



A Timed Off-Switch for Dynamic Control of Gene Expression in *Corynebacterium Glutamicum*

Daniel Siebert^{1,2}, Josef Altenbuchner³ and Bastian Blombach^{1,2*}

¹ Microbial Biotechnology, Campus Straubing for Biotechnology and Sustainability, Technical University of Munich, Straubing, Germany, ² SynBiofoundry@TUM, Technical University of Munich, Straubing, Germany, ³ Institute of Industrial Genetics, University of Stuttgart, Stuttgart, Germany

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*Correspondence:

Bastian Blombach
bastian.blombach@tum.de

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Dynamic control of gene expression mainly relies on inducible systems, which require supplementation of (costly) inducer molecules. In contrast, synthetic regulatory circuits, which allow the timed shutdown of gene expression, are rarely available and therefore represent highly attractive tools for metabolic engineering. To achieve this, we utilized the VanR/P_{vanABK}* regulatory system of *Corynebacterium glutamicum*, which consists of the transcriptional repressor VanR and a modified promoter of the *vanABK* operon (P_{vanABK}*). VanR activity is modulated by one of the phenolic compounds ferulic acid, vanillin or vanillic acid, which are co-metabolized with D-glucose. Thus, gene expression in the presence of D-glucose is turned off if one of the effector molecules is depleted from the medium. To dynamically control the expression of the *aceE* gene, encoding the E1 subunit of the pyruvate dehydrogenase complex that is essential for growth on D-glucose, we replaced the native promoter by *vanR/P_{vanABK}** yielding *C. glutamicum* ΔP_{aceE}::*vanR-P_{vanABK}**. The biomass yield of this strain increased linearly with the supplemented amount of effector. After consumption of the phenolic compounds growth ceased, however, *C. glutamicum* ΔP_{aceE}::*vanR-P_{vanABK}** continued to utilize the residual D-glucose to produce significant amounts of pyruvate, L-alanine, and L-valine. Interestingly, equimolar concentrations of the three phenolic compounds resulted in different biomass yields; and with increasing effector concentration, the product spectrum shifted from pyruvate over L-alanine to L-valine. To further test the suitability of the VanR/P_{vanABK}* system, we overexpressed the L-valine biosynthesis genes *ilvBNCE* in *C. glutamicum* ΔP_{aceE}::*vanR-P_{vanABK}**, which resulted in efficient L-valine production with a yield of about 0.36 mol L-valine per mol D-glucose. These results demonstrate that the VanR/P_{vanABK}* system is a valuable tool to control gene expression in *C. glutamicum* in a timed manner by the cheap and abundant phenolic compounds ferulic acid, vanillin, and vanillic acid.

Keywords: *Corynebacterium glutamicum*, dynamic expression control, lignin, L-valine production, ferulic acid, vanillin, vanillic acid, pyruvate dehydrogenase complex

INTRODUCTION

Controlling gene expression is one of the key tasks to manipulate the metabolism of a host cell for application in a biotechnological process. Regarding the targeted induction of gene expression in prokaryotes, several systems are well-established and widely applied, such as LacI/P_{tac}, AraC/P_{araBAD}, PrpR/P_{prp}, RhaR-RhaS/P_{rhaBAD}, and T7 RNA polymerase-based versions (Lee and Keasling, 2005; Terpe, 2006; Brautaset et al., 2009). While these systems are usually easy to handle and well-suited for lab scale studies, their application in the industrial environment is often hampered by, e.g., the price or availability of the effector molecule (Ferreira et al., 2018; Cardoso et al., 2020). Moreover, since most effector molecules show a high stability and cannot easily be removed from the culture broth, dynamic control of gene expression is still challenging and therefore subject of recent studies in different organisms (Jayaraman et al., 2018; Baumschlager et al., 2020; Wiechert et al., 2020; Glasscock et al., 2021).

Corynebacterium glutamicum is a Gram-positive facultative anaerobic organism that grows on a variety of sugars, organic acids, and phenolic compounds as single or combined carbon and energy sources (Eggeling and Bott, 2005; Merckens et al., 2005; Nishimura et al., 2007; Takeno et al., 2007; Becker and Wittmann, 2019). The organism is regarded as powerhouse for large-scale production of amino acids (if mentioned here, always the L-form is meant), such as glutamate and lysine at a level of 6 million tons per year (Becker et al., 2018). Moreover, many studies exploited *C. glutamicum* for the production of commodity chemicals, such as the biofuels isobutanol, ethanol, and *n*-propanol (Inui et al., 2004; Blombach and Eikmanns, 2011; Blombach et al., 2011; Yamamoto et al., 2013; Siebert and Wendisch, 2015; Lange et al., 2018; Hasegawa et al., 2020), the diamines cadaverine and putrescine (Kind and Wittmann, 2011; Wendisch, 2017), and other amino acids such as histidine (Schwentner et al., 2019) and valine (Oldiges et al., 2014; Schwentner et al., 2018). This impressive progress in metabolic engineering of *C. glutamicum* is based on wealth of knowledge about the central metabolism, physiology, and regulation of relevant pathways, and the development of systems biology approaches and genetic engineering tools (Eggeling and Bott, 2005; Burkovski, 2008; Yukawa and Inui, 2013; Inui and Toyoda, 2020; Wang et al., 2021). Although numerous tools to manipulate the metabolism of *C. glutamicum* have been developed and standard heterologous inducible promoter systems are established and optimized (Goldbeck and Seibold, 2018; Gauttam et al., 2019), the set of available native promoters to drive synthetic and dynamic expression control is still small (Wiechert et al., 2020). Recently, Wiechert et al. (2020) constructed a GntR-dependent metabolic toggle switch that is regulated by the effector gluconate and can be applied to dynamically control gene expression in *C. glutamicum* (Wiechert et al., 2020).

