently 125 μl of a 0.1% solution of crystal violet in water was added to each well. The plates were incubated at room temperature for 15 min and then washed with water. 125 μl of 30% acetic acid in water was added to each well to solubilize the crystal violet and it was incubated for 15 min. The volume was transferred to a new flat-bottomed microtiter dish. Absorbance was read with a Synergy HT plate reader (BioTek, Winooski, VT) at 550 nm.

To determine the statistical significance of differences in biofilm formation between the different treatments and the initial condition, we organized the data as contingency tables including the number of positive and negative strains for biofilm formation for the initial time and each treatment, and applied Barnard's exact tests. To correct multiple testing, a Bonferroni correction was applied.

Results

To characterize the changes in diversity of cultivated Proteobacteria related to pseudomonads associated with nutrient enrichment in a water system, we performed an *in situ* mesocosm experiment with three experimental manipulations, adding phosphate (P), phosphate and nitrogen (NP), and phosphate and excess nitrogen (NNP), as well as an un-enriched control. The experiment is part of a bigger project that is described in detail elsewhere (Lee et al., in press). Cultures were obtained from surface water and sediment in two sampling events, prior to the experimental manipulations, and after 21 days, and a total of 960 isolates were analyzed for interaction phenotypes such as biofilm formation, and antibiotic resistance. A subsample of the isolates was characterized by 16S rRNA sequence.

Nutrient Concentrations

Nutrient conditions during the experiment were characterized by low concentrations of P but relatively high concentrations of N that increased in NP and NNP treatments. Despite nutrient enrichment, N:P ratios remained quite stoichiometrically imbalanced. Except for the NNP treatment, total dissolved phosphorus and soluble reactive phosphorus were significantly different respect to control. Total phosphorus, total dissolved phosphorus and the N:P ratio were significantly different in all enriched treatments. Total dissolved nitrogen was significantly different only in the nitrogen amended treatments (**Table 2**). Details of dynamics and fate of N and P in the mesocosms are given elsewhere (Lee et al., in press).

Phylogenetic Diversity and Responses to Treatments

The phylogenetic relationships of partial 16S rRNA gene sequences (700 bases) of the 152 strains were determined by queries against the Ribosomal Database Project (Cole et al., 2009) using SeqMatch and Classifier (Wang et al., 2007). The phylogenetic results showed that the strain collection was dominated by Proteobacteria (145 isolates/95.4%), with members from the gamma- (137/90.1%) and alpha- (8/5.3%) subdivisions (**Table 1**), consistent with the use of the PIA medium for isolation. We

TABLE 2 | Nutrient concentrations.

Treatment	DOC	TDN	Seston P	TP	TDP	SRP	N:P
T0	2177 ± 51.84	130 ± 5.98	1.21 ± 0.08	1.79 ± 0.20	0.58 ± 0.17	0.06 ± 0.02	47.26 ± 1.95
Control	2799.17 ± 212.81	182.77 ± 9.51	1.09 ± 0.11	2.47 ± 0.24	1.39 ± 0.25	0.31 ± 0.09	49.12 ± 6.04
NP	3005 ± 227.63	202.48 ± 12.11*	3.03 ± 0.26*	4.17 ± 0.62*	1.15 ± 0.40	0.27 ± 0.12	36.39 ± 6.34*
NNP	3198.81 ± 144.87*	226.32 ± 25.63*	3.29 ± 0.68*	4.15 ± 0.71*	0.86 ± 0.25*	0.19 ± 0.08*	36.25 ± 6.20*
P	3010.65 ± 227.30	192.05 ± 17.88	2.71 ± 0.33*	3.95 ± 0.59*	1.24 ± 0.28	0.33 ± 0.10	28.26 ± 3.75*

Dissolved organic carbon (DOC), total dissolved nitrogen (TDN), total phosphorus (TP), total dissolved phosphorus (TDP) and soluble reactive phosphorus (SRP) concentrations in the pond's water and in each treatment after the nutrient enrichment. All values are in $\mu M\ L^{-1}$ with the exception of N:P which represents the elemental stoichiometry of seston. Each value represents the average and one standard deviation. The "" symbol denotes significant change from the control.*

also found several Actinobacteria (7/4.6%). As described by King et al. (1954), this medium includes Irgasan®, a broad spectrum antimicrobial substance that is not active against *Pseudomonas* spp. The medium also enhances the formation of pigments by *Pseudomonas* by adding magnesium chloride and potassium sulfate. *Pseudomonas* species were found in all un-enriched water cultures as well as in almost all enriched samples, with the exception of the NP treatment. *Pseudomonas* was the most abundant genus for the water isolates and overall for all samples (35.5% of the isolates). *Halomonas* was the most abundant genus among the sediment strains, representing 30.9% of the isolates.

Using the aligned sequences, a maximum likelihood tree was constructed (**Figure 1**). For any given treatment, groups of closely related, almost identical strains dominated the samples, even though they were obtained from different replicate mesocosms. Previous to the experimental manipulations, only gamma-proteobacteria were retrieved from the water samples, which were dominated by *Pseudomonas*. Other genera were present, including *Aeromonas*, *Shewanella*, *Citrobacter*, *Rheinheimera*, and *Halomonas*. With the addition of phosphorus (P treatment), many closely related *Pseudomonas* were retrieved. In the NP treatment, all the obtained isolates belonged to the genus *Aeromonas*. For the high N:P treatment (NNP), a group of *Pseudomonas* became abundant and two *Aeromonas* isolates were obtained.

Phylogenetic differences were observed between the abundant strains obtained from the different habitats (water vs. sediment) but also between the strains corresponding to the different treatments. At the beginning of the experiment, the sediment samples were dominated by another gamma-proteobacterium, *Stenotrophomonas*, as well as an alpha-proteobacterium, *Rhizobium*. Some Actinobacteria were also isolated from these initial samples, belonging to the genus *Brachybacterium*. With P enrichment, *Rhizobium* was also found, as well as an Actinobacterium, from the genus, *Kocuria*. However, a larger change in the sampled community was found in all treatments including nitrogen along with P (NP and NNP), where *Halomonas* (gamma-proteobacteria) was largely dominant.

Antibiotic Resistance

We analyzed the prevalence of antibiotic resistances among the 960 isolates. The antibiotics analyzed were Carbenicillin, Kanamycin, Tetracycline, Streptomycin and Gentamicin (Supplementary Table 1; Supplementary Figures 1, 2). Carbenicillin was the most common form of resistance among the isolates. The Carbenicillin resistance was found in 55.2% of the water isolates at time zero and in 68.3% of the total water isolates after the experiment, and in 28.6 and 90.5% of the total sediment isolates, at time zero and after the experiment, respectively. Tetracycline was the least common form of resistance, being represented in 10.4 and 0.8% of the water isolates and 7.1 and 1.0% of the sediment isolates before and after the experiment, respectively. Kanamycin resistance was not observed in sediment samples after the experiment. Overall, there was a statistically significant decrease in the number of resistant strains after the experiment (Bernard's exact test; $p < 0.05$; **Figure 2**; **Table 3**), in all three fertilization treatments and in the control, with the marked exception of Carbenicillin, for which resistance increased significantly in NNP and P treatments in water and in all treatments in sediment.

Biofilm Formation

To further characterize the ecological traits of the studied strains, biofilm formation was analyzed for 923 isolates. In the water samples, the enriched isolates exhibited an increase in the proportion of the ability to form biofilm (**Figure 3**; Supplementary Figure 1). In a structured environment, interactions can be enhanced by biofilm formation. As expected, sediment strains generally had a greater tendency to produce biofilm that those isolated from water. However, in sediment, differences between before and after the treatments were not significant (Barnard's exact test; $p > 0.05$; **Table 4**), except for the NNP treatment, for which no biofilm-producing strains were detected (**Figure 3**; Supplementary Figure 2). In the case of water, a significant increase in the percentage of biofilm-producing strains was observed for all treatments involving nutrient additions, namely P, NP, and NNP (Barnard's exact test; $p < 0.05$; **Table 4**).

Discussion

In this study, the effect of experimental nutrient enrichment in a shallow, nutrient-deficient pond was analyzed to assess changes in the composition of the cultivable microbial community as a function of the N:P ratio of enrichment, as well as the modifications in the interaction network among cultivable isolates. The selective medium PIA was found to be highly selective to gamma-proteobacteria, a class previously reported as abundant

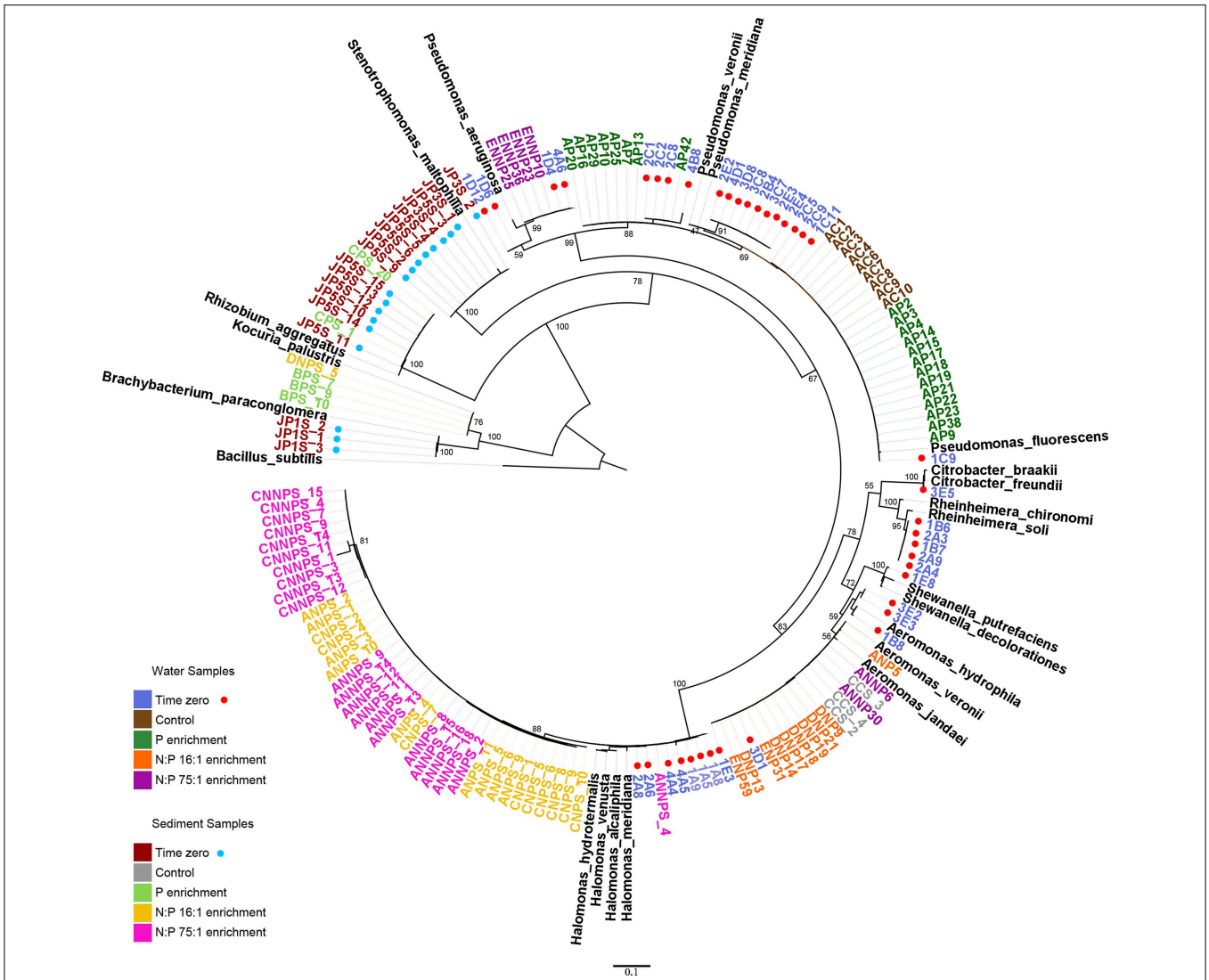

FIGURE 1 | Phylogenetic relationships among 16S ribosomal RNA genes. Sequences from the 152 isolates (highlighted in colors) and 21 reference strains. The coded name of the strains corresponds to the treatments and the environment from which they were isolated. Water samples: #[A-E]#, mesocosm T0, XC#, control treatment, XP#, phosphorus enrichment, XNP#, N:P 16:1 treatment, XNNP#, and N:P 75:1 treatment. Sediment samples: JPXs_#, mesocosm T0, XCs_#, control treatment, XPs_#, phosphorus treatment, XNPs_#, N:P 16:1 treatment, XNNPs_#, and N:P 75:1 treatment, where # is a number and X a letter from A to E, each one represents a replicate.

at Churince as well as other sites at CCB (Souza et al., 2006; Escalante et al., 2009; Bonilla-Rosso et al., 2012) and it has not been analyzed in detail. Unfortunately, a great amount of the isolates could not be identified, as it seems that Cuatro Ciénegas bacteria produce compounds that inhibit the PCR reaction, compounds that remain despite the use of several DNA extraction methods. To analyze the community response to the nutrient enrichment without the cultivation bias, 16S libraries from water and sediment samples, from May to June 2011, were also sequenced. The analysis of the community showed, in agreement with this study, a shift in the community composition with the disappearance of several bacterial genera after the increased nutrient availability (Elser et al., 2014, in preparation).

Our hypothesis that Lagunita is P-limited was supported, as it was found that all added P that remained in the water column was immobilized into seston (**Table 2**). This is reflected by the fact that TDP and SRP were not significantly different respect to the control, but TP and phosphorus in seston were. This difference was also observed in the N:P ratio. With respect to total dissolved nitrogen, it was found that both nitrogen-amended treatments were significantly different but the phosphorus-amended treatment was not. This supports that our observations were not due to an isolation bias.

Based on the 16S rRNA sequences from the cultivated strains, we found that two different groups of gamma-proteobacteria responded to increases of nutrient availability in water and

sediment environments, *Pseudomonas* and *Halomonas*, respectively. The strains characterized differed among the different treatments in the physiological characteristics analyzed, biofilm formation and antibiotic resistance. In contrast, genera such as *Shewanella*, *Citrobacter*, and *Rheinheimera* in water, as well as *Stenotrophomonas* and *Brachybacterium* in sediment were not isolated from the fertilized mesocosms, although they were present at time zero. Moreover, some rare genera, such as *Kocuria*, which was not documented at time zero, was found after the enrichment. However, we note that this rare genus of Actinobacteria has been obtained in past work at CCB (Cerritos et al., 2011), so it is not foreign to this environment. Several genera isolated in this study, had not been previously reported to grow in PIA medium (McCaig et al., 2001; Rajkowski and Rice, 2001; Falcone-Dias et al., 2012; Weiser et al., 2014).

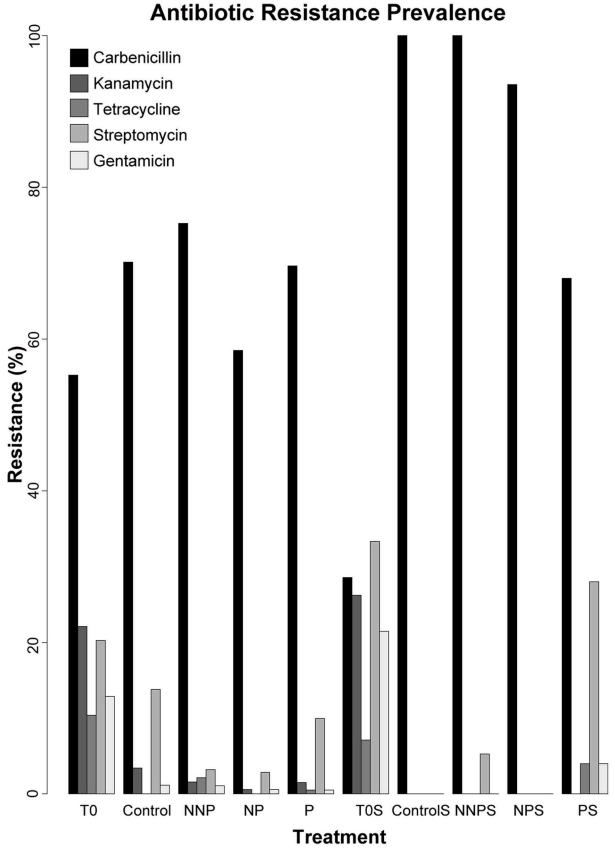

FIGURE 2 | Prevalence of antibiotic resistance among 960 isolates. The isolates analyzed for each treatment were as follows. T0—163, Control—87, NNP—186, NP—176, P—201, T0S—42, CS—11, NNPS—38, NPS—31, and PS—25.

All these changes in bacterial groups, which were isolated during different times in the experiment, suggest that an important community change at the bacterial level took place after the enrichment, however it should be taken carefully given the sampling method. It can be speculated that the original community in Lagunita was dominated by *K*-strategists, which rely on long-term survival on limited resources (Pianka, 1970; Fuchs et al., 2000; Singer et al., 2011). After the enrichment, the microbial community was dominated by faster-growing gammaproteobacteria, which can be considered *r*-strategists, rapidly exploiting nutrient patches and then dying or becoming dormant after substrate exhaustion. Indeed, *Pseudomonas* has been previously characterized in general as a *r*-strategist, as pseudomonads rapidly colonize and grow on nutrient-rich environments (Juteau et al., 1999; Margesin et al., 2003), due to its metabolic versatility (Clarke, 1982; Hallsworth et al., 2003; Domínguez-Cuevas et al., 2006). This shift is important as it has been reported that *K*-strategists are expected to allocate more energy and interact in a broader way with their environment, for example developing strategies to cope with their environment, than to grow (Fontaine et al., 2003).

Although the experiment showed a reduction in diversity of the isolated strains and interactions as a whole, details of these responses of strains to the individual treatments were largely idiosyncratic and seemed independent of their phylum. For example, the group that includes strain AP29, affiliated with

TABLE 3 | Antibiotic resistance.

Treatment	n	Carbenicillin		Kanamycin		Tetracycline		Streptomycin		Gentamicin	
		Resistant (%)	*p*	Resistant (%)	*P*	Resistant (%)	*p*	Resistant (%)	*p*	Resistant (%)	*p*
Water T0	163	55.21		22.09		10.43		20.25		12.88	
Water Control	87	70.11	0.09	3.45	4.60E-4	0.00	7.23E-3	13.79	0.93	1.15	7.23E-3
Water NNP	186	75.27	3.30E-4	1.61	4.30E-9	2.15	4.94E-3	3.23	1.81E-6	1.08	3.00E-5
Water NP	176	58.52	1.00	0.57	6.04E-10	0.00	4.00E-5	2.84	1.40E-6	0.57	2.00E-5
Water P	201	69.62	0.02	1.49	7.91E-10	0.50	5.00E-5	9.95	0.02	0.50	2.97E-6
Sediment T0	42	28.57		26.19		7.14		33.33		21.43	
Sediment Control	11	100	3.00E-4	0.00	0.38	0.00	1.00	0.00	0.10	0.00	0.38
Sediment NNP	38	100	1.53E-11	0.00	2.54E-3	0.00	0.41	5.26	6.99E-3	0.00	0.01
Sediment NP	31	93.55	4.38E-8	0.00	8.15E-3	0.00	0.63	0.00	1.31E-3	0.00	0.02
Sediment P	25	68.00	6.64E-3	0.00	0.02	4.00	1.00	28.00	1.00	4.00	0.27

Percentages of strain resistant for different antibiotics, and their significance with respect to the initial condition (T0) in both water and sediment, according to Barnard's exact tests, with Bonferroni correction for multiple tests. Percentages with p-values less than 0.05 are considered significantly different than the corresponding to T0 values.

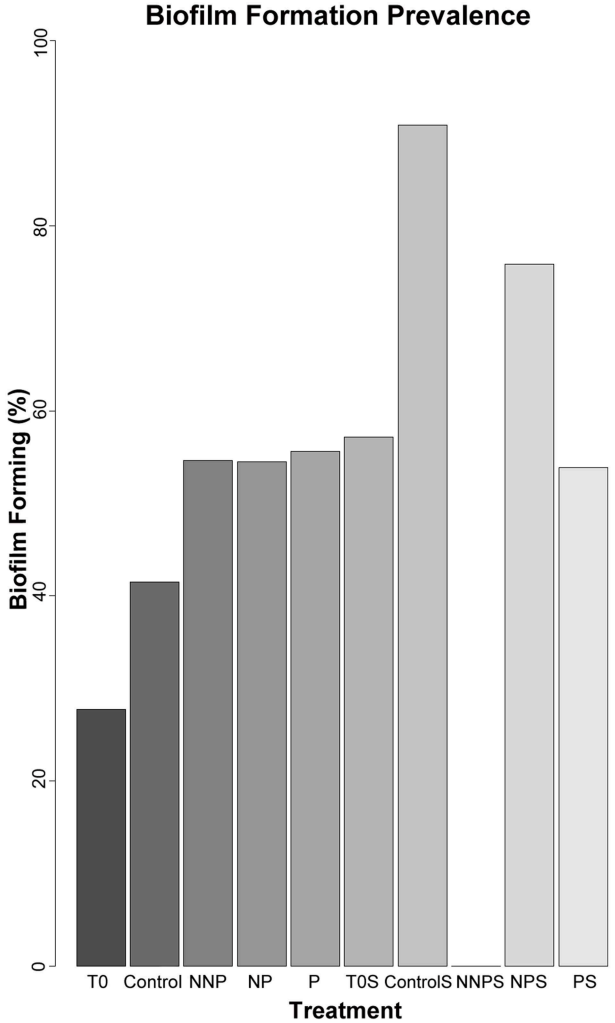

FIGURE 3 | Relative frequency of biofilm forming strains among 923 isolates. The isolates analyzed for each treatment were as follows. T0—157, Control—84, NNP—174, NP—167, P—196, T0S—42, CS—11, NNPS—38, NPS—29, and PS—25.