Corynebacterium glutamicum is able to grow on the lignin-derived phenolic compounds ferulic acid (FA), vanillin (Van), and vanillic acid (VA) and, compared with other microbial systems, is less susceptible to growth inhibition by these molecules (Shen et al., 2012; Ding et al., 2015; Becker and

Wittmann, 2019). The degradation of Van and VA proceeds *via* the vanillate pathway yielding protocatechuic acid (PCA), which is subsequently converted by the β -ketoacid pathway to acetyl-CoA and succinyl-CoA entering eventually the tricarboxylic acid (TCA) cycle [Figure 1 (Merckens et al., 2005; Brinkrolf et al., 2006; Shen et al., 2012; Kallscheuer et al., 2016; Okai et al., 2017)]. Within the vanillate pathway, FA might be converted in a cascade *via* Van and VA to PCA (Merckens et al., 2005; Brinkrolf et al., 2006; Shen et al., 2012). However, FA utilization essentially requires the action of the β -oxidative deacetylation pathway yielding VA [Figure 1 (Kallscheuer et al., 2016)]. The vanillate utilization genes are transcribed as *vanABK* operon from the promoter P_{vanABK}, which is under control of the PadR-like repressor VanR. This transcriptional regulator is *in vitro* inhibited only by VA but not by FA, Van, or PCA; whereas *in vivo* the presence of FA, Van, and VA leads to a de-repression of the *vanABK* operon [Figure 1 (Morabbi Heravi et al., 2015)]. The crystal structure of VanR of *C. glutamicum* was recently elucidated (Yao et al., 2020) and the VanR operator sequence, which is located downstream of the P_{vanABK}-10 region, has been identified (Morabbi Heravi et al., 2015).

In this study, we utilized the VanR/P_{vanABK} regulatory system to dynamically control gene expression in *C. glutamicum*. As a proof of concept, we show that the expression of the *aceE* gene, encoding the E1 subunit of the pyruvate dehydrogenase complex (PDHC), can be switched off in a timed manner, which was achieved by replacing the native *aceE* promoter with a modified VanR/P_{vanABK} system (subsequently indicated as VanR/P_{vanABK}*). This dynamically controlled off-switch essentially relies on the utilization of one of the compounds, FA, Van, or VA (Figure 1), and was applied to control biomass formation and to induce the production of pyruvate and its derived products alanine and valine.

MATERIALS AND METHODS

Microorganisms, Media, and Cultivation Conditions

All *C. glutamicum* strains and plasmids used in this study are given in Table 1. The *Escherichia coli* strain DH5 α (Hanahan, 1983) was used as shuttle organism for all cloning purposes. For cultivation of *E. coli* as well as for the precultures of *C. glutamicum*, 2x TY complex medium (Green and Sambrook, 2012) was applied. The growth experiments with *C. glutamicum* were all conducted aerobically with a modified version of CGXII minimal medium (Buchholz et al., 2014) either with 5 g ammonium sulfate L⁻¹, if nothing else is mentioned, or 20 g L⁻¹, set to a pH of 7.4 and 20 g glucose L⁻¹ as standard carbon source. Because of the phenotype of *aceE*-deficient strains (Schreiner et al., 2005), 5 g acetate L⁻¹ (as potassium salt) was supplemented in precultivation steps for all *C. glutamicum* strains in this study. The standard conditions for liquid cultures were 37°C (*E. coli*) and 30°C (*C. glutamicum*) in an orbital shaker (\emptyset 25 mm, Multitron[®]2, INFORS GmbH, Einsbach, Germany) at 180 rpm applying glass tubes with 5-mL medium or 500-mL cultivation flasks with four baffles containing 50 mL medium. Solid medium

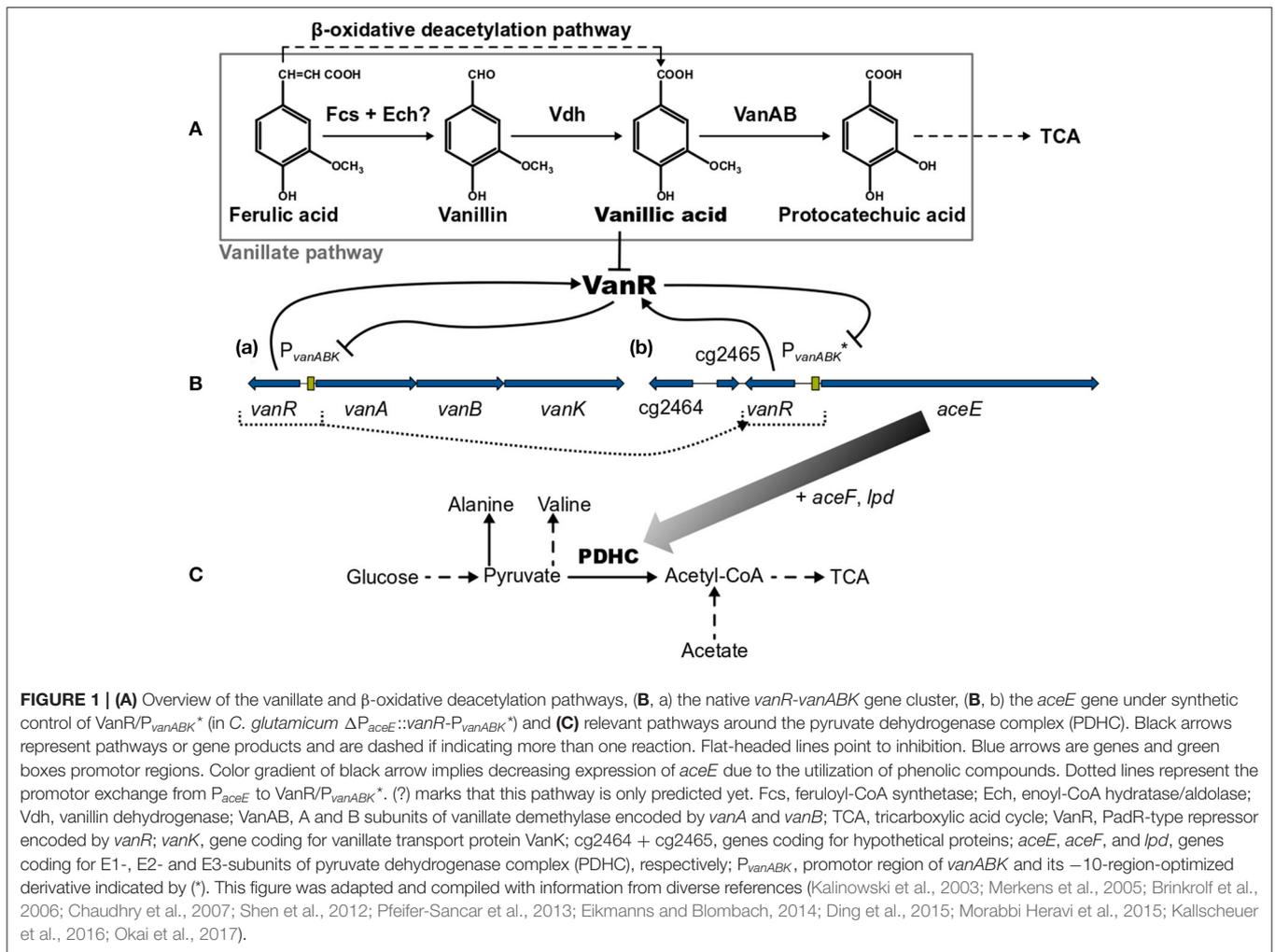


FIGURE 1 | (A) Overview of the vanillate and β -oxidative deacetylation pathways, **(B, a)** the native *vanR-vanABK* gene cluster, **(B, b)** the *aceE* gene under synthetic control of $VanR/P_{vanABK}^*$ (in *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$) and **(C)** relevant pathways around the pyruvate dehydrogenase complex (PDHC). Black arrows represent pathways or gene products and are dashed if indicating more than one reaction. Flat-headed lines point to inhibition. Blue arrows are genes and green boxes promoter regions. Color gradient of black arrow implies decreasing expression of *aceE* due to the utilization of phenolic compounds. Dotted lines represent the promoter exchange from P_{aceE} to $VanR/P_{vanABK}^*$. (?) marks that this pathway is only predicted yet. Fcs, feruloyl-CoA synthetase; Ech, enoyl-CoA hydratase/aldolase; Vdh, vanillin dehydrogenase; VanAB, A and B subunits of vanillate demethylase encoded by *vanA* and *vanB*; TCA, tricarboxylic acid cycle; VanR, PadR-type repressor encoded by *vanR*; *vanK*, gene coding for vanillate transport protein VanK; *cg2464* + *cg2465*, genes coding for hypothetical proteins; *aceE*, *aceF*, and *lpd*, genes coding for E1-, E2- and E3-subunits of pyruvate dehydrogenase complex (PDHC), respectively; P_{vanABK} , promoter region of *vanABK* and its -10-region-optimized derivative indicated by (*). This figure was adapted and compiled with information from diverse references (Kalinowski et al., 2003; Merkens et al., 2005; Brinkrolf et al., 2006; Chaudhry et al., 2007; Shen et al., 2012; Pfeifer-Sancar et al., 2013; Eikmanns and Blombach, 2014; Ding et al., 2015; Morabbi Heravi et al., 2015; Kallscheuer et al., 2016; Okai et al., 2017).

TABLE 1 | Strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Reference or source
C. glutamicum strains		
WT	Wild type (ATCC13032)	Abe et al., 1967; Ikeda and Nakagawa, 2003
$\Delta P_{aceE}::vanR-P_{vanABK}^*$	Wild type with insertion of the native <i>vanR</i> gene and the optimized <i>vanABK</i> promoter sequence (indicated by asterisk, derived from pJOE7747.1) in exchange for the <i>aceE</i> promoter sequence	This study
Plasmids		
pK19 <i>mobsacB</i>	Mobilizable <i>E. coli</i> vector for the construction of deletion and insertion mutants, Km^R , <i>sacB</i> , <i>lacZ</i> α with multiple cloning site, <i>oriV</i> , <i>oriT</i>	Schäfer et al., 1994
pK19 <i>mobsacB</i> - $\Delta P_{aceE}::vanR-P_{vanABK}^*$	pK19 <i>mobsacB</i> to replace the native <i>aceE</i> promoter sequence with the native <i>vanR</i> gene and the optimized <i>vanABK</i> promoter sequence (indicated by asterisk, derived from pJOE7747.1)	This study
pJOE7747.1	<i>oriP</i> BR322, <i>oriP</i> CG1, <i>rop</i> , Km^R , <i>vanR</i> 192-P <i>vanR</i> -P <i>vanABK</i> -eGFP- <i>terr</i> nB (optimized-10 region of P <i>vanABK</i>)	Morabbi Heravi et al., 2015
pJC4	<i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, derivative of pACYC177-pHM1519 hybrid pZ1, <i>ori</i> _{p15A} , <i>ori</i> _{pCG1} , Km^R	Menkel et al., 1989; Cordes et al., 1992
pJC4- <i>ilvBNCE</i>	pJC4 with coding sequence for native <i>ilvBNCE</i> genes from <i>C. glutamicum</i> WT	Radmacher et al., 2002