TABLE 4 | Biofilm formation.

Treatment	*n*	Biofilm forming (%)	*P*
Water T0	155	27.74	
Water Control	82	41.46	0.13
Water NNP	174	54.60	3.07E-6
Water NP	167	54.49	4.37E-6
Water P	196	55.61	5.78E-7
Sediment T0	43	57.14	
Sediment Control	11	90.91	0.16
Sediment NNP	38	0.00	5.82E-8
Sediment NP	31	75.86	0.50
Sediment P	26	53.85	1.00

Percentages of strain forming biofilm, and their significance with respect to the initial condition (T0) in both water and sediment, according to Barnard's exact tests, with Bonferroni correction for multiple tests. Percentages with p-values less than 0.05 are considered significantly different than the corresponding to T0 values.

Pseudomonas, responded to the P treatment, while another group of *Pseudomonas*, which includes strain ENNP10, responded to the NNP treatment. However, another *Pseudomonas* group was abundant at the beginning of the experiment and remained abundant in the control, as well as in the P treatment (group including strains AP9, AC10, and 1C11) (**Figure 1**). The sudden availability of P in P-deficient environment could favor the dominance of the *Pseudomonas* genus, which has been reported to solubilize phosphate (Park et al., 2009; Parani and Saha, 2012). In sediment samples it was observed in both N+P treatments that *Halomonas* exhibited the greatest response. This observation is consistent with the fact that the *Halomonas* genus has been identified as capable of denitrification and may have taken advantage of the added NO_3 in the NP and NNP treatments (Mormille et al., 1999; Guo et al., 2013).

The production of chemical compounds as bacteriocins and/or antibiotics is a common mechanism of antagonism among microorganisms (Riley and Gordon, 1999; Lenski and Riley, 2002; Riley and Wertz, 2002; Kirkup and Riley, 2004; Hibbing et al., 2010; Kohanski et al., 2010; Majeed et al., 2011, 2013; Pérez-Gutiérrez et al., 2013; Aguirre-von-Wobeser et al., 2014). In this study, we observed a general decrease in the antibiotic resistance in both the water and sediment environment, for all antibiotics except Carbenicillin, after the experiment (**Table 3**). This decrease likely reflects modifications in microbial survival strategies under different conditions, including the control were wind movement of both water and sediment, was restricted by the mesocosm tubes. Given the antagonistic network previously documented among *Pseudomonas* bacterial isolates from CCB (Aguirre-von-Wobeser et al., 2014), this antagonism could be due to the competition for resources. Following "microbial market logic" (Werner et al., 2014), without the acute nutrient limitation, the cost of producing antibiotics to repel competitors for a scarce resource is no longer beneficial in the case of increased P and the ideal N:P ratio, while in the NNP treatment, the community is so perturbed by the further limitation of P in relation to N that antagonism or cooperation through biofilm formation is no longer an economic option. On the other hand, in a rock—paper—scissors (RPS) model behavior scenario, that can be applied only for structured environment (Kirkup and Riley, 2004; Nahum et al., 2011) such as sediment or biofilm, the strains that produce toxins (*C*) kill sensitive strains (*S*), which outcompete resistant strains (*R*), which in turn outcompete C (Czarán et al., 2002; Kirkup and Riley, 2004). In this RPS game, the resistant and producer strains spend resources to keep the resistance, which in an enriched environment may be no longer needed, thus those strains are outcompeted by the sensitive strains (Kerr et al., 2002). Interestingly, this shift from a collection of isolates with a high prevalence of resistance to several antibiotic tested, to a collection dominated by sensitive strains was observed in both habitats: water and sediment (**Figure 2**), suggesting that the fact of predicting the neighborhood, as required for the rock-paper-scissor model, is not a requisite for antagonism, at least in the analyzed system.

Market logic suggests that local environments determine trade connections (Werner et al., 2014), and biofilm formation is a way to ensure a local environment both in water and sediment. The mechanisms of bacterial biofilm formation are processes by which single cells coordinate and implement the formation of complex surface-attached communities (Davey and O'Toole, 2000). Bacteria that reside within the biofilm are to some extent isolated from environmental stresses, such as desiccation or nutrient limitation (Danhorn et al., 2004). Complex interactions, in particular mutualistic behaviors, are expected in a biofilm, since the secretion of the matrix that forms the biofilm is a form of public good that will increase the survival of the coexisting partners and will avoid the presence of cheaters (Werner et al., 2014).

Biofilm-forming strains were found in our experiment in both habitats at time zero and in most of the enrichment treatments, suggesting that this cooperation strategy is more ingrained in the whole CCB microbial community than antagonistic interactions promoted by antibiotics, where rare species cannot afford to pay its costs. Biofilm formation was not present only in the sediments of the NNP treatment where P was further limited in relation to N. This treatment was dominated by strain of *Halomonas*. The NP treatment was also dominated by closely related strains of *Halomonas* that were biofilm-forming (Supplementary Figure 2). This differential response suggests that a wide range of strategies to cope with environmental limitations is present in CCB microbes, even within a single genus.

As the Black Queen Hypothesis (BQH) suggests, certain biological functions are not only expensive, but are also broadly distributed in the community, since they are public goods. Hence, the majority of the community can afford to lose those functions if they are at least a proportion of helpers that produce such public good (Morris et al., 2012). As a consequence, for a microbial market, we also need "price" differences and supply, as well as "demand" variation. In this study, we observed a shift in the composition of the bacterial community, from a diverse community prior the enrichment to a community dominated by few genera such as *Pseudomonas* in water or *Halomonas* in sediment after the addition of N+P. As previously stated, the change was not only related to the community composition, but also to its physiological characteristics as antibiotic resistances and the ability of biofilm formation. These changes could be explained with this microbial market theory as the supplies changed with the experiment the balance between cost and benefit.

According to the market theory, the benefit of trade depends not only on the interacting partners but also on the available supply of commodities from other sources and it is expected that biotic or abiotic conditions influence the demand for a particular service (Werner et al., 2014). Based on an analysis of Illumina tags of 16S rRNA (Elser et al., 2014, in preparation), our study site exhibits a large bacterial diversity dominated by alphaproteobacteria and bacteroidetes. In the original community, we expected to find a strong competition among the members of this community. Under the original condition of extreme low P and N availabilities, it is predicted that specialization will be favored. All this considering, it is not surprising that when this particularly diverse and fragile network of interactions was perturbed by the mesocosm conditions and the nutrient input, the community structure and its biological market equilibrium changed, reducing its overall diversity, not only in the few cultured genera that we could follow, but in the overall community (Elser et al., 2014), suggesting that the resilience of this extremely oligotrophic oasis depends precisely on the permanence of such unbalanced stoichiometry.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00246/abstract

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Theoretical analysis of the cost of antagonistic activity for aquatic bacteria in oligotrophic environments

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Many strains of bacteria produce antagonistic substances that restrain the growth of others, and potentially give them a competitive advantage. These substances are commonly released to the surrounding environment, involving metabolic costs in terms of energy and nutrients. The rate at which these molecules need to be produced to maintain a certain amount of them close to the producing cell before they are diluted into the environment has not been explored so far. To understand the potential cost of production of antagonistic substances in water environments, we used two different theoretical approaches. Using a probabilistic model, we determined the rate at which a cell needs to produce individual molecules in order to keep on average a single molecule in its vicinity at all times. For this minimum protection, a cell would need to invest 3.92×10^{-22} kg s^{-1} of organic matter, which is 9 orders of magnitude lower than the estimated expense for growth. Next, we used a continuous model, based on Fick's laws, to explore the production rate needed to sustain minimum inhibitory concentrations around a cell, which would provide much more protection from competitors. In this scenario, cells would need to invest 1.20×10^{-11} kg s^{-1}, which is 2 orders of magnitude higher than the estimated expense for growth, and thus not sustainable. We hypothesize that the production of antimicrobial compounds by bacteria in aquatic environments lies between these two extremes.

Keywords: Bacterial antagonism, diffusion, community ecology, microbial ecology, microbial physiology

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Introduction

The bacterial production of molecules with antagonistic activity toward other strains is believed to be involved in complex inter-strain interactions at the local scale (Cordero et al., 2012; Pérez-Gutiérrez et al., 2013; Aguirre-von-Wobeser et al., 2014). The role of antagonistic molecules as a means of gaining competitive advantage by killing other cells, has been extensively studied *in silico* (Pagie and Hogeweg, 1999; Czárán et al., 2002; Czárán and Hoekstra, 2003) and in controlled laboratory experiments (Kerr et al., 2002). Alternatively, other types of interactions have been proposed, where these molecules change the behavior of target cells, to elicit biofilm formation, dispersal and other responses (Ratcliff and Denison, 2011). Chemical-mediated competitive interactions can result in local extinction of sensitive strains unless there is a tradeoff paid by the producing strain. An example is the growth penalty resulting from the metabolic cost of

the biosynthesis of antagonistic compounds, which results in a disadvantage for the antagonist producing strain in the presence of resistant strains (Kerr et al., 2002; Conlin et al., 2014). If resistant strains pay a metabolic cost for the resistance lower than the cost of being antagonistic, these interactions may result in non-transitive cycles, described as a rock, scissors and paper model (Pagie and Hogeweg, 1999; Frean and Abraham, 2001). If these cycles are stable, permitting that each strain controls another from becoming dominant, coexistence of the strains is favored, while the selective advantage of being antagonistic is maintained. In a different scenario, where an antagonistic strain is able to completely out-compete all sensitive strains, the production of antagonistic mechanisms would soon become disadvantageous (as no competitor strain would survive), and any non-antagonistic (but resistant) mutants could easily be favored by natural selection, since they would not need to spend resources on producing antagonism-molecules in a competitor-free environment.

From the literature exploring rock, scissors and paper models, a consensus view has emerged where a structured environment seems to be necessary for the competitive advantage to result in stable coexistence of resistant, sensitive and antagonistic strains (Durrett and Levin, 1998; Czárán et al., 2002; Kerr et al., 2002; Czárán and Hoekstra, 2003; Greig and Travisano, 2008; Conlin et al., 2014), and therefore, to encourage the stable prevalence of antagonistic compound production. Indeed, antagonistic interactions are extremely common in densely populated, highly structured environments like soils (Vetsigian et al., 2011; Pérez-Gutiérrez et al., 2013) or microbial mats (Long et al., 2013). Surprisingly, bacterial antagonistic activity has also been found in strains isolated from natural water samples with no apparent structure (Lo Giudice et al., 2007; Aguirre-von-Wobeser et al., 2014). Although it is well known that at least some aquatic environments do have structure at the microscopic level (Azam, 1998), they are clearly less stable than sediment environments, and it is not known if that structure is enough to sustain the rock, scissors and paper model dynamics.

Antagonistic mechanisms usually involve the release of molecules to the environment, which can be potentially costly in terms of resources. This poses a paradox for bacterial strains, since each strain needs to invest resources to become more competitive, and this often is needed precisely when resources for growth are scarce. Indeed, higher nutrient availabilities favor antagonistic strains in competition models (Hulot and Huisman, 2004). Bacterial antagonism mechanisms are varied (Konisky, 1982; Michel-Briand and Baysse, 2002; Rebuffat, 2012). In some cases, like pyocins, a single molecule of the antagonistic substance is sufficient to kill a competing cell (Michel-Briand and Baysse, 2002). For instance, one single pore-forming molecule can cause enough depolarization of the membrane of a cell for it to loose viability (Michel-Briand and Baysse, 2002).

One problem that antagonistic strains could face in aquatic environments is the diffusion of antagonistic molecules away from the producing strains. Molecules will wander in random Brownian motion, which will result in the diffusion of the released molecules away from the cell. Therefore, there could be an important cost of producing these molecules, which is expected to depend strongly on the required production rate, according with the target concentration at the vicinity of a cell. In this paper, we propose a model to analyze the order of magnitude of the investment a cell must do to maintain different concentrations of antagonistic molecules. We consider the case where only one molecule of an antagonistic substance in the proximity of the cell is required, as a lower bound of resource allocation, and the case where a certain concentration of these molecules around it is needed, as an upper bound. Based on the results of simulations, we hypothesize that the production of antimicrobial compounds by bacteria in aquatic environments lies between these two extremes. Finally, we put forward the hypothesis that many of these strains regulate the production of antagonistic substances, for example in response to nutrient availability.

Overview of Mathematical Approaches

Two approaches were used to establish theoretical boundaries on the costs that cells incur in the production of antagonistic compounds that are released to the environment.

Probabilistic Mathematical Modeling

In a first scenario, we considered that a cell would need to keep at least one molecule of an antagonistic substance in order to gain a minimum amount of protection from competing cells (**Figure 1**, left side). With only one molecule on average in the vicinity of a cell, one cannot use mathematical approaches that depend on defined concentrations and gradients, like differential equations. Instead, the problem needs to be approached probabilistically, modeling the fates of individual molecules. After large numbers of simulations, the distribution of the behavior of individual molecules was analyzed using a mathematical model of the distribution, chosen for theoretical reasons, as explained below. We used a probabilistic model based on the Inverse Gaussian Distribution to explore the rate at which a cell would need to produce antagonistic molecules to maintain on average a single molecule in its vicinity at all times.

To simulate diffusion of molecules, simulation experiments were performed to explore the distribution of the time needed for particles to reach a threshold radius, when randomly drifting in Brownian motion. Particles were simulated as if they were released at the surface of the cell, and were allowed to take steps, either toward the cell or away from it. If a particle moved against the cells surface, it was reflected back to its original position. The simulations were performed in one dimension, assuming perfect radial symmetry around an idealized spherical model cell. Five sets of simulations were performed with threshold step-numbers 100, 200, 300, 400, and 500. In each case, 1000 particles were simulated, and the time needed to reach the thresholds was recorded. Inverse Gaussian Distributions were fitted by maximum likelihood using Matlab® (The Mathworks, USA).

Continuous Mathematical Modeling

In a second scenario, we considered that a cell would preferably maintain a concentration of antagonistic molecules around itself that could effectively inhibit the growth of potential competitors (**Figure 1**, right side). For this analysis, since it could be

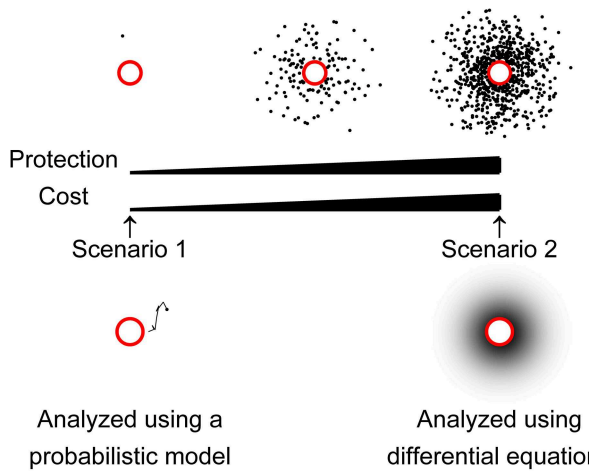

FIGURE 1 | Schematic illustration of the scenarios considered in this study. Cells are shown as red circles, and antagonistic molecules as black dots. In the lower left side, an example of a random trajectory of a molecule is represented as a black line. In the lower right side, a gradient represents concentrations of antagonistic molecules, with darker shades indicating higher concentrations. On the left side, a cell keeping on average one molecule of an antagonistic substance in its vicinity is considered. As shown, this would grant the cell an absolute minimum of protection against competing cells, and the associated production cost is considered a lower boundary for resource investment. Note that in these conditions, competing cells still could be close to the producing strain and not encounter the antagonistic molecule. These production costs were calculated using *in silico* simulations in this work, analyzing the random movements of individual molecules, and modeling the distribution of many such simulations (probabilistic modeling). On the right side, another extreme scenario is illustrated, where a cell would obtain full protection by maintaining certain concentration of antagonistic molecules around itself. The ideal concentration was arbitrarily chosen to be the minimum inhibitory concentration, as explained in the text. A cell in an intermediate scenario is also shown for illustration, although it is not explicitly investigated in this work.

described in terms of concentrations, we used differential equations. For this purpose, we used known solutions to Fick's second law (one of the most important mathematical models of diffusion) to explore the rate at which a cell would need to produce antagonistic molecules, to sustain concentrations that could inhibit the growth of other strains (Ling et al., 2010).

Mathematical Models

Probabilistic Mathematical Model to Estimate the Average Time a Particle Stays Close to its Producing Strain

For the production of antagonistic substances in aquatic environments to be effective, it is reasonable to assume that these molecules need to be close enough to the producing strain to affect cells that are actual potential competitors. We are interested in determining the timescale in which, on average, a molecule produced by a cell is expected to stay close to it, at a small enough distance to potentially interact with competing strains.

What constitutes the vicinity of a cell in terms of competition could be approached in several ways. Cells taking up nutrients from the environment by diffusion create a nutrient concentration gradient around themselves, where the concentration is lowest near the cell surface, and increases monotonically up to the concentration of the environment at a certain radius. Here, we consider two cells of different strains to be competing if the regions of lowered nutrient concentrations overlap. Assuming that nutrients are depleted completely at the cells surface, the concentration of any nutrient around a spherical cell with radius a (distances are expressed in meter units throughout this work; for clarity, units are indicated when variables are defined) is simply (Pasciak and Gavis, 1974):

$$C(r) = C_\infty \left(1 - \frac{a}{r}\right) \quad (1)$$

expressed as a function of the distance r (radius) from the cells center in any direction, and where C_∞ (concentrations are expressed in mol L^{-1} throughout this work) is the concentration in the medium. Equation (1) states that the concentration of a nutrient at a given distance from a cell taking it up is a simple function of the radius of the cell and the concentration of the nutrient in the surrounding medium. If we consider the radius where the concentration is 95% of C_∞ to be the limit where competition is relevant, then a competitor of the same size is in the vicinity if it is at a distance $r = 2 \times 20 = 40$ or closer. This distance is the same for any chemical substance taken up by the cell, since Equation (1) does not depend on the diffusion coefficient, or any other property of the chemical involved.

We can assume that an antagonistic molecule released at the surface of a cell will wander randomly through Brownian motion in the medium, due to thermal energy. Random molecular movement has been the subject of extensive theoretical development for almost two centuries, for instance in the context of the study of diffusion. In the traditional view of this phenomenon, the macroscopic flow of molecules (or particles) in a medium with a concentration gradient is thought to be the accumulated result of movements of individual molecules, in steps of nanometer lengths and picosecond timescales, with random directions and mean square velocity v (m/s) given by (Berg, 1993):

$$\langle v^2 \rangle^{\frac{1}{2}} = \left(\frac{kT}{m}\right)^{\frac{1}{2}} \quad (2)$$

Where k is the Boltzman constant (aprox. $1.3806504 \times 10^{-23}$ J K^{-1}), T (K) is the absolute temperature and m (kg) is the mass of the molecule or particle. Thus, according to Equation (2), the average velocity of a particle depends on the temperature and on its mass.

The discrete nature of these molecular movements is thought to reflect the trajectory between collisions with one molecule of the surrounding medium to the next one; trajectories whose average length is known as the mean free path. According to this random walk model of diffusion, the individual solute molecules very rarely meet (given sufficiently low concentrations), and their trajectories and displacements are therefore considered independent. This assumption of traditional diffusion theory allows us to utilize the exact same theoretical framework to study the mean

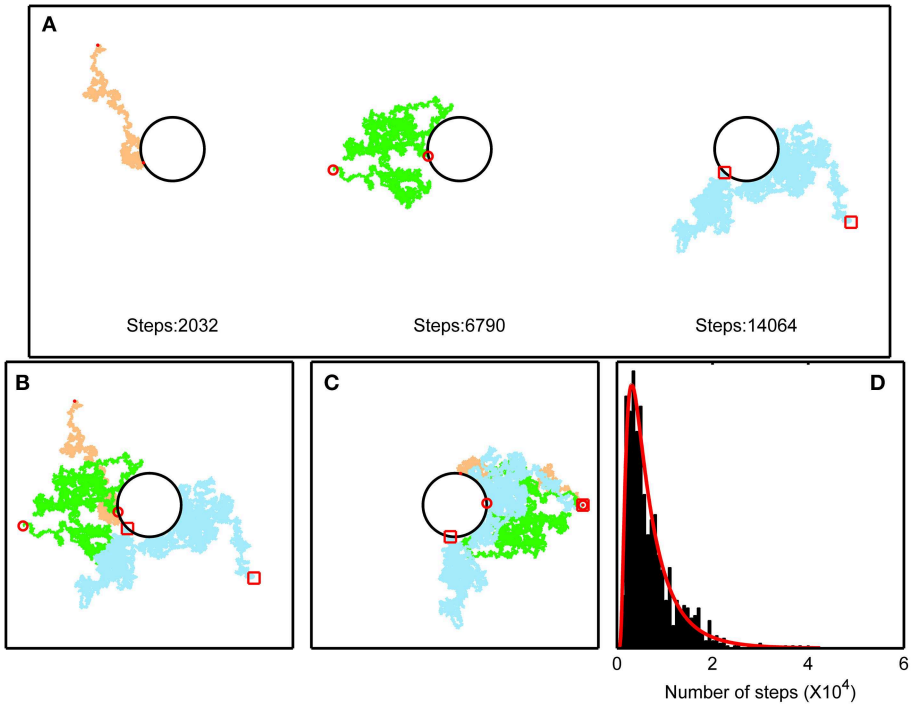

FIGURE 2 | Simulation to illustrate "random walk" movements of a particle away from a cell (black circles) that produced it. (A) Trajectories of three different particles produced by the same cell at different time points are shown as colored continuous lines (different colors are used to distinguish the three independent particles; red dots, circles and squares mark the start and end positions of the three independent particles), since the time they are released, until they reach a distance of 3 cell radiuses from the cell surface for the first time. Notice that they will approach the cell again with probability 0.5 at the next step, or drift further away. The number of steps taken to reach that position, is indicated for each case. Note that the length of each step is greatly exaggerated in this figure, as compared to the scales of real molecules and bacteria. **(B)** The trajectories are plotted around the same depiction of the cell, putting them in the same time-frame. **(C)** The trajectories are rotated, putting them in the same spatial frame, illustrating how these bi-dimensional trajectories can be studied in one dimension if the only quantity of interest is the distance from the cell. This same reasoning can be applied for a three-dimensional scenario. **(D)** Distribution of the number of steps taken to reach 3 cell radiuses in 1000 simulations with the same scales as in panel **(A)**. The red line indicates an Inverse Gaussian Distribution fitted to the data. Note that an equally well fit can be attained using a Birnbaum-Saunders Distribution, but the Inverse Gaussian is preferred for theoretical reasons.

displacements of individual molecules released at different times at a cells surface.