was prepared by adding 18 g agar-agar L⁻¹ to liquid medium in 9-cm Petri dishes, which were incubated at the above-mentioned temperature after inoculation. When appropriate, the medium was supplemented with 50 µg kanamycin mL⁻¹. For the dynamic control of gene expression, FA (product no: 128708; Sigma-Aldrich Chemie GmbH, Schnellendorf, Germany), Van (product no: 7887.1; Carl Roth GmbH + Co. KG, Karlsruhe, Germany), VA (product no: H36001; Sigma-Aldrich Chemie GmbH, Schnellendorf, Germany), or protocatechuic acid (product no: B24016; Alfa Aesar by Thermo Fisher Scientific, Kandel, Germany) was dissolved in DMSO to prepare 1 M stock solutions, which were applied in appropriate dilutions to reach the final concentrations given in the results. The seed train for all cultivation experiments with *C. glutamicum* was the following: cells out of glycerol cultures (30% glycerol and stored at -80°C) were streaked out on 2x TY-agar plates, which were incubated for 3 days. A single colony was used to inoculate 2x TY medium in a glass tube, which was incubated for 6–8 h. Then, the whole suspension was transferred into 2x TY medium in cultivation flasks and incubated overnight. The culture was centrifuged (4,000 × g, 10 min, room temperature), the cells were resuspended in CGXII medium and used to inoculate the main culture (flask or FlowerPlate, see below) to a start OD₆₀₀ (optical density at a wavelength of 600 nm) of 1. To follow the growth *via* determination of OD₆₀₀, a spectrophotometer (ULTROSPEC® 10, Biochrom, Holliston, MA, United States) was used, diluting the samples into a range of 0.1–0.3 OD₆₀₀ units. The cell dry weight (CDW) in gL⁻¹ was calculated by the conversion factor 0.23 × OD₆₀₀. The growth rate (h⁻¹) was determined *via* linear regression in a semi-logarithmic plot by maximizing the coefficient of determination (*R*²) in the exponential phase. Additionally, a microreactor system (BioLector® I, m2p-labs GmbH, Baesweiler, Germany) for microliter scale cultivation was employed. The seed train was the same as described above but using a cultivation volume of 1 ml in a 48-well FlowerPlate (product no: MTP-48-B; m2p-labs GmbH, Baesweiler, Germany) covered with a gas permeable sealing foil (product no: F-GP-10; m2p-labs GmbH, Baesweiler, Germany). The process parameters were set to 30°C, 85% humidity, and 1,000 rpm shaking frequency. The growth was followed by measurement of the backscatter light at 620 nm with a gain of 20. In all cultivation experiments 1-mL samples were taken at the time points given in the results and centrifuged (10 min; 21300 × g) and the resulting supernatants were stored at -20°C until further analysis.

Recombinant DNA Work and Construction of *C. glutamicum* Insertion Mutant

All oligonucleotides used in this study were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany) and are listed in **Table 2**. For purification of PCR products and isolation of plasmid and genomic DNA, the kit systems “NucleoSpin® Gel and PCR Clean-up,” “NucleoSpin® Plasmid,” and “NucleoSpin® Microbial DNA” were employed, respectively, as recommended by the manufacturer MACHEREY-NAGEL GmbH & Co. KG (Düren, Germany). All enzymes were purchased from

New England Biolabs GmbH [(NEB), Frankfurt am Main, Germany] and the appropriate reactions were carried out as recommended by NEB. For the construction of pK19*mobsacB*-Δ*P*_{aceE::vanR}-P_{vanABK}* the flanking regions were PCR-amplified from the genomic DNA of *C. glutamicum* with the primer pairs #1 + #2 and #3 + #4 and the fragment containing the native gene *vanR* with its native promoter and the optimized *vanABK* promoter sequence from pJOE7747.1 with the primer pair #5 + #6 *via* Phusion® High-Fidelity DNA Polymerase. These fragments were cloned into SmaI-linearized pK19*mobsacB* *via* Gibson assembly (Gibson, 2011), preparing the assembly master mix in-house with purchased enzymes and applying the assembled plasmid for transformation of *E. coli* DH5α with the calcium chloride method (Green and Sambrook, 2012). Positive clones were identified by colony PCR using the Quick-Load® Taq 2X Master Mix adding primers #7 + #8, and the purified plasmids were further validated by restriction enzyme digestion with KpnI and sequencing of the inserted fragments *via* “TubeSeq Service” at Eurofins Genomics (Ebersberg, Germany). The plasmid pK19*mobsacB*-Δ*P*_{aceE::vanR}-P_{vanABK}* was then used for transformation of electrocompetent *C. glutamicum* wild-type cells (Tauch et al., 2002) *via* electroporation (van der Rest et al., 1999). The integration of the plasmid was tested phenotypically *via* kanamycin resistance and sucrose sensitivity on 2x TY agar plates, and the whole recombination procedure *via* double cross-over events was carried out as described previously (Schäfer et al., 1994). The successful exchange of the *aceE* promoter sequence against the native *vanR* with its native promoter and the optimized *vanABK* promoter sequence in the *C. glutamicum* wild-type background was proven *via* colony PCR applying primer pair #12 + #13 and further confirmed by “TubeSeq Service” as described above, providing the purified product from the colony PCR for sequencing. The resulting strain *C. glutamicum*Δ*P*_{aceE::vanR}-P_{vanABK}* was further modified by transformation with pJC4 (Cordes et al., 1992) or pJC4-*ilvBNCE* (Radmacher et al., 2002), confirming the presence of the appropriate plasmid *via* colony PCR with primer pair #17 + #18 or #16 + #17, respectively.

HPLC Measurement

In this study, the concentration of all compounds besides that of biomass were determined *via* high-performance liquid chromatography (HPLC) employing a 1260 Infinity II system (Agilent Technologies, Waldbronn, Germany). All columns were purchased from Agilent Technologies and only HPLC-grade solvents were used. In the case of glucose and pyruvate measurements, the system was equipped with a Hi-Plex H column (7.7 × 300 mm, 8 µm) protected by a Hi-Plex H guard cartridge (3 × 5 mm, 8 µm) held at 50°C. The isocratic, mobile phase was 5 mM sulfuric acid (H₂SO₄) in water with a flow rate of 0.4 mL min⁻¹. The signals were acquired *via* refractive index detector (RID) held at 50°C. This method (Ball et al., 2011) was adapted from the manufacturer Agilent Technologies as well as the procedure for the analysis of the primary amino acids alanine and valine (Agilent Technologies, 2020). Therefore, an AdvanceBio Amino Acid Analysis (AAA)

TABLE 2 | Oligonucleotides used in this study.

#	Name	Sequence (5' → 3')
1	Pace_ex_upstrm_fw_pK19	<i>GTCGACTCTAGAGGATCCCCGCTCGAAAGCAACGAGTGAAATA</i>
2	Pace_ex_upstrm_rv	<i>CAAAGCTGTAGACGCATGCAGCTCACTGGGATTTTCAGGTATCC</i>
3	Pace_ex_dwnstrm_fw	<i>TAGTTCTTTAGGAGTTCCATATGGCCGATCAAGCAAAACTTG</i>
4	Pace_ex_dwnstrm_rv_pK19	<i>TGAATTCGAGCTCGGTACCCGTGCACCATCCTGGTCCTTC</i>
5	vanRP_fw_Pace_ex	<i>TACCTGAAATCCCAGTGAGCTGCATGCGTCTACAGCTTTGATAC</i>
6	vanRP_rv_Pace_ex	<i>AGTTTTGCTTGATCGGCCATATGGAAGCTCTAAAGAACTATATAACCAAATACCT</i>
7	pK19lacZ_fw	<i>ATGACCATGATTACGCCAAGCTTG</i>
8	pK19lacZ_rv	<i>TTAGCAGCCCTTGCGCC</i>
9	pK19lacI_fw	<i>TCAGTGAGCGAGGAAGCG</i>
10	aceP_ex_fw_2	<i>CGGAGCGTCGGCCAC</i>
11	aceP_ex_rv	<i>GGTGATGCGTGGCCCTG</i>
12	vanRP_fw_Pace_ex_2	<i>TTTTCTCATGCATACCCAATGCGTAC</i>
13	vanRP_rv_Pace_ex_2	<i>AGCTCGCTTGATAGGCTGC</i>
14	vanR_fw	<i>GTGGGCTTTGTGTGGAGTGG</i>
15	vanR_rv	<i>CTAAAGGGTATCGAGTAGTTTCAAGCC</i>
16	fw_ilvC-2_seq	<i>GAGTTGCGTGGCTACCTC</i>
17	fw_pJC4_seq	<i>CGATTGAAGACCGTCAAC</i>
18	rev_pJC4_seq	<i>GTCATCAGACCAAGGAG</i>

Italic letters: Overlapping sequence for Gibson assembly.