Due to the random distribution of the solvent molecules, in any frame of reference in a three dimensional environment, the displacement of diffusing particles is known to be independent for each of the three spatial dimensions (Berg, 1993). For convenience, we set the frame of reference arbitrarily parallel to the maximum displacement of each particle when they reach radius r (**Figures 2A–C**). Note that this is similar to setting the time frame of reference for every particle at the time they were excreted at the surface of the cell. Thus, we can model the dispersion of particles as a one-dimensional random walk (Crank, 1975). In other words, since the components of random movements that are not normal (perpendicular) to the cells surface in the direction that randomly generates the maximum displacement, do not contribute to drive the molecules away from the cells, only normal displacement in that direction need to be modeled (note that we only need to model one dimension, along the radius, although the particles actually move in three dimensions). Since we assume that the molecules are not absorbed again at the cells surface, we consider it a reflected random walk, as the molecules are reflected when they collide with the cells surface. With sufficiently large numbers of particles, the probability of finding a particle at a distance r from the origin at time t follows a normal distribution (Berg, 1993):

$$p(r,t) = \frac{1}{\sqrt{4\pi Dt}} e^{-\frac{r^2}{4Dt}} \quad (3)$$

where D ($m^2\ s^{-1}$; note that, to use a single unit system, we do not follow the common practice of expressing D as $cm^2\ s^{-1}$) is the diffusion coefficient, and the variance is $2Dt$. So, the expected number of particles will decline with the distance from the cell, and the variations around this expected number will be greater with higher diffusion coefficients.

To model the time needed for a collection of molecules released at the cells surface to reach a threshold distance from the cell (say 40 cell radiuses), we can consider each step to be a normally distributed random variable, as described by Equation (3). The distribution of the number of steps needed by particles to reach a threshold distance $r = b$ can be modeled statistically under certain premises. In the case of a random walk with positive drift (for example when a current is present) it can

be described by an Inverse Gaussian Distribution, which has a probability density function (Folks and Chhikara, 1978):

$$p\,(t,\,\mu,\,\lambda) = \left(\frac{\lambda}{2\pi t^3}\right) e^{\frac{-\lambda(t-\mu)^2}{2\mu^2 t}} \tag{4}$$

for $t > 0$, where μ is the mean number of steps needed to reach distance b, and λ is a shape parameter which, together with the mean, defines the variance as μ^3/λ. The time to reach a threshold radius in a reflected random walk has also an Inverse Gaussian Distribution (**Figure 2D**), even when no drift is present (Abate and Whitt, 2011). Note that the problem is described mathematically as a one-dimensional, focusing on the relevant dimension, being the distance of the particle from the cell, as explained above. A simple simulation study shows that μ is equal to n_b^2 where n_b is the number of steps needed to reach b (**Figure 3**). Although we lack mathematical proof of this empirically observed relation, it allows us to express the average number of steps needed to reach arbitrary radius b as a function of useful quantities, as follows. If each step takes an average τ s, the mean time to reach b is $\langle t_b \rangle = \tau\, n_b^2$, that is, the average number of steps times the average time they take. From the definition of velocity as $v = \delta/\tau$, and the definition of the diffusion coefficient as $D = \delta^2/2\tau$, one can express τ in terms of known quantities as:

$$\tau = \frac{2D}{v^2} = \frac{2Dm}{kT} \tag{5}$$

which gives us a simple expression for the average step time, and we can express the average step size as:

$$\delta = \tau v = \tau \left(\frac{kT}{m}\right)^{\frac{1}{2}} = \frac{2Dm}{kT}\left(\frac{kT}{m}\right)^{\frac{1}{2}} = 2D\left(\frac{m}{kT}\right)^{\frac{1}{2}} \tag{6}$$

The number of steps to reach b is equal to $n_b = b/\delta$ which, with $b = 40a$, equals $n_b = 40a/\delta$. Thus:

$$\langle tb \rangle = \tau {n_b}^2 = \tau\ 1600\frac{a^2}{\delta^2} = \frac{2Dm}{kT} 1600a^2 \frac{1}{4D^2}\frac{kT}{m} = 800\frac{a^2}{D} \tag{7}$$

Using Equation (7), we can calculate the average time for molecules to reach a distance of 40 cell radiuses from the cell, by knowing their diffusion coefficients. Diffusion coefficients for small molecules have an order of magnitude of 10^{-9} $m^2 s^{-1}$ while for small proteins they tend to range from 10^{-10} to 10^{-11} $m^2 s^{-1}$ (Erickson, 2009). For example, for an s5-type pyocin from *Pseudomonas aeruginosa* which can target other strains from the same species (Ling et al., 2010), we can calculate the diffusion coefficient using the Einstein-Stokes relation:

$$D = \frac{kT}{6\pi\eta r} \tag{8}$$

where η is the viscosity, which for water at 291.15 K (18°C) is about 0.001009 Pa s, and the radius of the protein can be estimated (Erickson, 2009):

$$r = 6.6 \times\ 10^{-11} M^{\frac{1}{3}} \tag{9}$$

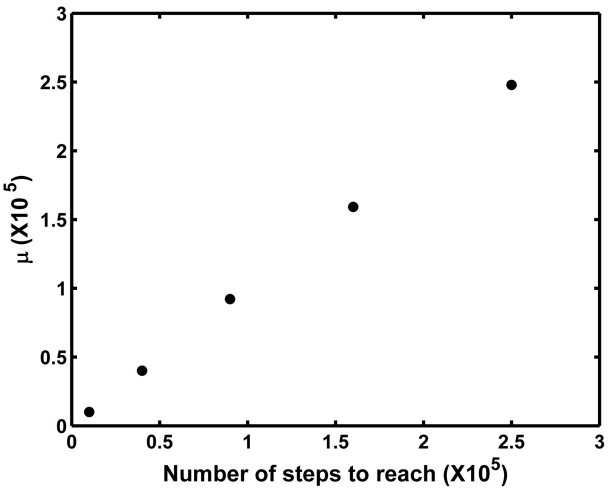

FIGURE 3 | Simulation experiment showing the relation between parameter μ of the Inverse Gaussian Distribution, and the square of the number of steps n_b^2 needed to reach a radius n_b from the surface of the cell, measured in step-lengths. For each point, 1000 particles were allowed to wander with steps of fixed length and random direction, simulating Brownian motion, until they reached the radius indicated in the x axis, and the number of steps taken were recorded. The values correspond to parameter μ, obtained by fitting the Inverse Gaussian Distribution to the corresponding 1000 times. We notice that this relation can most likely be obtained analytically, but do not count with a mathematical proof at the moment.

where M is the molecular weight M, which for this protein is 57.6 kDa (Ling et al., 2010). Equation (8) gives an estimated diffusion coefficient of $D = 8.29 \times 10^{-10}$. For a cell with radius $a = 0.5 \times 10^{-6}$m (0.5 μm), Equation (7) estimates the average time for a molecule of the said pyocin to stay close to the producing cell to be about 0.24 s.

Thus, in order to maintain at least one molecule of an antagonism molecule, of similar size, at a distance close enough to be able to affect a competitor, a cell would need to produce them at a rate of 4.1 molecules s^{-1}, or 6.88×10^{-24} mol s^{-1}. We regard this figure as the minimum investment that would make any sense in an aquatic environment (**Figure 1**, left side). However, in order to be really protected, and gain competitive advantage, a cell would need to maintain a concentration of antagonism molecules around itself (**Figure 1**, right side). Therefore, we are also interested in the rate of synthesis that a cell would need to sustain in order to keep certain concentration of antagonism molecules around it.

Fick's Laws Based Model to Determine the Production Rate to Sustain a Minimum Inhibitory Concentration Around a Cell

In the second approach, we considered the cost for the cell to maintain a certain concentration around itself to inhibit potential competitors. For this approach, to model individual molecules becomes impractical, but the continuous gradients provided by large numbers of molecules allow us to use differential equations of known diffusion theory (**Figure 1**, right side). The change in concentration C of a substance at a distance r in the presence of

a gradient can be described by Fick's second law, which around a sphere has the following form (Crank, 1975; Berg, 1993):

$$\frac{\partial C}{\partial t} = \frac{D}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial C}{\partial r}\right) \tag{10}$$

Equation (10) is only valid when the number of molecules produced is high enough to be adequately described as a continuum concentration. There is no general solution (lacking unknown integration constants) for Equation (10), but solutions depending on particular conditions of a given setting can often be found. These conditions are termed boundary conditions, and aid in determining the values of integration constants to obtain particular solutions for the problem in hand. For instance, if a spherical cell is producing a substance and releasing it a constant rate at the surface, Equation (10) can be solved assuming a steady state with boundary conditions $C_\infty = 0$ (no antagonistic molecules exist in the surrounding medium, and a current I (mol s^{-1}) originating at the cells surface (the production of the molecules by the cell), where C_∞ is the concentration of the molecule in the medium away from the cell. This problem has known solution:

$$C(r) = \frac{I}{4\pi Dr} \tag{11}$$

Equation (11) conveniently expresses the concentration of the antagonistic molecules as a function of the distance from the cell, according the constant production rate and the diffusion coefficient. Since the concentration is lowest at the outer extreme of the region of interest, by taking $r = 40a$, we make sure that the concentration is high enough in the whole region between this radius and the surface of the cell:

$$C_{r=40a} = \frac{I}{160\pi Da} \tag{12}$$

Now we need a value for the ideal concentration in the interest region. For this purpose, we use the concept of minimum inhibitory concentration (*MIC*), which is the minimum concentration that yields a noticeable decline in growth of sensitive strains. This minimum concentration required to inhibit a potential competitor depends on the specific antimicrobial substance and the particular sensitive strain. Studies can be found that measure this quantity (e.g., Ling et al., 2010) and report empirically determined *MIC* values. Using this concept, we substitute $C_{r=40a}$ for *MIC* and get:

$$I = MIC\ 160\pi Da \tag{13}$$

Values of *MIC* are typically in the order of the tens of microgram per milliliter. For example, for the s-type pyocin mentioned in the preceding section, a measured *MIC* of 0.0126 kg/m^3 against a specific target *P. aeruginosa* strain has been reported (Ling et al., 2010). Using the diffusion coefficient calculated with Equation (8), Equation (13) gives a value of $I = 2.1 \times 10^{-13}$ mol s^{-1}, or 1.26×10^{11} molecules per s.

Cost of Producing and Releasing an Antagonistic Substance

Under the assumption that antagonism molecules have the same elementary composition as the average stoichiometry of the cell, the production and release of those molecules would be limited by the same nutrient(s) as growth. So, comparing the mass range of these molecules and the mass and growth rates of typical bacteria, we can estimate if the production rates we calculated in the previous sections are sustainable.

To obtain the dry weight of a spherical bacterium, we can use an empirical relation between the cells volume and the dry weight W (kg $cell^{-1}$), valid for cells larger than 0.025 μm^3 (Romanova and Sazhin, 2010):

$$W = 133.745 \times 10^{-12} V^{0.438} = 133.745 \times 10^{-12}\left(\frac{4}{3}\pi a^3\right)^{0.438} \tag{14}$$

For example, for a cell with radius a $= 0.5 \times 10^{-6}$m, the dry weight would be approximately 1.32×10^{-18} kg. To sustain this biomass, at a growth rate of 1 division per day (1.16×10^{-5} s^{-1}), the cell would need to produce biomass at a rate of 1.14×10^{-13} kg s^{-1}. For the pyocin discussed above (Ling et al., 2010), the mass of a single molecule would be 9.56×10^{-23} kg. It was determined that a cell would need to produce molecules at a rate of 4.1 s^{-1} to have, on average, a single molecule of an antagonistic compound in its vicinity at all times (Scenario 1), which amounts to 3.92×10^{-22} kg s^{-1}. Compared to the cost of biomass production for growth, this is about 9 orders of magnitude lower. However, to produce enough molecules to sustain a minimum inhibitory concentration, a cell would need to produce molecules at a rate of 1.26×10^{11} s^{-1}, or 1.20×10^{-11} kg s^{-1}. This value is two orders of magnitude larger than the cost of biomass production.

Discussion

Many bacterial species produce substances to hamper the growth and proliferation of others in densely populated or highly structured environments (Vetsigian et al., 2011; Long et al., 2013; Pérez-Gutiérrez et al., 2013). We are concerned with the theoretical challenge posed by the presence of antagonistic mechanisms in water environments as well (Long and Azam, 2001; Lo Giudice et al., 2007; Aguirre-von-Wobeser et al., 2014). Moreover, these mechanisms can be found in highly oligotrophic environments like the Churince system in Cuatro Ciénegas (Aguirre-von-Wobeser et al., 2014) where there is not only low structure, but the cell densities are much lower, and the probability of encounter with other cells is very low, and where molecules can easily diffuse away from the producing strain. We specifically addressed this last issue, since we were interested in calculating the order of magnitude of the frequency of production of antagonism molecules that would make sense for a cell to sustain. We considered two different scenarios:

1. A cell that would only need to keep a single molecule of an antagonistic substance in its close proximity most of the time. This scenario is supported by the observation that for

some antagonistic compounds, a single molecule can kill a competing strain (Michel-Briand and Baysse, 2002).
2. A cell that would need to keep a minimum inhibitory concentration around itself.

Since the two scenarios are best described by discrete and continuous mathematical models, respectively, we used different theoretical approaches to further our understanding of the cost of antagonism in a water environment. First we analyzed the situation where a single molecule around a producing strain could be advantageous. In this scenario, where a minimum production of antagonistic molecules is expected, we found the cost of production in terms of biomass to be nine orders of magnitude lower than the metabolic cost of net growth. However, with such a low release of antagonistic substances, the cell would need to rely on the chance encounter of the antagonistic molecule with the target strain, which could be highly random. Therefore, we regard this absolute minimum antagonism molecule production as not likely to be enough for an effective protection from competitors.

Then we analyzed the cost of sustained production of antagonistic molecules to create a minimum inhibitory concentration around the producing strain. To achieve this, the cell would need to sustain a constant production of antagonistic molecules in high enough quantities to replenish all the molecules that constantly diffuse away. As expected, the calculated cost contrasted sharply with what was found in the previous case. The rate of molecule production the cell would need to sustain turned out to be even higher than the cost of growth by net biomass synthesis, by two orders of magnitude, when considering one doubling event per day. This means that the cell would need to spend much more resources for antagonism molecule production than for growth. In real life, it is not likely that the cells invest continually these high amounts of resources. For example, *Escherichia coli* cells carrying different colicin-producing plasmids show a growth reduction of approximately 20% (Gordon and Riley, 1999).

The models used in this study were developed to understand antagonistic substance production in aquatic oligotrophic environments with low, possibly negligible structure. However, they could be adapted to scenarios where structure is more prominent, such as soils, root-associated communities, snow environments, eutrophic aquatic environments, marine-snow rich oceanic waters, among others, to understand the role of diffusion in such environments.

Although it is possible that the cells synthesize antagonistic compounds at an intermediate rate between the two extremes considered, another likely scenario is that these mechanisms are regulated by differential gene expression, and are only turned on under certain conditions. Bacteria are known to be very flexible in their gene expression profiles in varying environmental conditions (e.g., Aguirre-von-Wobeser et al., 2011; Dugar et al., 2013). Modulation of the production of antagonistic substances in response to environmental conditions has been observed (Bruhn et al., 2007). A recent study has explored regulation of colicin gene expression in *Escherichia coli*, and found a very dynamic regulation of antagonism (Hol et al., 2014). Therefore, we hypothesize that regulation of antagonistic molecule synthesis is an important strategy for cells to produce these compounds in the environment when they are cost-effective.

It is a highly complex question to establish at which concentrations of nutrients it makes sense for a bacterium to produce antagonistic substances, and it falls outside the scope of this article. However, to have some insight on this problem, the costs of production of these molecules for aquatic bacteria calculated in this work could be inserted in growth models that consider maintenance costs, like those discussed in van Bodegom (2007). Additionally, such studies would need to include several experimentally obtainable parameters, like growth kinetic parameters of producing and sensible strains and growth penalties for competing strains with different concentrations of antagonistic substances.

A possibility that cannot be ruled-out is that at least some cells have proteins in their external surface that could reversibly attach to antagonism molecules. In that case, the times in which the molecules would stay in the cells vicinity could greatly increase. To the best of our knowledge, this question remains unanswered.

We conclude that by using theoretical approaches and observations from the literature, we found that the production of antagonism by bacteria in non-structured environments is a complex phenomenon that is likely to be highly regulated. Nutrient availability, cost of production of molecules with antagonistic activity, diffusion of these molecules and cell motility are likely forces participating in the process.

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Antagonistic interactions are sufficient to explain self-assemblage of bacterial communities in a homogeneous environment: a computational modeling approach

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Most of the studies in Ecology have been devoted to analyzing the effects the environment has on individuals, populations, and communities, thus neglecting the effects of biotic interactions on the system dynamics. In the present work we study the structure of bacterial communities in the oligotrophic shallow water system of Churince, Cuatro Cienegas, Mexico. Since the physicochemical conditions of this water system are homogeneous and quite stable in time, it is an excellent candidate to study how biotic factors influence the structure of bacterial communities. In a previous study, the binary antagonistic interactions of 78 bacterial strains, isolated from Churince, were experimentally determined. We employ these data to develop a computer algorithm to simulate growth experiments in a cellular grid representing the pond. Remarkably, in our model, the dynamics of all the simulated bacterial populations is determined solely by antagonistic interactions. Our results indicate that all bacterial strains (even those that are antagonized by many other bacteria) survive in the long term, and that the underlying mechanism is the formation of bacterial community patches. Patches corresponding to less antagonistic and highly susceptible strains are consistently isolated from the highly-antagonistic bacterial colonies by patches of neutral strains. These results concur with the observed features of the bacterial community structure previously reported. Finally, we study how our findings depend on factors like initial population size, differential population growth rates, homogeneous population death rates, and enhanced bacterial diffusion.

Keywords: bacterial antagonism, ecological modeling, community emergence, spatial patterns, bacterial biodiversity, cuatro cienegas

1. Introduction

Microbial ecosystems have proved to be excellent frameworks to understanding ecological systems (Prosser et al., 2007). New ecological theories have arisen from microbial ecology due to its simplicity, controllability, replicability and experimentally-required times (Jessup et al., 2004). Remarkably, much of that progress has been achieved by means of simplified theoretical models (Momeni et al., 2011). Most of these models account for the interaction of only a few microbial

populations. This is an advantage, because simple models can be more easily studied both numerically and analytically, but also a limitation, because such models oversimplify biological reality. Nowadays, thanks to the rapid increase of computer power, it is possible to investigate the dynamics of larger sets of interacting populations (Costello et al., 2012).

Organisms in an ecosystem are affected by abiotic and biotic factors. Abiotic elements refer to non-living chemical and physical factors in the environment, whereas biotic factors are living or once-living organisms in the ecosystem. The influence of abiotic factors on a community spatial-temporal dynamics has been thoroughly studied (Dunson and Travis, 1991). Conversely, biotic factors have been mostly disregarded. Nonetheless, recent studies have evidenced that biotic interaction networks play very important roles (Hooper et al., 2005), specially in terms of biodiversity maintenance and energy flow (Raes and Bork, 2008; Eisenhauer et al., 2012).

Diverse biotic interactions have been experimentally described in nature, and some others have been hypothesized from mathematical modeling results (Evans et al., 2013). At microbial scales, an outstanding panoply of interaction mechanisms has been observed (Prasad et al., 2011), favoring competition over cooperation (Foster and Bell, 2012). Antagonistic interactions (also known as interference competition) have been studied by Czárán et al. (2002), using the simplified model: Killer (K), Resistant (R) and Sensitive (S). This interaction loop implies an associated metabolic cost (and a concomitant growth-rate reduction) for being either an antibiotic producer (K) or a resistant strain (R). Thus, in terms of proliferation, S outcompetes R and R outcompetes K. This model, which is able to sustain bacterial populations, gave rise to new studies aimed at figuring out how microorganisms use and evolve bacteriocin and antagonistic molecules to sustain biodiversity in structured ecosystems (Kerr, 2007).

The shallow water system of Churince, located in Cuatro Cienegas, Mexico, sustains an impressive microorganism biodiversity, mainly due to its geological history (Souza et al., 2006). Even with scarce nutriments in the water, microorganisms have proliferated and evolved to use any available component around them, as has been seen in other oligotrophic environments (Kuznetsov et al., 1979). It is well known that some bacteria, such as *Bacillus spp.*, are able to synthesize antagonistic molecules (Abriouel et al., 2011). Cuatro Cienegas' bacteria are not an exception because, as previous studies show, these organisms use a variety of bacteriocin molecules to annihilate competing neighbors (Pérez-Gutiérrez et al., 2013; Aguirre-von Wobeser et al., 2014). All of this, together with the fact that the sediment of this natural setting is static and homogeneous regarding its physicochemical conditions, make it an excellent candidate to study how biotic interactions affect the spatial-temporal arrangement of microbial populations.

In a previous study (Pérez-Gutiérrez et al., 2013), we isolated 78 bacterial strains (most of them from the genus *Bacillus*) from 5 different sampling sites across Churince pond, and tested them for one-to-one antagonistic interactions. Among others, we obtained the following results which are not self evident from the point of view of the most commonly accepted ecological theories:

- Bacterial strains are not homogeneously distributed across the pond, in spite of the pond's physical-chemical conditions being homogeneous and pretty much stationary.
- Antagonistic interactions are more frequent across sampling sites than within them.

In Pérez-Gutiérrez et al. (2013), we hypothesized that microscopic microbial antagonistic interactions may be responsible for shaping bacterial communities at the macroscopic scale, and that this may suffice to explain the above-enlisted observations. The present work is aimed at proving the feasibility of such hypothesis from a mathematical modeling perspective. To that end we decided to model the pond as a square grid, each of whose cells represents a small area that can be colonized by at most one bacterial strain. The dynamics of the grid cells are then modeled as a set of rules derived from the antagonism matrix experimentally determined in Pérez-Gutiérrez et al. (2013). We decided to employ this modeling strategy because of its adequacy given the available experimental data, and because it has been employed to demonstrate how local interactions can give rise to complicated global patterns, like those observed in natural systems (Gardner, 1970; Hogeweg, 1988; Iwasa et al., 1998; Sarkar, 2000; Wootton, 2001; Wolfram, 2002; Deutsch and Dormann, 2005). To our knowledge, this is the first study in which such a problem is tackled with an antagonistic network involving a large number of interacting strains.

2. Materials and Methods

2.1. Bacterial Collection

In this work we make use of the interaction network of a set of 78 bacterial strains isolated and studied by Pérez-Gutiérrez et al. (2013). The strains in this set were isolated from 5 different samples taken from the superficial sediment of Churince pond in Cuatro Cienegas, Mexico. Since the isolating methodology involved subjecting the samples to thermal shock, all of the isolated strains came out to be thermo-resistant, and most of them belong to the genus *Bacillus*. All 78×78 pairs of bacterial strains were cultured in Petri dishes to test for antagonistic interactions. The resulting antagonism matrix is reported in Pérez-Gutiérrez et al. (2013) and reproduced in **Figure 1**. In this figure, bacterial strains are organized in decreasing order according to their *Aggressiveness Index* (number of other strains antagonized by a given strain, minus number of other strains antagonizing it). The ID numbers given in this work to all strains, the labels employed by Pérez-Gutiérrez et al. (2013), and the corresponding aggressiveness indexes are tabulated in **Table 1**.

2.2. Computational Algorithm

In order to simulate the evolution of a bacterial community interacting according to the antagonism matrix reported in Pérez-Gutiérrez et al. (2013), we developed a computational algorithm as follows:

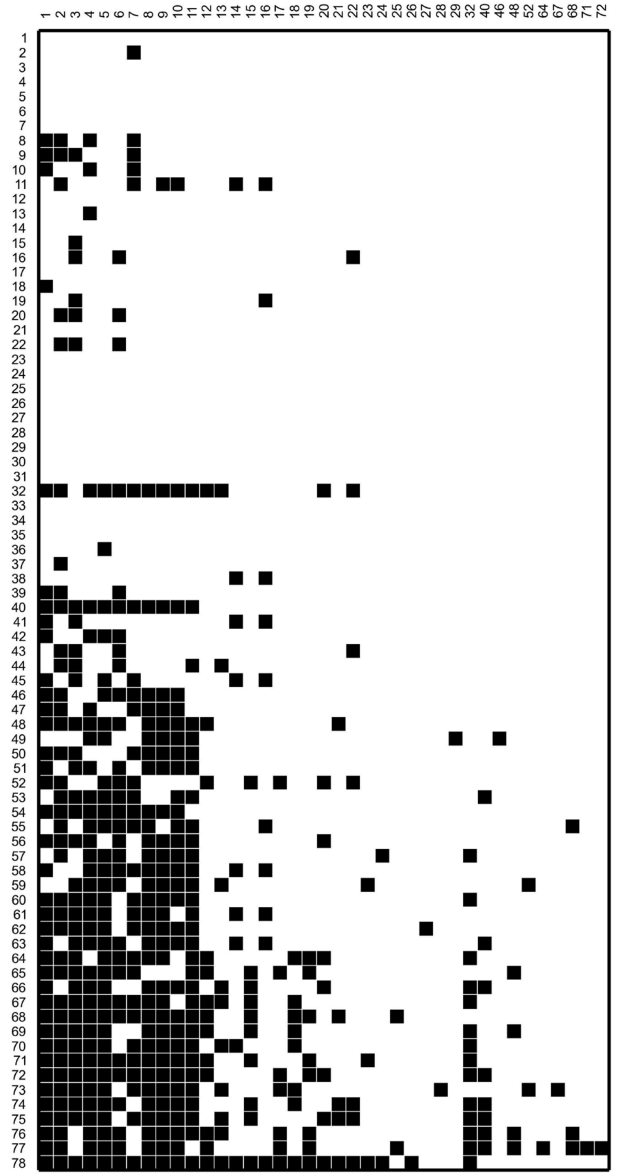

FIGURE 1 | Antagonism matrix for the bacterial strains considered in this work. A black box indicates that the bacterial strain in the corresponding column antagonizes that in the box row. Some strains do not appear in the matrix columns because they antagonize no other bacterial strain. The bacterial strains were labeled in decreasing order according to their aggressiveness index AI (numbers of other strains antagonized by it, minus number of strains antagonizing it). In **Table 1**, we give the original label and the AI number of each strain.

TABLE 1 | ID numbers, labels, and aggressiveness indexes of the investigated bacterial strains.

ID	Label	AI	ID	Label	AI	ID	Label	AI
1	CH95a	38	27	CH38c	1	53	CH44	−9
2	CH21	37	28	CH452b	1	54	CH26a	−10
3	CH90	35	29	CH159b	1	55	CH19a	−10
4	CH150a	35	30	CH39a	0	56	CH149a	−10
5	CH156	34	31	CH448a	0	57	CH155a	−10
6	CH43	31	32	CH452a	0	58	CH28	−11
7	CH22	29	33	CH99B	0	59	CH135a	−11
8	CH144a	28	34	CH88	0	60	CH98b	−11
9	CH144b	28	35	CH112a	0	61	CH84	−11
10	CH145	28	36	CH29	−1	62	CH33	−11
11	CH109a	25	37	CH93	−1	63	CH447	−12
12	CH154a	13	38	CH26b	−2	64	CH161d	−13
13	CH37	9	39	CH160c	−3	65	CH138	−13
14	CH113a	9	40	CH87b	−3	66	CH158b	−13
15	CH148	9	41	CH25	−4	67	CH145b	−14
16	CH112b	7	42	CH140a	−4	68	CH159a	−14
17	CH448b	7	43	CH111	−4	69	CH449a2	−14
18	CH23	7	44	CH157b	−5	70	CH20a	−14
19	CH20b	6	45	CH45	−6	71	CH162	−15
20	CH450	5	46	CH36	−7	72	CH446	−16
21	CH30	5	47	CH449a1	−7	73	CH40	−16
22	CH41b	4	48	CH160a	−8	74	CH445	−16
23	CH449b	3	49	CH451b	−8	75	CH451a	−17
24	CH24	2	50	CH91b	−8	76	CH163b	−17
25	CH19b	2	51	CH81a	−8	77	CH153a	−19
26	CH164b	1	52	CH142	−8	78	CH34	−26

In the present work, bacterial strains were ordered (assigning each of them an ID number) in decreasing order according to their aggressiveness index AI (numbers of other strains antagonized by it, minus number of strains antagonizing it). We also include the label given to each strain in Pérez-Gutiérrez et al. (2013).

1. A square grid of 200 × 200 cell represents the pond soil. Each cell in the grid corresponds to a small surface that can be either empty or colonized by at most one bacterial strain.
2. The grid is initialized by randomly distributing a fixed number of individual colonies of each bacterial strain among the grid cells.
3. After initializing the grid, the state of all the grid cells is updated according to the following rules:

   (a) Let i denote the current grid cell. Randomly chose one of its 8 neighboring cells and denote it by j.
   (b) If both the i- and j-th grid cells are empty at the n-th time step, the i-th cell remains empty at time $n + 1$.
   (c) If the i-th grid cell is empty and the j-th grid cell is occupied by a given strain at time n, the i-th cell is colonized at time $n + 1$ with probability $P_g(\mu_j)$ by the strain in the j-th cell, μ_j. Function $P_g(\mu_j)$ is specified in the Results section for different simulations.
   (d) If the i-th grid cell is occupied at time n by strain μ_i, it becomes empty at time $n + 1$ with probability $P_d(\mu_i)$. The corresponding probability distribution is specified in the Results section for different simulations. If this does not happen, consider the following two possibilities:

   - If both grid cells (i and j) are occupied at time n and the strain in the j-th grid cell antagonizes the strain in the i-th grid cell, then the i-th grid cell gets empty at time $n + 1$.
   - If both grid cells (i and j) are occupied and no antagonism exists, or if the i-th grid cell is occupied and the j-th grid cell is empty, the i-th cell remains the same.

4. Step 3 is iteratively repeated a fixed number of times.

2.3. Generation of Random and Experimental-like Antagonism Matrices

In order to test how the system dynamics depends on the architecture of the antagonism interaction-network, we constructed alternative antagonism matrices (and employed them to repeat the analysis of the system dynamics) following two different strategies:

- Random antagonism matrices were constructed by randomly generating N unidirectional antagonistic interactions between the 78 isolated bacterial strains, with N the number of links in the original matrix. We took care of avoiding self-antagonism.
- To construct what we call experimental-like antagonism matrices, we first noted that, according to the original matrix, bacterial strains can be classified as high-level, medium-level and low-level antagonistic strains (a k-means algorithm, MacQueen, 1967 was employed to carry out this classification). Then, we made use of a Markov-Chain Monte Carlo algorithm (Gilks, 2005) to randomly generate N links in such a way that the resulting antagonism matrix has the same number of high-level, medium-level, and low-level antagonistic strains as the original matrix.

3. Results

3.1. Emergency of Community Patches Provides a Survival Mechanism to Low-Level Antagonistic Bacteria

To investigate whether, as claimed by Pérez-Gutiérrez et al. (2013), antagonistic interactions have a lead role in the spatial distribution of bacterial communities, as well as in the diversity differences found across sites, we took the antagonism matrix reported therein, and used it to implement the algorithm described in the Section 2. Recall that, in such algorithm, a 200×200 square grid represents the pond, and each cell in the grid corresponds to a small surface in the pond soil that can either be empty or colonized by at most one strain. Initially, the grid was "inoculated" with 50 individual colonies of every bacterial strain, randomly distributed across the grid (see **Figure 2A**). That is, the grid was seeded with $78 \times 50 = 3900$ cells in total. The system was then iteratively evolved in time for 500 round cycles of the algorithm. In these initial simulations, we assumed for all strains (μ) that the probability that the bacterial strain in a grid cell colonizes a neighboring empty cell in an algorithm step is 1 ($P_g(\mu) = 1$), and that the probability that an inhabited grid cell is emptied because the corresponding colony dies is zero ($P_d(\mu) = 0$). To clarify the role of antagonism in the evolution of the system, an aggressiveness index was defined as follows: the aggressiveness index of a given bacterial strain, $AI(\mu)$, equals the number of other strains antagonized by it, minus the number of other strains that antagonize it.

In **Figure 2** we present the results corresponding to a single simulation. We carried out several simulations and obtained equivalent results in all cases. Namely, an emergent spatial arrangement of bacterial communities in patches spans the grid after a few iteration steps, and eventually a stationary state in which the patches no longer change is reached (see **Figure 2B**). We note that the formation of patches is consistent with the fact that different strains were isolated from different samples (Pérez-Gutiérrez et al., 2013), despite the habitat homogeneity. Moreover, we can see in this particular simulation, but this is a consistent observation, that patches of vulnerable strains are always surrounded, and shielded from the most aggressive strains, by patches of

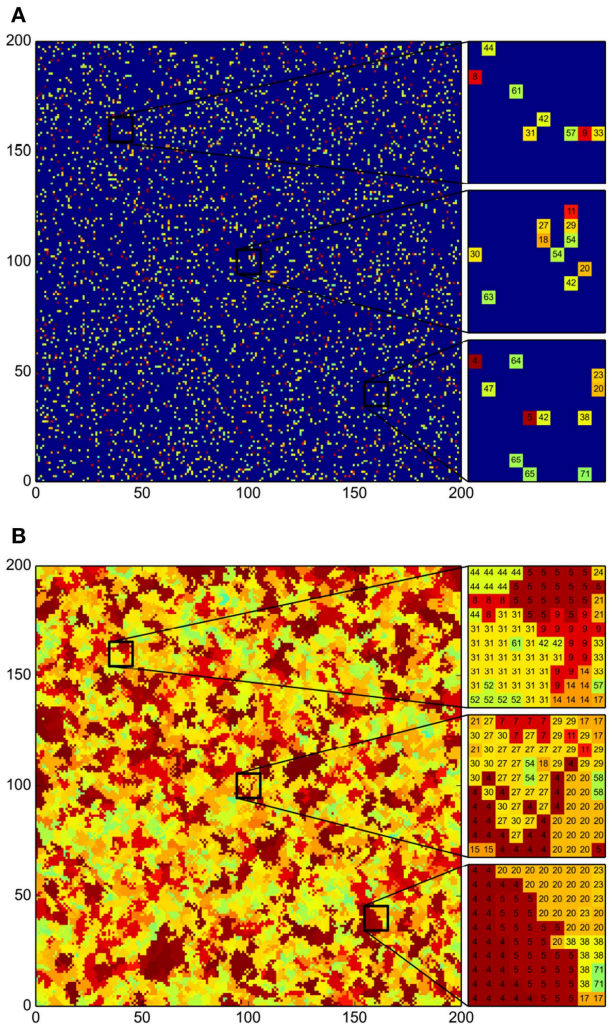

FIGURE 2 | Emergence of community patches as a result of bacterial growth. Color indicates strain *Aggressiveness Index* (*AI*). The less aggressive bacteria are represented in light green and the most aggressive ones in maroon; deep blue denotes an empty grid cell. The *AI* of the *i*-th bacterial strain is computed as the number of other strains antagonized by it minus the strain count that antagonize it. **(A)** Initial distribution of the 78 bacterial strains. For each strain 50 initial individual colonies were distributed randomly. **(B)** Final distribution after 500 iteration steps of the simulation algorithm. Total coverage of the surface and emergence of bacterial patches isolating populations can be observed. Beside both graphs we present close-ups of selected regions across the grid, with the ID number of the strain inhabiting each grid cell indicated within (see **Table 1**).

other strains that are both resistant to the aggressive ones and non-antagonistic toward vulnerable bacteria. This result suggests a mechanism for the survival of low-antagonistic and vulnerable bacteria, but also offers a plausible explanation for the observation that antagonistic interactions within a sampling site are on average less frequent than interactions across sites (Pérez-Gutiérrez et al., 2013).

To have a deeper understanding of the system dynamics we measured patch sizes in the stationary state of 100 different simulations. The results are summarized in **Figure 3A**. We can appreciate there that a positive correlation exists between the mean patch size and the aggressiveness index of a given strain. That is, more aggressive bacteria tend to form larger patches, whereas the patches of more vulnerable bacteria are consistently smaller. We further measured the total population of every strain at the end of 100 different simulations and computed the corresponding averages. A scatter plot representing mean final population size vs. aggressiveness index is shown in **Figure 3B**. Observe that total final population is also positively correlated with the aggressiveness index.

Finally, we followed the evolution in time of all the strain populations and the results (averaged over 100 different simulations) are shown in **Figure 3C**. Observe that, initially, all the inoculated colonies grow steadily, but eventually, a stratification of them according to their *AI* value becomes apparent. As far as we understand, this happens because reduction of the distance between colony borders increases the frequency of conflicts. After about 200 simulation steps, a stationary regime, in which the population size of all bacteria strains remains constant, is reached.

3.2. Bacterial-Community Spatial Structure is Disrupted Under Constant Perturbation and all Vulnerable Strains are Driven to Extinction

Since the spatial arrangement of bacteria in stagnant community patches appeared to be necessary for the preservation of bacterial diversity, we carried out an alternative experiment with constant shuffling (mixing) of the grid cells. In these experiments, we carried out simulations as previously described (see **Figure 4A**), but every 10 simulation steps the grid cells were randomly shuffled (different inter-shuffling times were also considered, but all of them led to equivalent results). We observed that all susceptible bacterial strains (most of them with low antagonistic levels) become extinct, and they are replaced by larger populations of medium- and high-level antagonist strains (see **Figures 5B,C**). Furthermore, the surviving bacteria do not form patches. Instead, single-grid-cell colonies are randomly distributed (in a well mixed fashion) across the grid (see **Figures 4B**, **5A**). Even though we shuffled the grid at regular times, the first shuffling events have the most notorious effects: they rapidly cause the extinction of the sensitive strains. After this, the populations of surviving bacteria reach a stationary value, independently of the changing spatial distribution (see **Figure 5C**). A correlation between stationary populations and *AI* values is still present for the surviving strains (see **Figure 5B**).

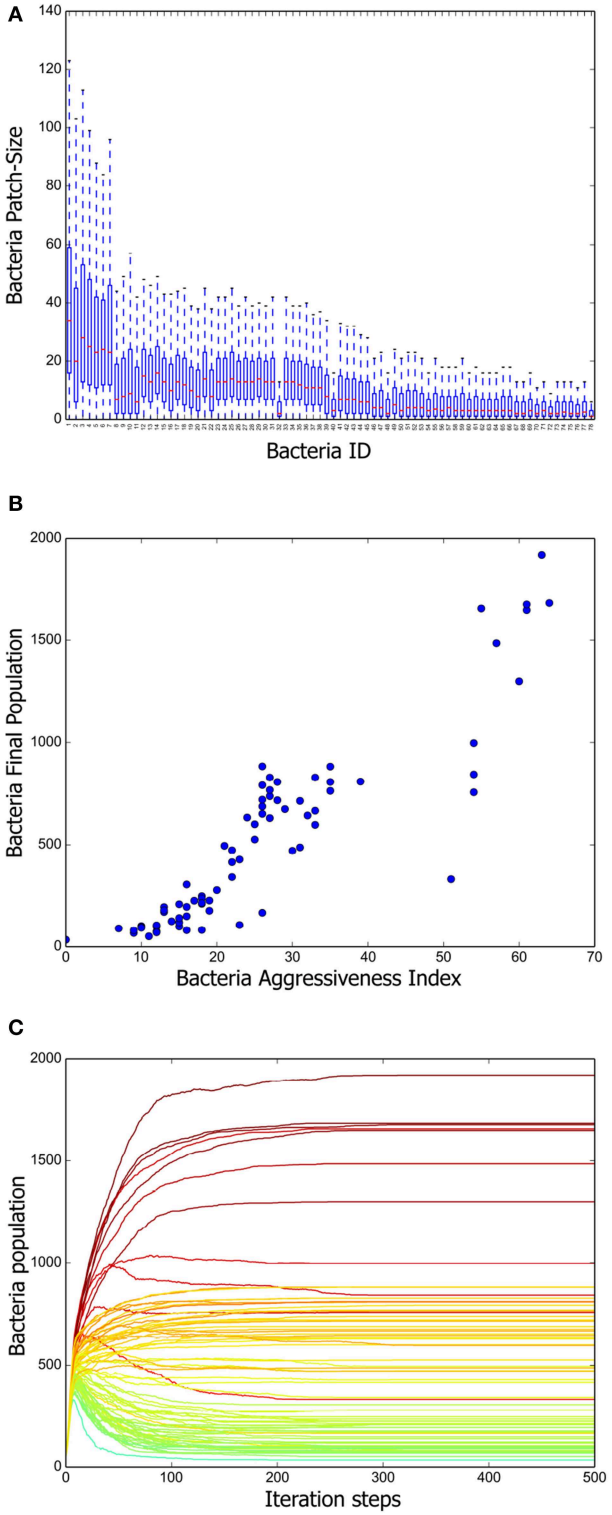

FIGURE 3 | Positive long-term correlation between *AI* and bacterium population. (A) Box plot summarizing the patch-size statistics of 100 simulations. Bacterial strains are shown in decreasing order according to their AI value. Note that a positive correlation exists between aggressiveness index and average patch size. **(B)** Correlation between stationary bacterial

(Continued)

FIGURE 3 | Continued
populations, averaged over 100 simulations, and *AI* values. A constant was added to the previously defined *AI* so that it attains positive values for all strains. **(C)** Growth curves for all the bacterial strains tabulated in **Table 1**. The AI value corresponding to each strain is indicated by means of the same color code as in **Figure 2**. Notice that, initially, all strains populations increase monotonically. However, once the grid becomes saturated, antagonistic interactions make the population of low *AI* strains decrease.