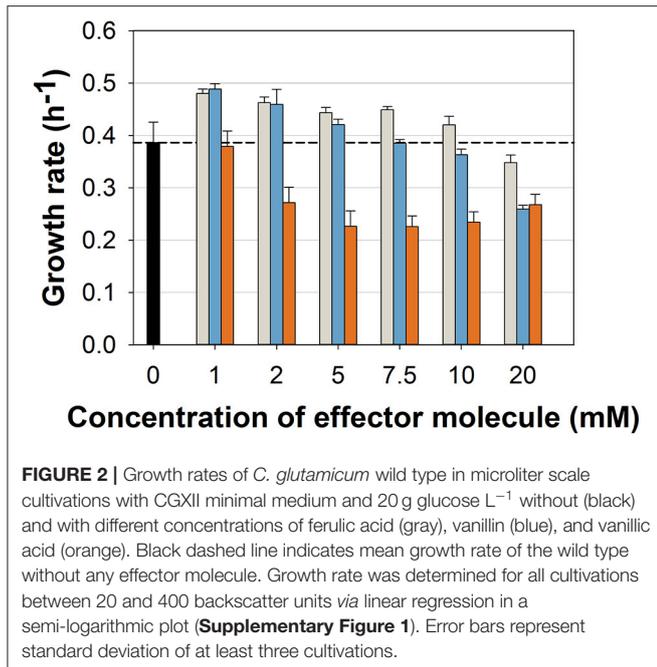
column (4.6 × 100 mm, 2.7 μm) protected by an AdvanceBio AAA guard column (4.6 × 5 mm, 2.7 μm) was installed and heated to 40°C. The separation was carried out *via* a gradient with an aqueous, polar phase (10 mM Na₂HPO₄, 10 mM Na₂B₄O₇, pH 8.2) and a non-polar phase (45 vol% acetonitrile, 45 vol% methanol, 10 vol% water). For detection of the primary amino acids, an automated online derivatization with *ortho*-phthalaldehyde (OPA) was conducted, and signals were acquired *via* fluorescence detector (FLD) at an excitation wavelength of 340 nm, an emission wavelength of 450 nm, and a PMT gain of 10. As internal standard, 100 μM norvaline was added to each sample. The measurement of the phenolic compounds FA, Van, and VA was adapted from Mollerup Andersen and Batsberg Pedersen (1983) and Merkens et al. (2005) using the same column described in the amino acids analysis. For the separation, a gradient from 95% 30 mM formic acid in water decreasing to 65% within 15 min was adjusted, applying as second phase pure methanol with an overall constant flow rate of 1 mL min⁻¹. FA and Van were detected at 280 nm and VA at 264 nm in a diode-array detector (DAD). For all HPLC analysis peak detection, integration and calculation of the final concentrations were carried out with the software “OpenLab CDS ChemStation Edition Rev. C.01.10 [236]” (Agilent Technologies, Waldbronn, Germany), which was also employed for the instrument controls. For the calibration curves, external reference standards were prepared for each compound ranging from 1 to 200 mM with seven points (glucose and pyruvate), 10 to 400 μM with seven points (alanine and valine), or 0.01 to 25 mM with 11 points (phenolics). Sample preparation was carried out by thawing of the supernatants, additional centrifugation (10 min; 21300 × g) and transfer of the

resulting supernatants into HPLC glass vials. The samples were diluted if appropriate.

RESULTS

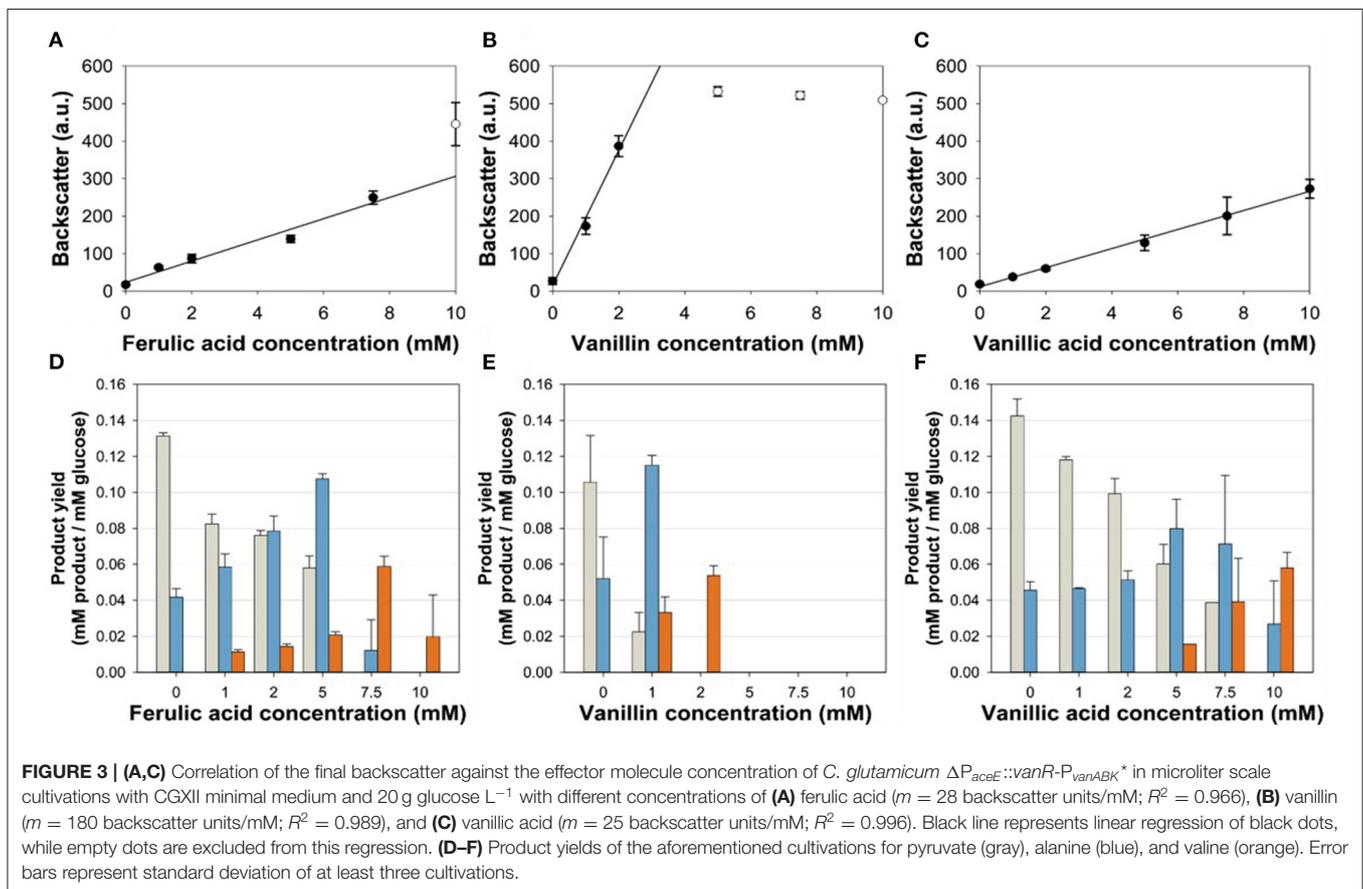
Influence of the Phenolic Compounds Ferulic Acid, Vanillin, and Vanillic Acid on Growth of *C. glutamicum*

While the general ability to utilize ferulic acid, vanillin, and vanillic acid as sole carbon source for growth was already shown (Merkens et al., 2005; Brinkrolf et al., 2006), we aimed at first to investigate which concentrations of these potential antimicrobial compounds may hamper the growth of the *C. glutamicum* wild type. We cultivated the wild type strain in a microliter scale (Biolector[®] I, M2PLabs, Baesweiler, Germany) with the CGXII minimal medium and 20 g glucose L⁻¹ supplemented with different concentrations of FA, Van, and VA. Interestingly, FA and Van at concentrations up to 10 mM did not inhibit growth (**Figure 2**). In contrast, low concentrations (0–5 mM) of both phenolic compounds even increased the growth rate by maximal 24 and 27%, respectively. Only at 10 and 20 mM of FA and Van growth was negatively affected. VA showed the strongest inhibitory effect with 2 mM already leading to a decrease of 30% in the growth rate. While the finally reached backscatter values were almost the same, the lag phase was prolonged with higher concentrations of the supplemented substances (**Supplementary Figure 1**). Based on these results, we conducted the characterization of the strain *C. glutamicum* Δ*P_{aceE}::vanR-P_{vanABK}** with concentration up to 10 mM for all the three compounds.



Adjusting Growth of *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ With the Effectors Ferulic Acid, Vanillin, and Vanillic Acid

To test the suitability of the VanR/ P_{vanABK}^* regulatory system to dynamically control the gene expression in *C. glutamicum*, we constructed *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ via exchange of the native *aceE* promoter by P_{vanABK}^* (Morabbi Heravi et al., 2015) and an additional copy of *vanR* (Figure 1). P_{vanABK}^* carries a single point mutation in the -10 region (CAATAT → TAATAT) leading to a 73-fold induction compared with its native version (Morabbi Heravi et al., 2015) and we anticipated that the regulatory circuit drives *aceE* expression as long as the effector molecule is present in the medium. After utilization, growth should arrest due to the abolished *aceE* expression and, consequently, the loss of PDHC activity. *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ was cultivated in a microliter scale (Biolector® I, M2PLabs, Baesweiler, Germany) with the CGXII minimal medium and 20 g glucose L⁻¹ supplemented with 0–10 mM of the potential effector molecules FA, Van, and VA. Without an effector in the medium, *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ showed negligible growth (Supplementary Figure 2). In the presence of the phenolic compounds, we observed a linear correlation between the introduced effector concentration and the finally reached biomass, but equimolar amounts led to different reads in the backscatter (Figures 3A–C;



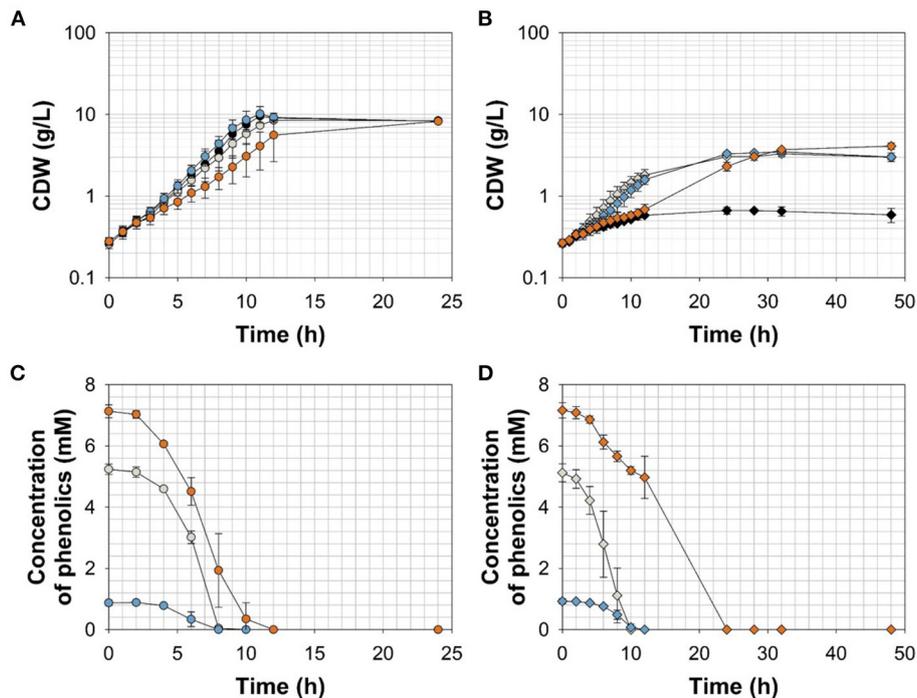


FIGURE 4 | (A,B) Growth and **(C,D)** effector molecule consumption of *C. glutamicum* wild type (circles) and *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (diamonds) in shaking flasks with CGXII minimal medium and 20 g glucose L⁻¹ without (black) and with ferulic acid (gray), vanillin (blue), or vanillic acid (orange). Error bars represent standard deviation of cultivations of at least three biological replicates.