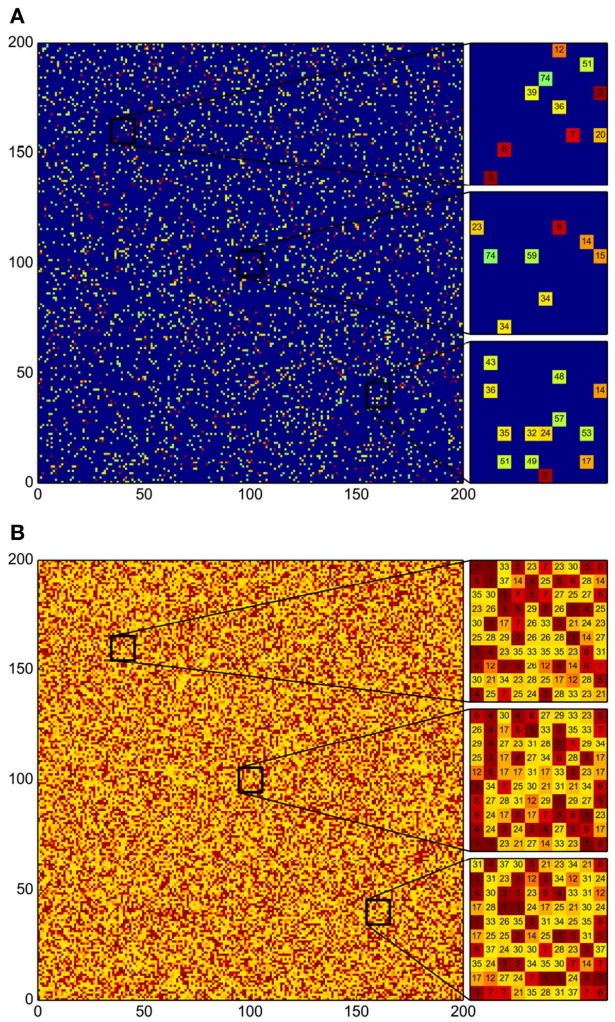

FIGURE 4 | Disruption of the emergent community patches by regularly shuffling of the grid. (A) Initial distribution of the 78 bacterial strains. For each strain 50 initial individual colonies were distributed randomly. **(B)** Final distribution after 500 iteration steps of the simulation algorithm. Every 10 iterations the grid was randomly shuffled. As in the simulations without shuffling, the grid ends up being completely occupied. However, a structure without patches emerges, and all susceptible bacterial strains are missing.

The above results indicate, to our consideration, that periodic shuffling of the grid cells not only precludes patch formation (by randomly relocating individual colonies of all strains) but also ensures that all susceptible strain colonies eventually get in contact with aggressive strains and are thus driven to extinction.

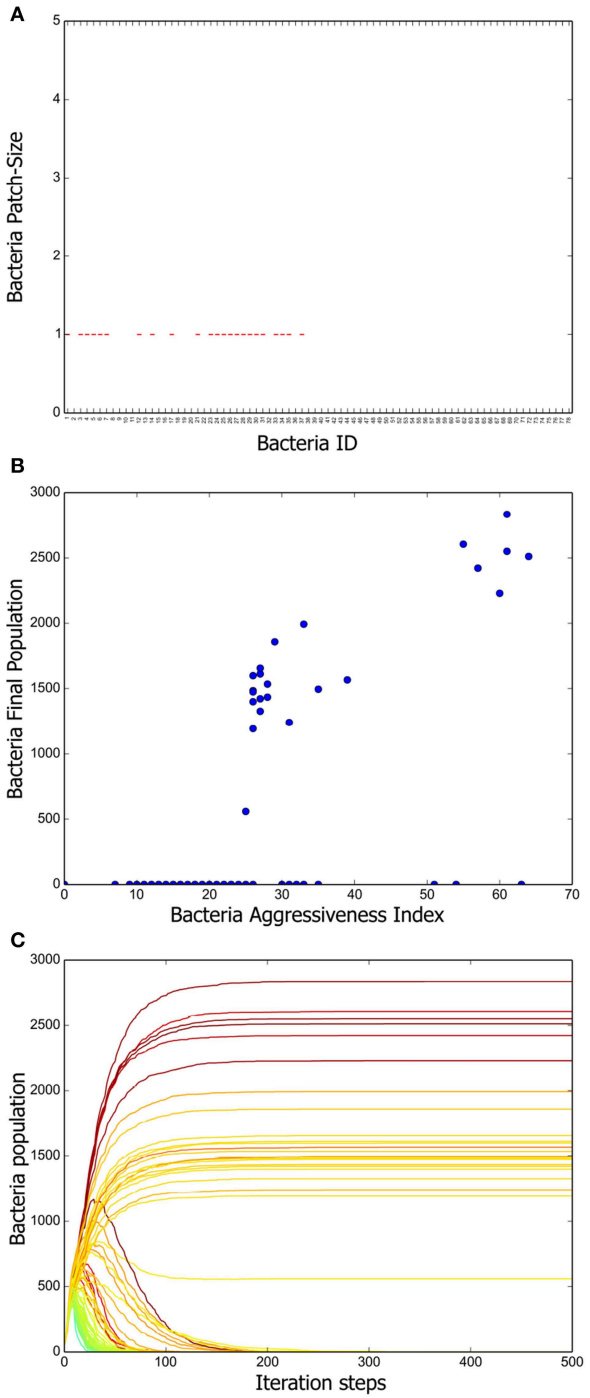

FIGURE 5 | Susceptible strains become extinct due to regular strain shuffling. (A) Box plot summarizing the patch-size statistics of 100 simulations. Bacterial strains are shown in decreasing order according to their AI value. Many strains become extinct and the patch size for all the surviving-strain colonies is exactly 1. That is, no patches are formed whatsoever. **(B)** Correlation between *AI* and stationary population levels. Observe that all susceptible strains (most of them with low antagonism levels) become extinct and that the surviving strains occupy the space left by those that become extinct. **(C)** Growth curves for all bacterial strains in this Figure. Notice that regular reshuffling of the grid does not affect bacterial population levels once a stationary state has established.

3.3. Influence of the Antagonism Matrix Architecture on the System Dynamic Behavior

To answer the question of whether the results discussed in previous subsections depend on the architecture of the antagonistic-interaction network (which is determined by the antagonism matrix), we generated random and experimental-like interaction matrices (see Section 2). Experimental-like interaction matrices have the same number of high-level, medium-level, and low-level antagonistic strains, and the distribution of exerted-antagonism and received-antagonism links are very similar to those of the experimentally obtained matrix. On the other hand, random antagonism matrices were built by linking, via antagonism interactions, couples of bacterial strains chosen at random (avoiding self-antagonism). The number of antagonism links in the random and the experimentally-obtained matrices are always the same.

When we repeated the simulations using experimental-like interaction matrices, we were able to recover all the previously described results, with and without shuffling. Nonetheless, the simulations with random interaction matrices rendered quite different results. Community patches can still be observed in the non-shuffling simulations, but the final bacterial population size is less disperse than in the simulations carried out with the experimentally-obtained or the experimental-like antagonism matrices. Furthermore, in the shuffling simulations, most bacterial strains become extinct and no correlation is observed between AI and the stationary population size (see Supplementary Material).

In conclusion, the architecture of the interaction network plays a very important role in the emergence of the community patch structure. In particular, the characteristic that seems to be essential is that bacterial strains can be classified in three different classes: aggressive (highly antagonistic and resistant to other strains), neutral (barely antagonistic and resistant to aggressive strains), and vulnerable (non-antagonistic and sensitive to aggressive strains).

3.4. Influence of Growth Rate and Initial Population Size on the System Dynamics

So far, we have assumed in our simulations that all bacterial strains grow at the same rate, whenever they have available space. Recall that in our model the growth rate of strain μ is determined by probability $P_g(\mu)$. Moreover, we considered equal initial populations in all of our simulations. In order to have a more complete picture of the studied biological phenomena, we repeated our simulations by taking into consideration distinct growth probabilities and initial populations.

Based on previously reported observations (Bohannan et al., 2002; Kneitel and Chase, 2004; Cadotte, 2007), who have shown that changes in the flow of metabolic energy and in the cellular machinery need to be done by a bacterium in order to become either highly antagonist or fast growing, we assumed that $P_g(\mu)$ is inversely proportional to $AI(\mu)$ (the strains with the largest and the smallest AI values have $P_g = 1$ and $P_g = 0.5$, respectively), and repeated the previously described simulations. According to our results, the new simulations differ from the previous ones in that low-level and medium-level antagonistic strains have now larger stationary populations, at the expense of high-level antagonistic strains (see Supplementary Material).

To account for variable initial populations, we took the final population distributions of the simulations described in Section 3.1, and used them to compute the initial strain populations of a new grid, with the constraint that the most populous strain initially occupies 1% of the grid cells. After running the simulations, the only notorious difference we observed, with respect to those corresponding to constant initial populations, is that having larger initial population has a positive effect on the corresponding stationary population. This happens regardless of whether we consider constant or variable growth rates (see Supplementary Material).

3.5. Non-Zero Death Rate may Increase the Chance of Survival of Less Antagonistic Bacteria

Finally, we considered in our model a death rate related to causes independent of bacterial antagonism. According to Servais et al. (1985); Pace (1988), in aquatic environments there are several events that generate the reduction of certain populations of bacteria, due primarily to: biotic factors (viral infections, starvation, senescence, and allelopathy) and abiotic factors (environmental changes such as radiation, temperature changes and toxic compounds). To take this into account we assumed that, in every step of the algorithm, a grid cell inhabited by strain μ has a probability $P_d(\mu)$ of being emptied, independently of growth and antagonism events.

Initially, we considered a constant $P_d(\mu) = 0.01$ for all strains. With this assumption, we were able to recover all the results described in previous sections, with the exception that the most favored strains (those with the largest stationary populations) are those with intermediate AI values. I.e., those strains that neither antagonize many other bacteria nor are susceptible to be antagonized by the most aggressive ones (see Supplementary Material). When we repeated the simulations with $P_d(\mu) = 0.1$, we obtained similar results, except that the strains that had the smallest stationary populations in the previous simulations were driven to extinction (see Supplementary Material). Interestingly, when a non-zero death rate is accounted for, the achieved stationary state is not stagnant any longer. Community patches are formed, but they do not remain the same once the stationary population levels are achieved and the grid is full. On the contrary, they slowly change their size and shape, and move around the grid.

4. Discussion and Conclusions

The computational modeling framework employed in this work has proved to be very useful to studying the spatial and temporal behavior of diverse biological phenomena (Ermentrout and Edelstein-Keshet, 1993; Alber et al., 2003; Rohde, 2005). Hence, we decided to employ it to test the hypothesis that macroscopic community patches can emerge as the result of microscopic individual interaction in a homogeneous environment. Our results not only confirm the feasibility of such hypothesis, but also show that these patches allow bacteria to minimize conflicts while preserving biodiversity.

We tested the robustness of our results by considering different initial-condition scenarios, as well as non-zero death rates and distinct growth rates for different bacterial strains. In all cases we obtained qualitatively equivalent results, thus confirming that the achieved conclusions do not depend on these factors.

On the other hand, it must be emphasized that the rules underlying the implemented computational algorithm are concomitant with low-motility bacteria growing in a homogeneous surface, interacting through pairwise-local-interactions. These restrictions are consistent with the environmental conditions observed in Cuatro Cienegas ponds (Johannesson et al., 2004), where oligotrophic constant conditions have given rise to a great diversity of bacteria who take advantage of any component in the media around them (Escalante et al., 2008; Cerritos et al., 2011) and compete with direct neighbors (Pérez-Gutiérrez et al., 2013; Aguirre-von Wobeser et al., 2014). However, we wondered to what extent having low-motility bacteria is a necessary condition for biodiversity preservation. To test this, we repeated our simulations but included periodical and random shuffling of the grid cells. Since we invariably observed that all susceptible strains become extinct, we conclude that having a slowly changing environment is mandatory for sustaining biodiversity when highly antagonistic, neutral, and highly susceptible strains share the ecosystem.

Previous studies have shown the importance of biodiversity in food-webs, being the web architecture the cause and effect of biodiversity prevalence (Sole and Montoya, 2001; Dunne et al., 2002; Ives and Carpenter, 2007; Allesina and Pascual, 2008), specially under perturbation scenarios (Girvan et al., 2005; Pascual and Guichard, 2005; Dunne and Williams, 2009; Baho et al., 2012). Counter-intuitively our results suggest that antagonism interaction networks may have a similar effect. Previous similar studies have been published (Silvertown et al., 1992; Kerr et al., 2002; Kirkup and Riley, 2004; Károlyi et al., 2005; Walshe, 2006), and some report a large repertoire of possible dynamic behaviors (Silvertown et al., 1992; Károlyi et al., 2005). However, to our knowledge, this is the first study in which a large set of experimental antagonism data is considered.

We are conscious that the model here introduced does not provide a detailed picture of the real-life system. Instead, it can best be regarded as a very simple cartoon or toy model. This is so because the model ignores dynamic aspects that play important ecological roles. For instance, positive interactions are well-documented in the case of biofilms, which tend to aggregate various types of bacteria and promote positive interactions among them (in this way, positive interactions can generate micro-habitats which introduce a level of physicochemical heterogeneity even in an otherwise rather stable and constant habitat). In this respect, we build our model following Einstein's advice that every theory (model) should be as simple as possible, but not simpler (i.e., not so simple that it does not represent reality any longer). To our consideration, given the amount of available experimental information, the present is the simplest possible model one can come out with to tackle the question of whether the antagonism matrix found by Pérez-Gutiérrez et al. (2013) can explain a heterogeneous bacterial community distribution in a homogeneous habitat.

Author Contributions

GO and MS conceived the project, designed the *in-silico* experiments, and interpreted data and results. RZ conducted the experiments and analyzed and interpreted data. All authors contributed to writing the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00489/abstract

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Species interactions differ in their genetic robustness

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Conflict and cooperation between bacterial species drive the composition and function of microbial communities. Stability of these emergent properties will be influenced by the degree to which species' interactions are robust to genetic perturbations. We use genome-scale metabolic modeling to computationally analyze the impact of genetic changes when *Escherichia coli* and *Salmonella enterica* compete, or cooperate. We systematically knocked out *in silico* each reaction in the metabolic network of *E. coli* to construct all 2583 mutant stoichiometric models. Then, using a recently developed multi-scale computational framework, we simulated the growth of each mutant *E. coli* in the presence of *S. enterica*. The type of interaction between species was set by modulating the initial metabolites present in the environment. We found that the community was most robust to genetic perturbations when the organisms were cooperating. Species ratios were more stable in the cooperative community, and community biomass had equal variance in the two contexts. Additionally, the number of mutations that have a substantial effect is lower when the species cooperate than when they are competing. In contrast, when mutations were added to the *S. enterica* network the system was more robust when the bacteria were competing. These results highlight the utility of connecting metabolic mechanisms and studies of ecological stability. Cooperation and conflict alter the connection between genetic changes and properties that emerge at higher levels of biological organization.

Keywords: cooperation, competition, genetic robustness, *E. coli*, *Salmonella*, community stability, metabolic modeling

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Introduction

Microbes often lead highly social lifestyles, engaging in interactions that span the gamut from cooperation to conflict (Mitri and Foster, 2013). These interactions determine the composition and function of microbial systems, and influence communities that are critical for both natural and applied processes. Microbes primarily interact through the compounds that cells remove from and excrete into the environment (Klitgord and Segrè, 2011). As such, the dynamics of species interactions are intimately connected to the behavior of physiological networks inside of cells. However, it is unclear how sensitive species interactions are to intracellular perturbations and vice versa. Specifically, how robust to mutation are emergent community properties when species are competing vs. when they are cooperating? And conversely, how do species interactions influence the physiological robustness of species?

There has been a great deal of work on both the robustness of genomes and the stability of communities, but little work connecting the two (Klitgord and Segrè, 2010). Genetic literature typically focuses on the likelihood that mutations will alter a phenotype. Indeed, connecting phenotypes to their underlying genotypes is a challenge that has spanned generations of scientists (Mather, 1941; Ho and Zhang, 2014), and has gained new momentum now that sequencing has enabled genome-wide association studies (Evangelou and Ioannidis, 2013). Another systems level approach that has proven highly useful is genome-scale libraries of gene knockout mutants (Winzeler et al., 1999; Baba et al., 2006). These libraries have allowed analysis of questions from the number of essential genes (Baba et al., 2006), to the average number of genes that affect phenotypes of interest (Ho and Zhang, 2014). While it is largely appreciated that environment mediates the connection of genotype to phenotype, there has been little systematic study of genetic perturbations in a community context (Klitgord and Segrè, 2010). Does the robustness of a genome change when it is involved in different ecological interactions? Do genetic perturbations have similar effects in different social contexts? Studying genomic robustness in a community context may provide further insight into the forces shaping genomic architecture.

Ecology literature focuses on the stability of communities to environmental perturbations. The relative stability of communities depends on the metric of interest. It is commonly held that competitive interactions should stabilize total community biomass through "compensatory dynamics" (Gonzalez and Loreau, 2009). If one species reduces in density, a competitor will increase in density to fill the available niche space (but see Loreau and de Mazancourt, 2013). In contrast, community composition is thought to be more stable in obligate mutualisms (Shou et al., 2007; Harcombe et al., 2014). If species are dependent on each other for essential metabolites this can create frequency dependent dynamics that push the system toward a stable equilibrium ratio of species. However, Kim et al. used a tri-partite system to demonstrate that uncoupled rates of consumption and production can lead to highly unstable dynamics (Kim et al., 2008). These approaches tend to ignore the genetic mechanisms underlying the interactions (Klitgord and Segrè, 2010). Do mutations frequently change the nature of ecological interactions? Are cooperative or competitive systems more robust to genetic perturbations? Studying the genetic basis of community stability can provide insight into the molecular mechanisms that determine community composition and function, and may improve prediction of community dynamics.

Genome-scale models of metabolism offer an opportunity to connect physiological mechanisms to the emergent behavior of species interactions. A stoichiometric, metabolic network can be used to define feasible patterns of steady state metabolite flow through a population of cells and optimization calculations can then identify the physiological fluxes that maximize the biomass production capacity or that satisfy other optimality criteria (Orth et al., 2010). This technique of flux balance modeling has had noted success at predicting the effect of environmental and genetic perturbations on cell growth (Segrè et al., 2002; Joyce et al., 2006; Orth et al., 2010). Harcombe et al. recently developed an extension of flux balance modeling that can simulate inter-species interactions as emergent properties of the uptake, excretion and environmental diffusion of metabolites (Harcombe et al., 2014). This flexible platform, called COMETS (computation of microbial ecosystems in time and space), accurately predicted the experimentally measured equilibrium ratio of 2 and 3-species microbial consortia. The impact of mutation on species interactions can now be tested by simulating growth of a mutant model in the presence of a model of another bacterial species.

Here we use metabolic modeling to determine the robustness of different types of pair-wise interactions. Specifically, we model interactions in a synthetic system involving *Escherichia coli* and *Salmonella enterica* that were previously engineered to depend on each other through the exchange of metabolites (Harcombe, 2010; Chubiz et al., 2014). In lactose minimal media, the species form an obligate mutualism as *S. enterica* relies on carbon byproducts from *E. coli* and *E. coli* in turn is dependent on methionine from *S. enterica* (**Figure 1A**). However, if acetate and methionine are added to the environment the species are no longer dependent on one another and instead compete for resources (**Figure 1B**). We investigated the impact of mutations (i.e., genetic perturbations) in each of these environments by systematically knocking out every reaction in the *E. coli* network one at a time and simulating community growth. We determined how each mutation influenced community properties, and compared the variance in properties when the species were competing or cooperating (**Figure 1C**). Based on the ecological principles discussed above, we expected that in the cooperative system species ratios would be robust to the effect of mutations, while in the competitive scenario total biomass would be robust. Finally, to investigate the generality of our findings we repeated the analysis by introducing mutations into the *S. enterica* model. The robustness of community phenotype to genetic perturbations has important implications for the dynamics of natural communities and informs design principles for engineered systems.

Materials and Methods

Experimental System

We tested the genetic robustness of an engineered model system involving *E. coli* and *S. enterica* (Harcombe, 2010; Chubiz et al., 2014). In this system *E. coli* is unable to produce its own methionine because *metB* has been deleted from the genome. *S. enterica* excretes methionine as a result of experimental evolution (Harcombe, 2010). To simulate the dynamics of this system we used previously published genome-scale metabolic models of *E. coli* (iJO_1366, Orth et al., 2011), and *S. enterica* (iRR_1083, Raghunathan et al., 2009). The *E. coli* model was modified by blocking flux through the reaction that corresponds to *metB,* and the *S. enterica* model was modified to excrete methionine (Harcombe et al., 2014). Methionine excretion was encoded by coupling a secretion flux to the biomass production flux in *S. enterica*. This modification caused the model to excrete 0.5 mmol/gDW of new biomass (Harcombe et al., 2014). It has been shown that these modified models generate growth dynamics that quantitatively

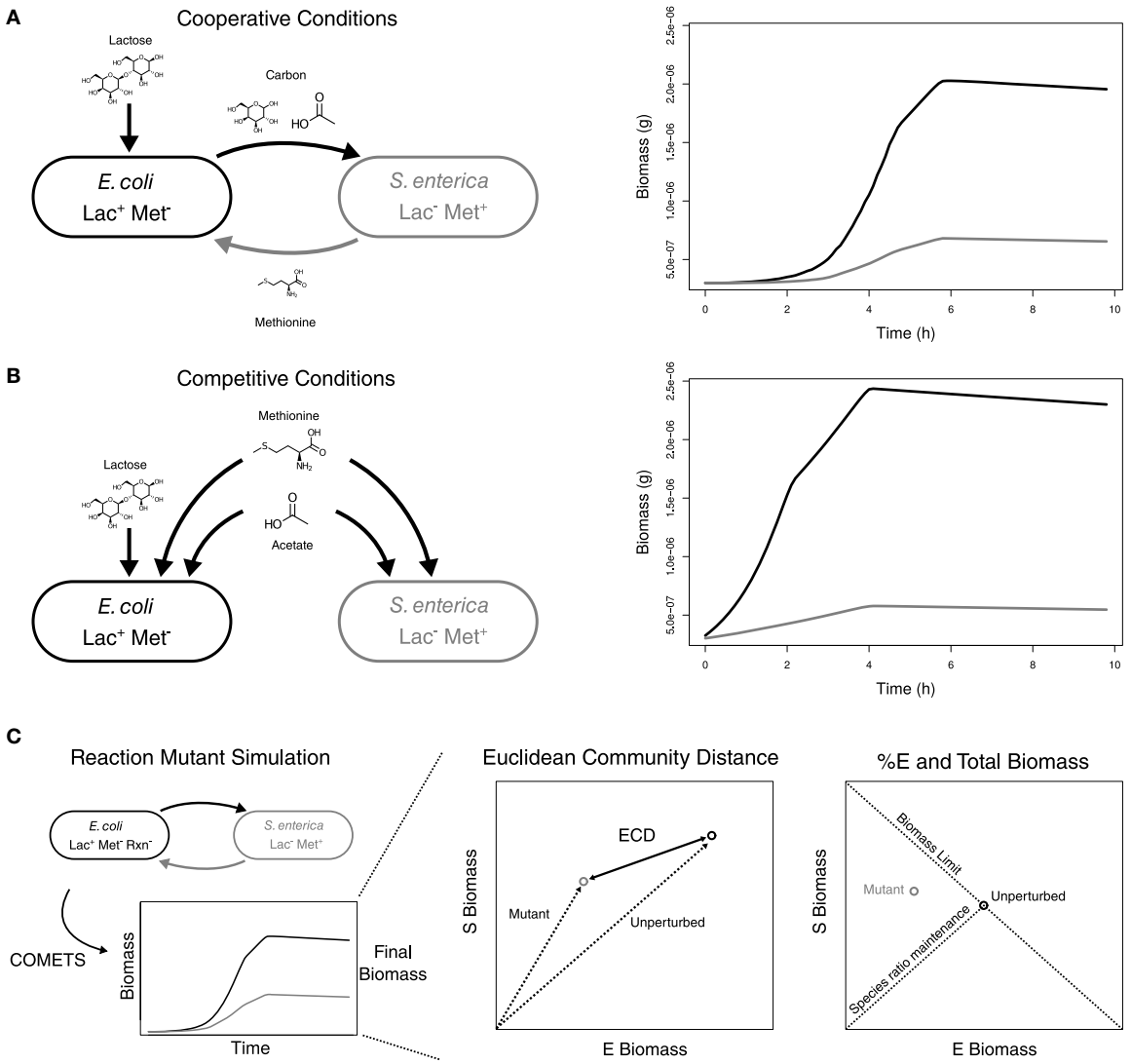

FIGURE 1 | Metabolic interactions in the unperturbed community and metrics for the analysis of the impact of perturbations. Growth conditions and metabolic interactions between *E. coli* and *S. enterica* with growth data from COMETS for **(A)** Cooperation and **(B)** Competition. **(C)** Diagram of simulation data, ECD calculation, and %E and BM determination. Also, indicated are expected correlations for maintenance of species ratios and theoretical limits for BM.

match laboratory observation (Harcombe et al., 2014). We refer to these initial modified models as unperturbed.

Libraries of genetically perturbed mutants were then computationally created for each species. A knockout mutant was created for each reaction in each species. Reactions were knocked out by setting the lower and upper bounds on the reaction in the model file to zero. This corresponds to 2583 mutants of *E. coli* and 1286 mutants of *S. enterica*. Note that this systematic blockage of individual reactions is slightly different than the approach of knocking out individual genes (Orth et al., 2011). Both knocking out individual reactions or all reactions associated with individual genes provide relevant information about the effect of genetic perturbations. We compared our results against previous computational experiments in which genes were removed and found that both displayed similar agreement with empirical observations. We discuss this comparison further in the results section.

Computer Simulations

The impact of each genetic perturbation was tested in a community context using the Computation of Microbial Ecosystems in Time and Space (COMETS) platform (Harcombe et al., 2014). Each perturbed mutant was paired with an unperturbed partner and the biomass of each species was assayed after 10 h of simulated growth. Simulations were initiated with 3×10^{-7} g biomass of each bacterial species. Growth was simulated in the minimal media used in previous work (Harcombe et al., 2014). The cooperative system was tested in an environment with 1.2×10^{-5} mmol lactose as the limiting reagent. The competitive environment was generated by adding unlimited acetate and methionine

to the lactose minimal media. In this case the limiting reagent became the oxygen, which was set at 6.2×10^{-5} mmol in all scenarios. Each simulation was performed in a one by one grid with edges set to 0.5 mm. The time step was 0.1 h. The Michaelis-Menten uptake parameters were set to a default of $K_m = 10\,\mu M$, and $V_{max} = 10$ mmol/g/h for all metabolites (Harcombe et al., 2014). The death rate was 0.01/h. COMETS simulations were performed on a 48-core computer with 64 GB of memory using COMETS version 1.3.3 (http://comets.bu.edu). Simulation data was parsed and tabulated with custom Python scripts (available on request).

Data Analysis

Three primary metrics were used to assess changes in community-level simulation outcomes. The first metric, which we term Euclidean community distance (ECD), was used to measure total community change. It is described by the following relationship:

$$ECD = 100^* \sqrt{\frac{(E - E_{Unperturbed})^2 + (S - S_{Unperturbed})^2}{E^2_{Unperturbed} + S^2_{Unperturbed}}}$$

where E is the simulated biomass of *E. coli* and S is the simulated biomass of *S. enterica* at the end of the simulation, i.e., 10 h after inoculation. Effectively, ECD is a percent deviation in biomass of all species relative to the observation in the absence of mutations (i.e., the deviation from our unperturbed community, **Figure 1C**). ECD = 0 for a genetically perturbed community in which both organisms reached the same biomasses observed in the unperturbed case.

While ECD gives an integrated view of the effects of mutations on community state, it does not give specific information on how the ratio of species (i.e., community composition) is influenced by genetic perturbation. To explore the robustness of community composition, we used the normalized percentage of *E. coli* in the community (%E), defined as:

$$\%E = \frac{E}{E+S} \Bigg/ \frac{E_{Unperturbed}}{E_{Unperturbed} + S_{Unperturbed}}$$

Finally, to investigate the robustness of community function we used a measure of community productivity. The normalized biomass (BM), was defined as.:

$$BM = \frac{E+S}{E_{Unperturbed} + S_{Unperturbed}}$$

As described in the results section, knockouts leading to changes in %E or BM values ≥1% of the unperturbed %E and BM were deemed to be substantive. The 1% cutoff was chosen because it eliminated small effects that arose from rounding or randomized calculation order in simulations.

To compare the robustness of cooperative and competitive communities for each of our metrics we used a mean-centered Levene test to evaluate the equality of variances in the face of mutation. If mutations in one context (i.e., cooperative or competitive) lead to a significantly smaller standard deviation in a community metric, that context was deemed more robust to genetic perturbation. The Levene test determines whether two groups have equal variance, and does not require the data to be normally distributed. Statistical significance was based on $P < 0.05$. The tests were implemented in R with the lawstat library. It should be noted that 2.2×10^{-16} is the lower limit of significance values in this package. Several of our tests returned values at this limit.

Results

Description of Growth in the Absence of Genetic Perturbation

The unperturbed *E. coli* and *S. enterica* followed similar growth trajectories in both cooperation and competition. *E. coli* was always the dominant community member, consistent with what was observed in prior simulations and experimental testing (Harcombe et al., 2014). In the lactose environment that required cooperation (**Figure 1A**) the community reached a total final biomass of 2.60×10^{-6} g, of which 75.1% was *E. coli*. Competition between species was created by providing excess acetate and methionine in the environment (**Figure 1B**). With the additional metabolites, the community reached a final biomass of 2.85×10^{-6} g, and the frequency of *E. coli* increased to 80.7%. The competitive scenario also allowed the bacteria to start growing sooner, as they could extract all necessary metabolites from the environment rather than waiting to obtain them from a partner.

Validating the Growth of Genetically Perturbed Mutants

To determine the validity of our genetic perturbation models we first tested our ability to predict essential reactions in monoculture by comparing against previous work. Essential reactions in our simulations were defined as those whose removal allowed the mutant to grow less than 2% relative to the unperturbed organism, consistent with common empirical approaches (Baba et al., 2006). *E. coli* was found to have 287 essential reactions when simulated in monoculture with lactose, acetate and methionine.

To determine the validity of our genetic perturbation predictions, we compared them against previous experimental and computational analyses. Typically, perturbation studies investigate the loss of genes rather than the loss of specific reactions, as investigated in the current study. In order to compare our predictions against previous work, we mapped each reaction back to a gene. We compiled a list of 975 genes whose removal would lead to the loss of at least one metabolic reaction. Of this list, 198 genes were deemed essential based on the reactions that we found to be necessary for growth. We compared our predictions of gene essentiality against previous experimental assays of growth of *E. coli* knockout mutants in minimal media (summarized in Orth et al., 2011). We found that our predictions on essentiality of a gene matched empirical observations 90% of the time. Previous computational work—in which genes, rather than reactions, were knocked out—found a similar 91% agreement with experimental work (Orth et al., 2011).

Genetic Robustness of *E. coli* in Different Ecological Contexts

We began by examining how ecological context influenced the robustness of a single organism. *E. coli* had fewer essential reactions when competing against *S. enterica* than when cooperating with it. When *E. coli* was competing, the same set of 287 reactions were found to be essential as when the bacteria was grown in monoculture; this seems reasonable given that the initial nutrients provided were the same. During cooperation, an additional 10 reactions were essential for growth. These reactions involved sugar transport (glucose and galactose exchange), glycolytic reactions (phosphoglycerate kinase, phosphoglycerate mutase, phosphoglyerate isomerase, and enolase), and maintenance energy. Metabolites must flow through these reactions in the initial stages of consortium growth, when *E. coli* is metabolizing carbon but not yet creating biomass. In essence the reactions help "jumpstart" the metabolic exchange between *E. coli* and *S. enterica*.

Mutations in non-essential reactions created greater variance in final *E. coli* biomass when the species were competing. Mutations in reactions that were essential in any context were excluded from the analysis. The variance between *E. coli* mutants was significantly bigger when the species were competing than when *E. coli* was cooperating with *S. enterica* ($\sigma_{\text{Cooperation}} = 5.54 \times 10^{-8}$ g, $\sigma_{\text{Competition}} = 9.67 \times 10^{-8}$ g, Levene Test $P = 1.76 \times 10^{-15}$).

Cooperation Minimizes Change in the Community

After analyzing robustness of a single organism in different ecological contexts, we moved on to analyze the impact of mutations on community properties. Loss of function mutations in *E. coli* generated considerable variation in final *E. coli* and *S. enterica* biomass (**Figures 2A,B**). We quantified the amount by which each mutation altered the community by calculating a normalized Euclidean community distance (ECD, see Materials and Methods) when the species were competing or cooperating (**Figures S1**, **S2**). We first left out of the analysis simulations involving essential reactions to better quantify the impact of non-lethal mutations on altering *E. coli* and *S. enterica* biomass across each environment. Interestingly, we found that the cooperative relationship displayed significantly lower sensitivity to loss of function mutations as compared to competition ($\sigma_{\text{ECD,Cooperation}} = 3.16$ and $\sigma_{\text{ECD,Competition}} = 4.73$, Levene Test $P < 2.2 \times 10^{-16}$, **Figure 2C**). Upon including essential reactions into the analysis, we obtained similar, statistically significant trends ($\sigma_{\text{ECD,Cooperation}} = 26.7$ and $\sigma_{\text{ECD,Competition}} = 27.1$, Levene Test $P < 2.2 \times 10^{-16}$).

Species Ratios are more Robust to Genetic Perturbations in the Cooperative Systems

The percent of *E. coli* in the community was more resilient to mutation when the bacteria were cooperating (**Figure 3**). In this analysis, the final fraction (in %) of *E. coli* was standardized to those observed in the absence of perturbation in each growth condition. Mutations had a significantly smaller impact on the distribution of %E in the cooperative system than in the competitive scenario ($\sigma_{\text{\%E,Cooperation}} = 0.023$, $\sigma_{\text{\%E,Competition}} = 0.031$, Levene Test $P = 6.47 \times 10^{-11}$). This trend holds whether or not essential genes are included in the calculations (Levene Test $P < 2.2 \times 10^{-16}$).

Consistent with these observations on variance, the number of mutations found to alter the final composition of cooperative communities was also lower (**Figure 3A**). When *E. coli* was competing with *S. enterica*, 111 non-essential reactions altered species ratios by more than 1% when removed, while only 14 knockouts influenced the mutualism (**Table S1**). The reactions whose deletions cause the biggest effects tend to overlap between the two cases, and there is a correlation between the effects of the perturbations under each growth condition ($R^2 = 0.73$). In both cases, loss of oxygen utilization in *E. coli* substantially shifted the community toward a greater percentage of *S. enterica*. Other reactions with large effects on community composition were ATP synthase, galactokinase and triose-phosphate isomerase.

The Total Community Biomass Is Not Stabilized by Competition

In contrast to expectation, competition did not make biomass more resilient to genetic perturbations (**Figure 4**). The effects of mutations on community biomass were indistinguishable whether the bacteria were cooperating or competing ($\sigma_{\text{BM,Cooperation}} = 0.0127$, $\sigma_{\text{BM,Competition}} = 0.0130$, Levene Test $P = 0.0634$, **Figure 4B**). Interestingly, however, if essential reactions are taken into consideration, biomass is more stable in a competitive environment ($\sigma_{\text{BM,Cooperation}} = 0.25$, $\sigma_{\text{BM,Competition}} = 0.21$, Levene Test $P = 1.88 \times 10^{-8}$).

The number of mutations that altered biomass was higher under competition than under cooperation. In the competitive environment 104 mutations changed the total biomass by >1% relative to the unperturbed system (**Table S1**). When the two bacteria were cooperating, only 51 knockouts had an impact on final productivity. The effects of reaction removal on total biomass were highly correlated between the two scenarios ($R^2 = 0.79$). In both cases the most significant outliers were ribose-5-phosphate isomerase, ATP synthase, and O_2 exchange.

In *S. enterica*, Cooperation Stabilizes Species Ratios but Not Total Biomass

To test the generality of our findings, we also examined the impact of knocking out each reaction in the *S. enterica* model and again simulating growth of each mutant when competing and cooperating with *E. coli*. Mutations in *S. enterica* created very different distributions of final species densities than mutations in *E. coli* (**Figure 5**). Notably, *S. enterica* mutants more closely followed the expectation of cooperation maintaining the species ratios and competition maintaining total biomass.

Communities in which *S. enterica* mutants were competing with *E. coli* showed less change in our composite distance metric ECD, than did communities of mutualists ($\sigma_{\text{ECD,Cooperation}} = 0.044$, $\sigma_{\text{ECD,Competition}} = 0.028$, Levene Test $P = 0.00622$, **Figure 5B**). However, competition increased the variance in species ratios ($\sigma_{\text{\%E,Cooperation}} = 0.014$, $\sigma_{\text{\%E,Competition}} = 0.019$, Levene Test $P = 0.00386$).

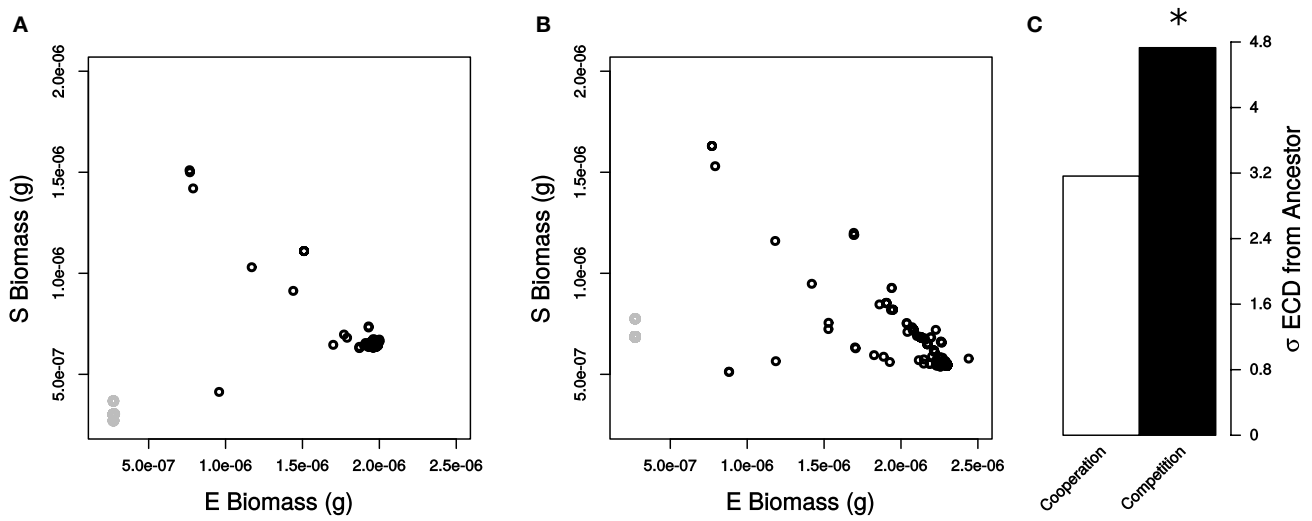

FIGURE 2 | Community state resulting from genetic perturbations in *E. coli*. Final biomass of each simulated species when bacteria were **(A)** cooperating and **(B)** competing. Non-essential reactions (black) and essential reactions (gray) are displayed in each plot. **(C)** Standard deviations of ECD values for all non-essential reactions for cooperation (white) and competition (black). Deviations in ECD were significant (star) by a Levene Test ($P < 2.2 \times 10^{-16}$).

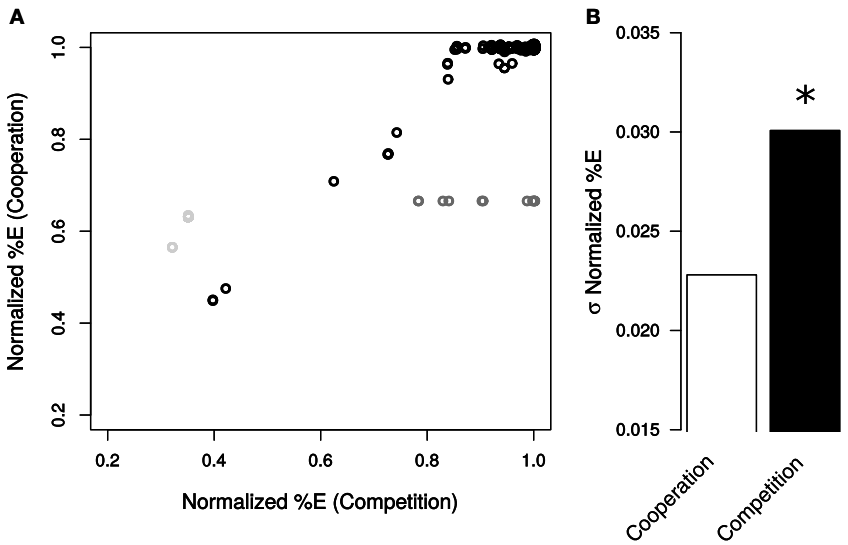

FIGURE 3 | Community composition resulting from genetic perturbations in *E. coli*. (A) Normalized %E values caused by each mutation in cooperation and competition environments. Non-essential reactions are displayed in black, essential reactions in dark gray, and reactions essential for cooperation only in light gray. **(B)** Standard deviation of normalized %E for all non-essential reactions for cooperation (white) and competition (black). Deviations in normalized %E were determined to be significant (star) by a Levene Test ($P = 6.47 \times 10^{-11}$).

Total biomass showed a different trend relative to what was observed when *E. coli* was perturbed. Loss of reactions in *S. enterica* created less biomass change in the competitive community than when the bacteria cooperated ($\sigma_{BM,Cooperation} = 0.042$, $\sigma_{BM,Competition} = 0.007$, Levene Test $P = 0.00179$). Mutations in *S. enterica* caused less variance in biomass during competition, and more variance in biomass during cooperation than mutations in *E. coli*. As a result, *S. enterica* followed the common assumption that competition should reduce the impact that perturbations have on community biomass.

Discussion

There has been a great deal of work on the robustness of genomes and parallel work on the stability of communities. However, there are scant examples of studies that link these two approaches (Klitgord and Segrè, 2010). We investigated the link between community and genetic robustness by using a novel platform that predicts community dynamics from the behavior of genome-scale metabolic networks. We found that the robustness of the *E. coli* genome changed significantly in different ecological contexts.

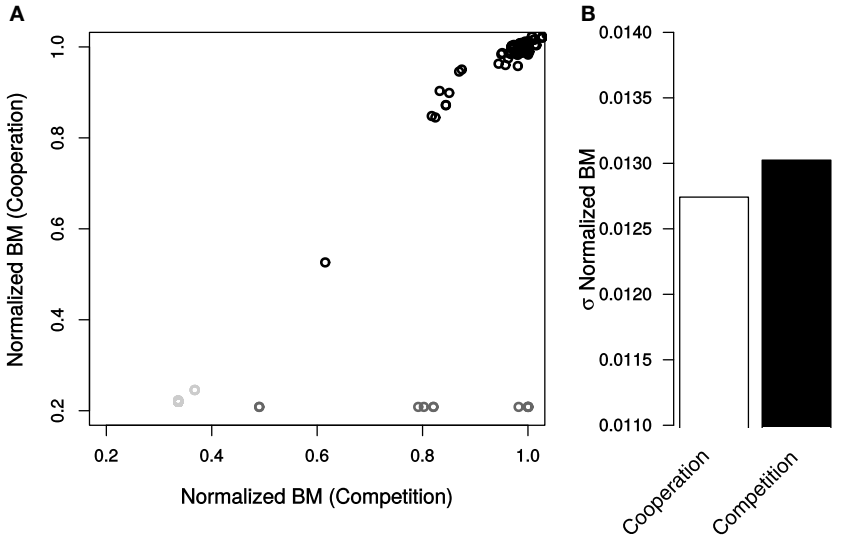

FIGURE 4 | Total community biomass resulting from genetic perturbations in *E. coli*. **(A)** Normalized BM values caused by each mutation in cooperation and competition environments. Non-essential reactions (black), essential reactions (dark gray), and reactions essential for only cooperation (light gray). **(B)** Standard deviation of normalized BM for all non-essential reactions. Deviations in normalized BM were determined to be not significant by a Levene Test ($P = 0.063$).

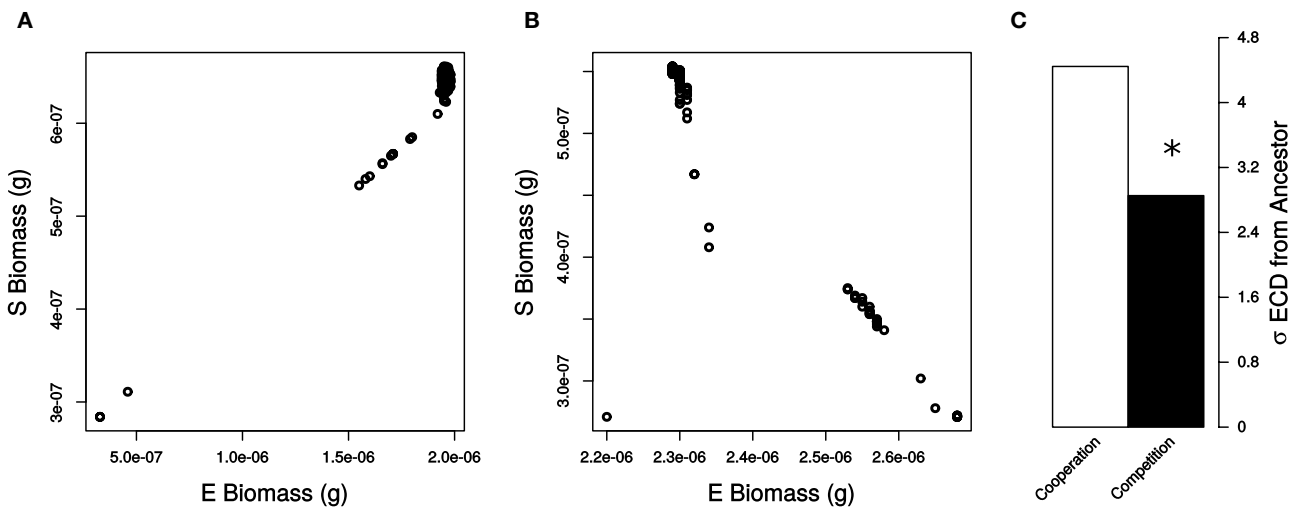

FIGURE 5 | Individual species biomass yields from COMETS simulations for (A) cooperation and (B) competition for all systematic reaction knockouts in *S. enterica* co-cultured with wild-type *E. coli*. Non-essential reactions (black) and essential reactions (gray) are displayed in each plot. **(C)** Standard deviations of ECD for non-essential reactions values for cooperation (white) and competition (black). Deviations in ECD were significant (star) by a Levene Test ($P = 0.0062$).

Perhaps more surprisingly, we found that cooperation stabilized the community against perturbations in *E. coli*. Cooperation reduced the variance in species ratios and lead to no more variance in total biomass relative to the competitive system. Interestingly, the opposite trends were observed on the community when perturbations were introduced to *S. enterica*. In this case, competition stabilized total biomass in the face of metabolic perturbations.

We found that mutations generated less variance in *E. coli* density when the bacterium was cooperating with *S. enterica* than when the two species were competing. This finding is consistent with previous work showing that genetic robustness of a genome can change in different environments (Baba et al., 2006). Furthermore, the direction of the change is reasonable. When *E. coli* is competing, mutations that slow its rate of growth should lead to a reduction in final yield, as *S. enterica* acquires more of the available resources. In contrast, when the species are cooperating *S. enterica* is less able to outpace its partner so *E. coli* mutants with slow growth can still reach a similar final density as the unperturbed strain even if it takes longer. Interestingly,

despite the difference in robustness there was still a strong correlation between the effects of knockouts in each growth condition. The correlation was substantially strengthened by the large effect mutations. One of the perturbations that had the largest effect was forcing *E. coli* to ferment. While knocking out oxygen transport is clearly not feasible, it is possible to modulate *E. coli* fermentation through ArcAB and FNR (Compan and Touati, 1994; Becker et al., 1996).

Beyond a single organism we were interested in the robustness of communities to mutation. We found that cooperative communities were robust to genetic perturbations in *E. coli*. The community state, as measured by ECD, was changed less by mutations in the cooperative system than the competitive system. This finding is consistent with the observation by both Shou et al. and Harcombe et al. that synthetic mutualisms are highly robust to starting ratios and will converge to an equilibrium community composition even from divergent initial conditions (Shou et al., 2007; Harcombe et al., 2014). However, they are in contrast to the findings by Kim et al. that precisely defined spatial structure was necessary to stabilize the species ratios in a tri-partite mutualism (Kim et al., 2008). This discrepancy in results is largely based on whether a species is capable of outcompeting its symbiotic partners. In the case that we modeled, and the cross-feeding yeast strains of Shou et al. frequency dependent dynamics ensued from the fact that species' growth rates were limited by excretions from the most rare member of the community. In contrast, in the system of Kim et al. species were able to outcompete the partners on which they relied. This lack of frequency dependence may be more common as the number of partners increases (May, 1972). Consistent with this assertion, two recent studies using randomly generated matrices of species interactions found that networks of mutualists were less stable than networks of competitors (Allesina and Tang, 2012; Mougi and Kondoh, 2012). In future work it will be informative to investigate how more complex networks of species respond to genetic perturbations.

Competition is often assumed to stabilize community productivity (Gonzalez and Loreau, 2009), however this expectation was contradicted by our observation that mutations in *E. coli* produced equivalent changes in total biomass in the two ecological contexts. Compensatory dynamics are thought to arise in competitive communities because if the abundance of one species is reduced another species will simply increase thereby maintaining biomass (though see Loreau and de Mazancourt, 2013). Our system did not behave this way in part because there was a substantial difference in the efficiency of the two species. In the competition scenario, the two species were competing over oxygen. A cell engaged in fermentation of a sugar (i.e., *E. coli*) can make far more biomass per molecule of oxygen than a cell that is consuming acetate. By constraining the ratio of *E. coli* to *S. enterica* the cooperative interaction allowed more utilization of the limiting nutrient by the more productive species. In support of this assertion if mutations in essential reactions in *E. coli* are included in the analysis then the opposite trend is observed. If *E. coli* is unable to grow, then growth of *S. enterica* increases community productivity and stabilizes biomass as generally predicted. Furthermore, if mutations are put in the less efficient *S. enterica* partner then the compensatory effects of competition are again observed. These results highlight the utility of connecting metabolic mechanisms to the study of community dynamics. Genome-scale metabolic models make it possible to determine how community dynamics will be influenced by changes in the efficiency of resource utilization.

Perturbations to *S. enterica* had a significantly different effect than mutations to *E. coli*. Part of this effect may be an artifact of model size. The *S. enterica* model contains fewer reactions (1286 vs. 2583 in *E. coli*) and therefore a different subset of mutations is being tested. Specifically, the smaller network concentrates reactions that are more directly involved in central carbon metabolism. More interestingly, the specificity of exchanged metabolites influences the robustness of the interactions. *E. coli* obtains exclusively methionine from *S. enterica*, while *S. enterica* can grow on any number of different carbon excretions. The mutations with largest effect in *E. coli* were those that altered the currency of metabolic exchange, such as galactokinase, which forced the cells to excrete unreduced forms of carbon. Supplying different carbon sources to *S. enterica* broke the constraint on species ratios in cooperation, and improved the resources available to *S. enterica* in the competitive environment. In contrast, mutations in *S. enterica* did not change the nature of the metabolic interactions in meaningful ways in either environment (i.e., mutations did not change the identity of the compound *S. enterica* provided to *E. coli*). This again highlights the utility of connecting metabolic mechanisms to species interactions to understand community behavior.

Cooperation and conflict drive dynamics in natural and engineered communities. To predict and ideally control the dynamics of these communities it will be necessary to understand how they respond to genetic perturbations. Here we investigate the connection between ecological and genetic robustness. Going forward it will be interesting to determine how evolution acts on the variation that mutation can generate in a community context. This will involve looking at the relative fitness of each mutation rather than the effects of each mutation independently. Genome-scale metabolic models provide a powerful approach for mechanistically investigating the feedback between ecological and evolutionary processes, as well as connecting the activity of genes to the function of ecosystems.

Author Contributions

WH and LC designed the experiment, analyzed the data and wrote the manuscript. BG generated data, analyzed data and helped with writing. DS helped with data analysis and writing of the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00271/abstract

Figure S1 | Histogram of ECD values for cooperation simulations.

Figure S2 | Histogram of ECD values for competition simulations.

Table S1 | Reactions identified as having >1% change in normalized %E or BM relative to the unperturbed state.

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Interactions between amphibians' symbiotic bacteria cause the production of emergent anti-fungal metabolites

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Amphibians possess beneficial skin bacteria that protect against the disease chytridiomycosis by producing secondary metabolites that inhibit the pathogen *Batrachochytrium dendrobatidis* (*Bd*). Metabolite production may be a mechanism of competition between bacterial species that results in host protection as a by-product. We expect that some co-cultures of bacterial species or strains will result in greater *Bd* inhibition than mono-cultures. To test this, we cultured four bacterial isolates (*Bacillus* sp., *Janthinobacterium* sp., *Pseudomonas* sp. and *Chitinophaga arvensicola*) from red-backed salamanders (*Plethodon cinereus*) and cultured isolates both alone and together to collect their cell-free supernatants (CFS). We challenged *Bd* with CFSs from four bacterial species in varying combinations. This resulted in three experimental treatments: (1) CFSs of single isolates; (2) combined CFSs of two isolates; and (3) CFSs from co-cultures. Pair-wise combinations of four bacterial isolates CFSs were assayed against *Bd* and revealed additive *Bd* inhibition in 42.2% of trials, synergistic inhibition in 42.2% and no effect in 16.6% of trials. When bacteria isolates were grown in co-cultures, complete *Bd* inhibition was generally observed, and synergistic inhibition occurred in four out of six trials. A metabolite profile of the most potent co-culture, *Bacillus* sp. and *Chitinophaga arvensicola*, was determined with LC-MS and compared with the profiles of each isolate in mono-culture. Emergent metabolites appearing in the co-culture were inhibitory to *Bd*, and the most potent inhibitor was identified as tryptophol. Thus mono-cultures of bacteria cultured from red-backed salamanders interacted synergistically and additively to inhibit *Bd*, and such bacteria produced emergent metabolites when cultured together, with even greater pathogen inhibition. Knowledge of how bacterial species interact to inhibit *Bd* can be used to select probiotics to provide amphibians with protection against *Bd*.

Keywords: *Batrachochytrium dendrobatidis*, emergent metabolites, amphibians, symbiotic bacteria, anti-fungal metabolites, host-associated microbial communities, interspecific competition, synergy

INTRODUCTION

Amphibians are experiencing global population declines and are the most threatened vertebrate class (Stuart et al., 2004; Hoffmann et al., 2010). Indeed, 41 percent of amphibian species are threatened. Among the many factors that contribute to amphibian declines, the fungal disease chytridiomycosis, caused by the pathogen *Batrachochytrium dendrobatidis* (*Bd*), is the largest disease threat to the biodiversity of any vertebrate group (Wake and Vredenburg, 2008; Hoffmann et al., 2010). Alarmingly, disease-induced declines occur in protected, pristine environments, such as national parks, where up to 92.5 percent of critically endangered amphibian species are affected by *Bd* (Kilpatrick et al., 2010).

Amphibians have several defenses against *Bd*, including symbiotic bacteria living on their skins (Harris et al., 2006). Numerous anti-fungal bacterial species have been cultured from amphibians (Lauer et al., 2007; Woodhams et al., 2007), and their presence is associated with disease resistance (Becker and Harris, 2010). Additionally, using culture-independent techniques, diverse host-specific bacterial communities have been observed on amphibians (McKenzie et al., 2011; Kueneman et al., 2013; Loudon et al., 2013; Walke et al., 2014). One promising mitigation strategy against *Bd* is to augment the bacterial communities on amphibians' skin to increase disease resistance (Harris et al., 2009b; Bletz et al., 2013). This approach of using bacterial probiotics has been successful in laboratory experiments and one field trial (Becker et al., 2009; Vredenburg et al., 2011).

Amphibian-associated bacteria can inhibit *Bd* by producing anti-fungal metabolites (Brucker et al., 2008a,b). Several anti-*Bd* metabolites are produced from amphibian skin bacteria, such as violacein and indole-3-carboxaldehyde (I3C) from *Janthinobacterium lividum* and 2,4-diacetylphloroglucinol is produced *Lysobacter gummosus* (Brucker et al., 2008a,b). Additionally, evidence from free-living salamanders in nature suggests that the concentration of bacterially produced metabolites on their skins can be high enough to kill pathogenic fungi,

such as *Bd* (Brucker et al., 2008b). Moreover, a positive correlation was found between the abundance of the amphibian symbiont *J. lividum* and violacein on red-backed salamanders; salamanders with more *J. lividum* and violacein had less morbidity due to chytridiomycosis (Becker et al., 2009).

A recent model on the assembly of beneficial microbiomes suggests that the microbiota's defensive function is a by-product of interspecific competition among microbial species (Scheuring and Yu, 2012). If microbial population densities reach the point where competition occurs and enough energy is available, defensive chemical production can be triggered that may defend the host. Without conditions of interspecific competition (pure culture), a weaker defensive function may occur. For example, bacteria cultured separately have been found to not produce anti-fungal compounds, but do produce anti-fungal compounds when cultured together (De Boer et al., 2007). In this study, we tested the hypothesis that conditions that favor interspecific competition will result in greater inhibition of *Bd* than under conditions of no interspecific competition. We hypothesized that greater inhibition of *Bd* would be caused by the production of emergent metabolites (i.e., metabolites produced in co-culture but not produced in mono-culture) that are only found with interspecific competition.

MATERIALS AND METHODS

COLLECTION AND IDENTIFICATION OF ANTI-BD BACTERIAL ISOLATES

The bacterial isolates in this study were cultured from red-backed salamanders (*Plethodon cinereus*) that were sampled from George Washington National Forest in October, 2011. Four bacterial isolates were chosen because they inhibit *Bd* in mono-culture. The bacterial isolates were maintained in culture in 16 × 125 mm test tubes with approximately 2 mL 1% tryptone broth and incubated in a shaking incubator at 25°C. Using traditional PCR, a portion of the 16S rRNA gene was amplified using the universal 8F and 1492R primers. Reaction conditions are given in Lauer et al. (2007). The DNA was sequenced (EuroFins MWG Operon; Huntsville, Alabama), and Genious (Biomatters Limited; Auckland, New Zealand) was used to assemble the forward and reverse sequences. The GenBank database was used to identify each bacterial isolate.

COLLECTION OF ANTI-BD BACTERIAL SPECIES' CELL-FREE SUPERNATANTS

To test bacterial isolates for *Bd* inhibition, we used a 96-well plate assay from Bell et al. (2013). Each bacterial species was re-cultured into a sterile 16 × 125 mm test tube containing 2 mL of 1% tryptone broth for 3 days and placed in an incubator at 26°C prior to the assay. Therefore, each culture had the same nutrient and environmental conditions for the same amount of time as a way to standardize growth conditions. After 3 days, cultures were noticeably turbid. To acquire cell-free supernatants (CFSs), each bacterial isolate was placed in a centrifuge tube and then homogenized with a vortexer. The bacterial isolates were then centrifuged for 5 min at 10,000 rpm. After centrifugation, the supernatants were filtered using a 0.22 μm filter (Millipore™ nitrocellulose membrane), and these solutions contained bacterial metabolites as well as other cell products. This starting solution was designated as the "high" concentration. "Medium" and "low" concentrations were 10- and 100-fold dilutions, respectively, of the "high" concentration, using 1X phosphate-buffered saline solution.

DETERMINATION OF THE INDIVIDUAL AND COMBINED EFFECTS OF ANTI-BD BACTERIAL ISOLATES ON BD INHIBITION

A concentration series of each isolate's CFS, alone and in combination, was used in pairwise combinations with *Bd* in a 96 well plate assay. All wells contained 50 μL of *Bd* zoospores at 2×10^6 zoospores/mL in 1% tryptone broth (10^5 zoospores/well). *Bd* zoospores were collected using the protocol of Harris et al. (2009b). To test the inhibitory effects of a single isolate, we added 25 μL CFS and 25 μL water to each well. To test the potential additive effects of two bacterial isolates grown separately, we added 25 μL of each isolate's CFS to each well. We called the latter treatment "separate then combined." Each of these treatments was replicated five times. Positive control wells received 50 μL of sterile water without any CFSs. Negative control wells received 50 μL of heat killed *Bd* zoospores (2×10^6 zoospores/mL), which were killed by placing them at 60°C for 20 min. Each of these treatments was replicated five times. Optical density (OD) readings at 490 nm were obtained at day 0 and day 5. The change in OD between days 0 and 5 indicated the amount of *Bd* growth in each well, with greater differences reflecting more *Bd* growth.

DETERMINATION OF THE EFFECTS OF INTERSPECIFIC COMPETITION ON BD INHIBITION

To assess the effects of interspecific bacterial competition on *Bd* inhibition, bacterial species were cultured in paired combinations for 3 days prior to assays with *Bd*. For these co-culture treatments, 5 μL of the original culture of each bacterial species was placed into a test tube containing 2 mL of 1% tryptone broth. Monocultures and additional "separate then combined" assays were done using the procedures stated above as comparisons. Bacterial culturing conditions were the same as the previous assay. The co-culture wells contained 50 μL of the CFS from paired combinations of bacterial species, and each treatment was replicated five times.

DETECTION AND ISOLATION OF EMERGENT METABOLITES

CFC's of monocultures and co-cultures of all isolates were extracted with ethyl acetate according to the method of Brucker et al. (2008a). The *Bacillus* sp. and *Chitinophaga arvensicola* combination was focused on since they resulted in the greatest *Bd* inhibition in co-culture. The resulting crude extracts were evaporated to dryness *in vacuo*, reconstituted in 0.75 mL methanol, and analyzed by reversed-phase, high performance liquid chromatography (HPLC, 25 μL injection) using a Shimadzu LC-20 liquid chromatograph equipped with an ACE C18 column (3 μm, 150 × 4.6 mm) according to the method of Umile et al. (2014). Comparison of the chromatograms showed the appearance of at least six metabolites in the co-culture that were not present in either monoculture (**Figure 3**). Metabolite peaks that were observed in co-cultures, but not present in either of the two individual cultures, were identified as emergent metabolites. These metabolites were isolated by semi-preparative HPLC [ACE C18

column (5 μm, 250 × 10 mm)] and assayed for bioactivity toward *Bd* (see below). To test this, each fraction was suspended in 5% DMSO solution, to facilitate the dissolution of metabolites. 10 μL of each metabolite solution was added to 40 μL of a *Bd* zoospore solution resulting in a final concentration of 10^6 zoospores/mL. The final concentration of each metabolite was ≈2 μg/mL. In addition, tests were performed without the use of DMSO as a co-solvent with low concentrations of each metabolite that were not quantified. These tests suggested that the compound eluting at 13.48 min ("Compound 3") was the most inhibitory (**Figure 4**).

CHARACTERIZATION AND IDENTIFICATION OF "COMPOUND 3"

An inhibitory compound eluting at 13.48 min ("Compound 3") was isolated using the semi-preparative HPLC described above and analyzed by ^{1}H NMR and liquid chromatography-mass spectrometry (LC-MS). ^{1}H NMR spectra were measured with a 300 MHz Varian spectrophotometer. UV-Vis measurements utilized a Shimadzu SPD-M20A diode array detector, and mass spectrometry used an Applied Biosystems SCIEX API 2000 triple quadrupole mass spectrometer operating in positive electrospray ionization mode.

BIOASSAY WITH METABOLITE AND BD

The IC_{50} for the emergent metabolite, tryptophol, against *Bd* strain JEL310 was determined using a 96 well plate method assay previously developed by Brucker et al. (2008a).

STATISTICAL ANALYSES

Analyses of variance (ANOVA) were used to test for the main effects of species' CFS on *Bd* inhibition. A significant interaction effect indicated that either synergy or antagonism between species' CFS was present. Dunnett's test was used for a posteriori tests to compare treatments to controls, and Tukey's test was used to compare each treatment to each other to identify an interaction effect (synergy or antagonism) when one was present.

RESULTS

BACTERIAL IDENTIFICATION

Isolates were determined to be a *Bacillus* sp., *Janithobacterium* sp., *Pseudomonas* sp., and *Chitinophaga arvensicola* (**Table 1**; sequences Genbank accession #'s KJ923801, KJ923802, KJ923803 and KJ923804 respectively). The genera of all four isolates were represented within the Illumina dataset presented in Loudon et al. (2013). In addition, OTUs within the family Pseudomonadaceae were within the core bacterial community and *Janthinobacterium lividum* was highly prevalent on red-backed salamanders.

"SEPARATE THEN COMBINED" ASSAYS

To determine the effect of combined bacterial isolates on *Bd* inhibition we used a "separate then combined" assay where isolates were first grown in mono-culture, and then combined together to challenge *Bd*. The six combinations of the four bacterial species' CFSs demonstrated several effects on the growth of *Bd* in comparison to the positive *Bd* control (Supplementary Table 1). The "separate then combined" results for *Pseudomonas* sp. and *Chitinophaga arvensicola* were not included due to failure of the positive controls; this resulted in 45 "separate then combined" combinations. Of the 45 combinations assayed, some produced an additive effect when assayed with *Bd*, some synergistically inhibited the growth of *Bd* in comparison to the positive control, and others had no effect on *Bd* growth.

Table 1 | Identities of anti-*Bd* bacterial isolates used in inhibition assays.

Identity	% match	*E*-value	Accession number
Bacillus sp.*	100	0	KJ923801
Pseudomonas sp.*	100	0	KJ923802
Janthinobacterium sp.*	99	0	KJ923803
Chitinophaga arvensicola	99	0	KJ923804

*Isolates were only identified to the Genus level.

Of the total of 45 isolate-CFS concentration combinations, 19 of these exhibited an additive relationship when assayed against *Bd*. These pairs showed an effect on *Bd* growth equal to the sum of the effects of the CFSs of each species. One example of an additive relationship was between the medium concentration of *Bacillus* sp. CFS and the low concentration of *Janthinobacterium* sp. CFS (**Figure 1A**). There was a tendency of high concentration combination of both isolates to have additive effects as well.

There were 19 isolate-CFS concentration combinations that inhibited *Bd* synergistically. These combinations inhibited *Bd* with a greater total effect than the additive relationship. Concentration combinations were identified as synergistic if their statistical interactions were significant. For example, the combinations of the medium concentration of *Bacillus* sp. CFS with the medium concentration of *Pseudomonas* sp. resulted in synergy (**Figure 1B**).

CO-CULTURE ASSAYS

The CFSs from co-cultured bacterial isolates showed greater inhibition of *Bd* than the "separate then combined" CFSs in some cases. Out of the six co-culture combinations, four resulted in synergistic inhibition of *Bd*, one resulted in additive inhibition of *Bd*, and one resulted in synergistic facilitation of *Bd* (Supplementary Table 2). The co-cultured combinations of *Bacillus* sp./*Janthinobacterium* sp., *Bacillus* sp./*Pseudomonas* sp., *Bacillus* sp./*C. arvensicola* (**Figure 2**) and *Janthinobacterium* sp./*C.* synergistically inhibited *Bd*. The co-culture combination of *Pseudomonas* sp./*C. arvensicola* additively inhibited *Bd.* Lastly, the combination *Pseudomonas* sp./*Janthinobacterium* sp. synergistically facilitated *Bd*.

The "separate then combined" assays that were repeated for a comparison with the co-culture assays sometimes differed from initial "separate then combined" trials. For the *Bacillus* sp./ *Pseudomonas* sp. combination, the "separate then combined" treatments from the two different trials were significantly different between trials. These comparisons were performed after standardizing the OD values by the positive controls from the two different assays.

EMERGENT METABOLITES

The co-culture between *Bacillus* sp. and *Chitinophaga arvensicola* produced seven isolated emergent metabolites (**Figure 3**) that

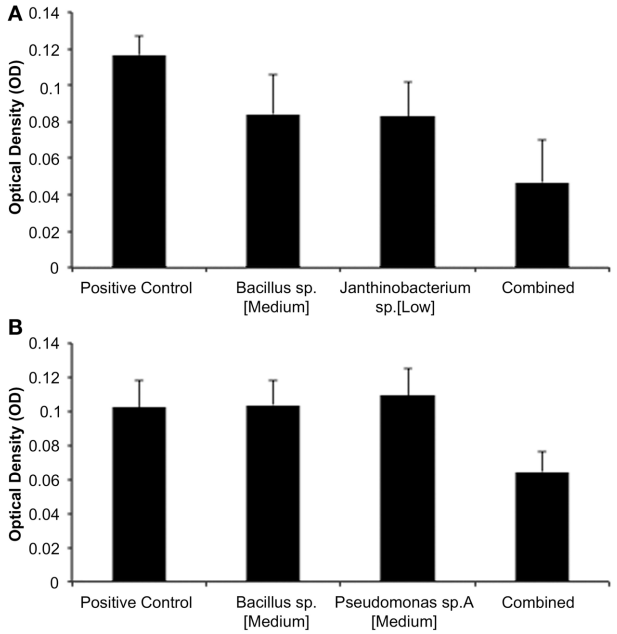

FIGURE 1 | (A,B) Representative results from "separate then combined" assays. **(A)** Additive *Bd* inhibition with a medium concentration of *Bacillus* and *Janthinobacterium* (ANOVA- *Bacillus* $p = 0.0011$; *Janthinobacterium* $p = 0.0008$; Interaction $p = 0.8277$). **(B)** Synergisytic inhibition of *Bd* with a medium concentration of *Bacillus* and *Pseudomonas* sp. *A* (ANOVA- *Bacillus* $p = 0.0512$; *Pseudo A* $p = 0.0239$; Interaction $p = 0.0025$).

were all inhibitory to *Bd* growth at an approximate concentration of 2 μg/mL. An additional test that did not use DMSO as a co-solvent and using low unquantified metabolite concentrations revealed that three compounds were inhibitory, with Compound 3 being the most inhibitory (**Figure 4**).

Compound 3 was identified as tryptophol by multiple chemical methods (UV-Vis spectroscopy, mass spectrometry, and ^{1}H NMR spectroscopy). Accordingly, isolated Compound 3 and an authentic tryptophol standard co-elute (13.48 min) and share a characteristic indole chromophore with peaks at 280 and 289 nm (**Figure 5**). Additionally, ESI-MS of a tryptophol standard and Compound 3 both show a characteristic 144 m/z peak corresponding to an $[M—H_2O]^+$ ion; finally, the ^{1}H NMR spectrum of isolated Compound 3 matches that of authentic tryptophol. Authentic tryptophol inhibited *Bd* (**Figure 6**) and displayed an IC_{50} of ∼100 ppm (∼600 μM).

The metabolite profiles for *Pseudomonas* sp. and *Janthinobacterium* sp. were also examined since this combination resulted in *Bd* facilitation. *Janthinobacterium* sp. had three metabolites that were present in the mono-culture that were not present in the co-culture; therefore, *Bd* was challenged with a different suite of metabolites than when challenged with the mono-cultures.

DISCUSSION

Combinations of bacterial isolate's CFSs exhibited several different relationships in the inhibition of *Bd*, demonstrating that the isolates, and the concentrations of an isolate's CFSs, are important factors in *Bd* inhibition. In some cases, the inhibition caused by two isolates CFSs had an additive effect on *Bd* growth inhibition. Other combinations inhibited *Bd* growth synergistically. Some combinations did not demonstrate any effect on *Bd* growth.

The *in vitro* model of combining CFSs can represent spatially separated isolates on amphibian skin whose secreted metabolites combine in the interstices between them. The different relationships among the pairwise combinations between the four isolates in high, medium and low concentrations demonstrate the variability of bacterial interactions that are likely found in nature. It is unknown how isolates are distributed on amphibian skins, much less their relative population densities, but it is likely to be variable even on a single host individual. In an additive relationship, the metabolites of both bacterial isolates are contributing equally to the inhibition of *Bd* growth. Synergistic relationships may be the result of anti-fungals with different modes of action (Ghannoum and Rice, 1999). As a hypothetical example, in our system a metabolite from the first bacterial isolate may puncture the cell wall of *Bd*, whereas the metabolites from the second bacterial isolate may inhibit translation.

Emergent metabolites were identified that were present in bacterial co-cultures, but not present in bacterial mono-cultures. CFSs from co-cultures were often more inhibitory of *Bd* than combining CFSs from mono-cultures. Co-culturing is a model of isolates on the skins that are in the same or close spatial location and are competing with each other by starting to produce additional inhibitory metabolites, and these new metabolites are one reason for greater inhibition of *Bd*. Furthermore, emergent metabolites may be synergistically interacting with the metabolites produced in mono-culture and other emergent metabolites to enhance their collective killing power. We determined that these emergent metabolites are anti-*Bd* and determined the identity and IC_{50} of the most inhibitory metabolite, tryptophol. Although it is inhibitory, it is much less potent than previously identified anti-*Bd* metabolites such as violacein (Brucker et al., 2008b). Tryptophol has been found to be produced by fungi such as *Ceratocystis adipose* (Guzmán-López et al., 2007), *Candida albicans* (Lingappa et al., 1969), *Pythium ultimum* (Rey et al., 2001), *Drechslera nodulosum* (Sugawara and Strobel, 1987), and *Zygosaccharomyces priorianus* (Rosazza et al., 1973). The mechanism that tryptophol uses to inhibit *Bd* is unknown. We hypothesize that these emergent metabolites are interacting to cause the greater *Bd* inhibition found in the co-culture assays. The strong inhibition of *Bd* caused by the co-culture CFSs supports the hypothesis that interspecific competition led to greater *Bd* inhibition than intraspecific competition alone (De Boer et al., 2007; Scheuring and Yu, 2012). Furthermore, it suggests that competing microbial species in amphibian skins would enhance disease protection.

One case of facilitation occurred in co-culture between *Pseudomonas* sp. and *Janthinobacterium* sp. This result is intriguing since when the bacteria were grown separately and then combined, they did not result in facilitation. Previous work

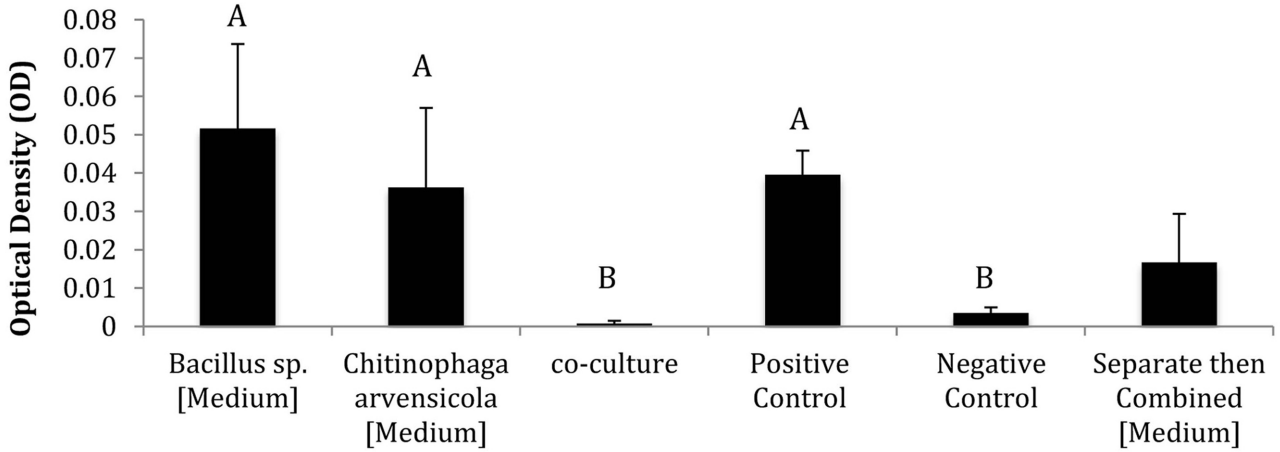

FIGURE 2 | Representative results of co-culture vs. mono-culture assay using *Bacillus* sp. and *Chitinophaga arvensicola* (Different letters indicate that treatments are significantly different at $p < 0.05$ level using Tukey's test). The "separate then combined" results for this isolate combination are also included as a comparison.

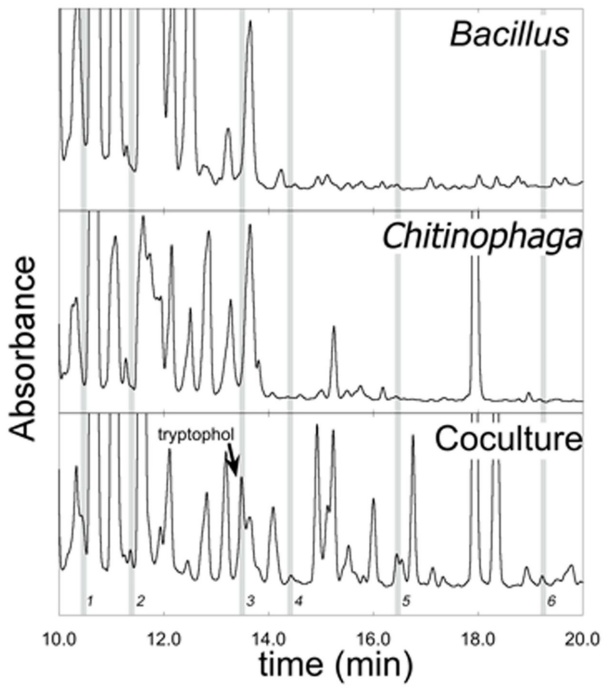

FIGURE 3 | LC-MS chromatograms of ethyl acetate extracts from monocultures of *Bacillus* and *Chitinophaga arvensicola* and a co-culture of the two. Gray bars indicate emergent metabolites found in the co-culture that were not present in either monoculture. (Absorbance axes not to common scale.) Metabolites are labeled 1–6; metabolite 7 has a retention time greater than what is shown in this figure and is therefore not present.

using a similar protocol to determine bacteria's effect on *Bd* found facilitation of *Bd* growth by some isolate's CFSs (Bell et al., 2013). *Pseudomonas* can produce a number of antifungal metabolites and *Janthinobacterium* has been widely studied as an anti-*Bd* bacterium, so facilitation of *Bd* was surprising. In our study, the co-culture lacked metabolites that were found in the *Janthinobacterium* mono-culture. This shift in metabolites may have led to the facilitation of *Bd*.

When the "separate then combined" assays were repeated, the results differed to some extent between trials. In particular, synergy between certain pairs of isolates in the first experiment was not always found in the second experiment. A possible reason for this is that isolates were under somewhat different densities in culture due to stochastic variation. As a result, isolates produced different concentrations of metabolites that then interacted differently when combined. An additional reason may be the age of the media used or the use of different batches of media that had slight chemical differences. Also, it is possible that mutation in laboratory stocks led to different outcomes in the assays. However, all of the results demonstrate that additive and synergistic interactions occur among amphibians' skin bacteria, which depend on a variety of factors, including bacterial species identify and chemical concentration.

The inhibitory interactions between different bacterial species are not the only chemical interactions that inhibit *Bd* within the amphibian skin system. Myers et al. (2012) found synergistic interactions between the antimicrobial peptides (AMPs) from the skin of the frog, *Rana muscosa*, and the known anti-*Bd* metabolite 2,4-diacetylphloroglucinol (2,4-DAPG), which is produced by the bacterium *Pseudomonas fluorescens*. It is likely that AMPs interact with other bacterially-produced metabolites to inhibit *Bd*, especially since we found that bacteria produce a high richness of metabolites. Bacteria also produce AMPs that may inhibit *Bd*, although we focused on smaller metabolites that are produced by bacteria.

Our results support the hypothesis that competition between different bacterial species causes greater *Bd* inhibition. Bacterial species compete through the production of defensive compounds, which leads to their competitive dominance. Therefore, the host obtains a defensive benefit as a by-product of microbial

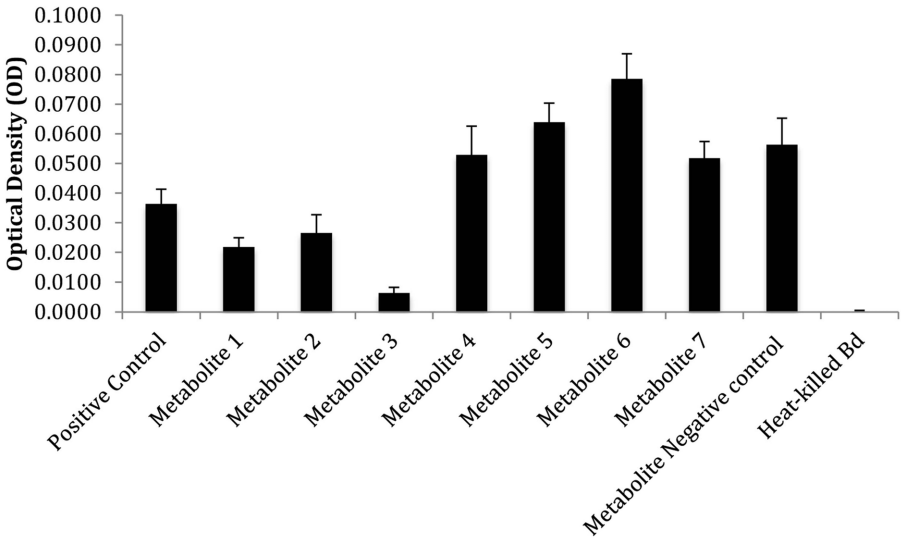

FIGURE 4 | Inhibitory properties of emergent metabolite fractions 1–7 at low unquantifiable concentrations. An additional control, "metabolite negative control," was also included, which was a fraction that contained no metabolites. The bars reflect standard deviation.

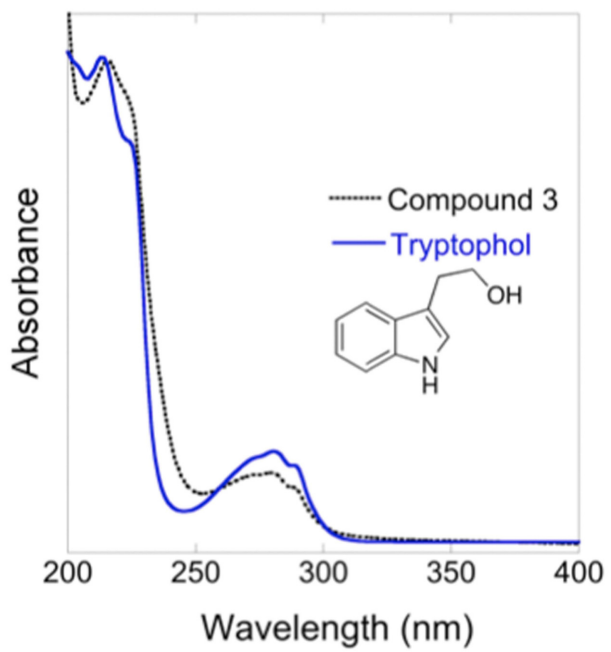

FIGURE 5 | UV-Vis spectra of isolated Compound 3 and an authentic tryptophol standard.

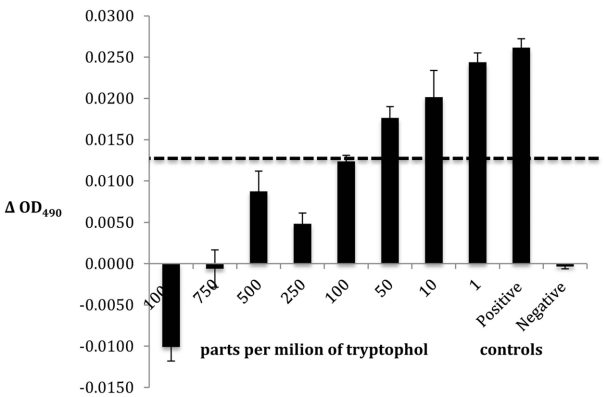

FIGURE 6 | *Bd* challenge assay to determine the IC_{50} of the emergent metabolite tryptophol. The bars reflect standard deviation.

competition. Results from theory suggest that beneficial, antibiotic-producing bacteria are favored over bacteria that do not produce such compounds as long as the host supplies adequate resources for antibiotic production (Scheuring and Yu, 2012). Although we expect that competition has driven the production of emergent metabolites and an overall greater inhibitory function, competition was not measured, and it is possible that other interactions, such as cooperation, led to our results.

This work reveals the complexity of microbial interactions and emphasizes the importance of considering such interactions when developing a bacterial probiotic as a conservation strategy. Furthermore, our results suggest that a bacterial probiotic with multiple bacterial species is more promising than a bacteria probiotic with a single bacterial species. Although a single trial to test bacterial probiotics with multiple bacterial species was unsuccessful on amphibians (Bletz et al., 2013), more work is needed to fully assess the potential of such therapy. Probiotic trials with a single isolate have been successful in some cases (Harris et al., 2009a,b), but not in others (Becker et al., 2011; Bletz et al., 2013). This variation could be explained by the probiotic interacting synergistically with the resident microbiota to inhibit *Bd* in the successful cases, but not synergistically inhibiting

Bd or even facilitating *Bd* the unsuccessful cases. Bacterial probiotics with multiple bacterial species have been successful in mouse intestines (Lawley et al., 2012). In this study, the successful combination of bacteria used in the probiotic was selected by trial and error. This strategy was successful, however not efficient. It is likely important to choose bacteria that will compete interspecifically and produce more inhibitory metabolites. We propose choosing bacteria that display additive or synergistic relationships when in combination with other isolates over a range of concentrations and be highly inhibitory when co-cultured. If a bacterial isolate does not have an effect on *Bd* growth and does not produce any effect when combined with a second bacterial isolate, then it should be excluded from further consideration as part of a probiotic solution. To test whether the addition of multiple probiotics on amphibians worked *in vivo*, the defensive function of the mucosome could be measured, which would determine if the chemicals on an amphibian are anti-fungal prior to infection by a pathogen (Woodhams et al., 2014). Our results provide support for the use of a co-culture method in probiotic development that may lead to a more effective probiotic solution.

In conclusion, we found that metabolites produced by amphibians' skin bacteria in monoculture can be combined, and they can interact synergistically with each other to inhibit *Bd*. In addition, we found that when these bacteria were co-cultured they were often even more inhibitory to *Bd*, and they produced emergent metabolites that were not made by the bacteria when in monoculture. Furthermore, we identified the emergent metabolite tryptophol, which was the most active emergent metabolite against *Bd*. These results will aid in developing combination of probiotics to mitigate the amphibian disease chytridiomycosis, in addition to contributing to our knowledge of bacterial and chemical interactions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2014.00441/abstract

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