Supplementary Figure 2). While Van concentration >2 mM was already sufficient to reach final biomass comparable to the wild type (**Supplementary Figures 1, 2**), >7.5 mM FA was required and even 10 mM VA was not enough. No growth occurred for this mutant strain if PCA was added as effector molecule in the same concentration range (data not shown). The cell cultures were harvested at the end of the cultivation and the supernatants were analyzed by HPLC to determine the amounts of glucose and the three metabolites pyruvate, alanine, and valine. The product yields for all three metabolites showed a strong dependence on the applied effector molecule concentration. With increasing effector concentrations, the product spectrum shifted from pyruvate over alanine to valine, and at about half-maximal biomass concentration, highest cumulated product yields were observed (**Figures 3D–F**).

Kinetic Analysis of *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ and the Wild Type

To further resolve the kinetics of growth and product formation in dependence of the effector molecules, we cultivated *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ and the wild type in CGXII minimal medium with 20 g glucose L⁻¹ at effector concentrations, which yielded around half of the maximal biomass concentration in comparison with the outgrown wild type. Thus, 5 mM FA, 1 mM Van, and 7.5 mM VA were applied (**Figure 4; Table 3**). The *C. glutamicum* wild type showed a growth rate of 0.35, 0.31, 0.39, and 0.23 h⁻¹ in the medium

without and with FA, Van, and VA (**Figure 4A; Table 3**), which is in accordance with the microscale cultivations (**Figure 2**). Furthermore, in the exponential growth phase, all the three phenolic compounds were consumed completely and in parallel to glucose (**Figure 4C; Supplementary Figure 3A**), which led to increased glucose-specific biomass yields correlating to the amount of additional carbon source provided by the phenolic compounds (**Table 3**). *C. glutamicum* did not secrete pyruvate, alanine, or valine into the culture broth. Interestingly, we observed the temporary accumulation of traces of VA in cultivations with FA (up to 0.1 mM VA) and Van (up to 0.03 mM VA; data not shown). *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ showed 40–60% decreased growth rates compared with the wild type in the presence of the respective phenolic compounds and negligible growth without an effector (**Figure 4B; Table 3**). Furthermore, applying the correlation from the microscale cultivation (**Figures 3A–C**) allowed to adjust the final biomass concentration and the cells grew only until the effector molecule was fully depleted (**Figures 4B,D**). Although growth ceased, the cells remained metabolically active and started to secrete pyruvate, alanine, and valine (**Supplementary Figures 3B–D; Table 3**) and the reached product yields (**Table 3**) are in good accordance with the microliter scale experiments (**Figures 3D–F**). Also, the temporary accumulation of traces of VA, as described for the wild type, could be observed if the strain *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ was cultivated with

TABLE 3 | Growth rates, biomass yields, and product yields of *C. glutamicum* wild type, *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$, and *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (pJC4-*ilvBNCE*) in shaking flasks with CGXII minimal medium and 20 g glucose L⁻¹ without and with ferulic acid, vanillin, or vanillic acid. Standard deviation was determined *via* cultivations of at least three biological replicates.

Concentration of effector molecule (mM)	Growth rate μ (h ⁻¹)	Biomass yield (g _{CDW} /g _{glucose})	Pyruvate yield (mol/mol _{glucose})	Alanine yield (mol/mol _{glucose})	Valine yield (mol/mol _{glucose})
<i>C. glutamicum</i> wild type					
–	0.35 ± 0.01	0.47 ± 0.01	b.d.	b.d.	b.d.
FA (5.2 ± 0.2)	0.31 ± 0.03	0.52 ± 0.02	b.d.	b.d.	b.d.
Van (0.9 ± 0.1)	0.39 ± 0.01	0.50 ± 0.04	b.d.	b.d.	b.d.
VA (7.1 ± 0.2)	0.23 ± 0.04	0.58 ± 0.03	b.d.	b.d.	b.d.
<i>C. glutamicum</i> $\Delta P_{aceE}::vanR-P_{vanABK}^*$					
–	n.d.	n.d.	0.18 ± 0.02	0.07 ± 0.01	b.d.
FA (5.1 ± 0.3)	0.19 ± 0.03	0.58 ± 0.09	0.08 ± 0.05	0.11 ± 0.02	0.01 ± 0.01
Van (0.9 ± 0.1)	0.16 ± 0.01	0.40 ± 0.01	0.06 ± 0.02	0.10 ± 0.01	0.02 ± 0.01
VA (7.2 ± 0.2)	0.10 ± 0.02	0.34 ± 0.03	0.07 ± 0.06	0.09 ± 0.01	0.02 ± 0.01
<i>C. glutamicum</i> $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (pJC4-<i>ilvBNCE</i>)					
–	n.d.	n.d.	0.10 ± 0.05	0.04 ± 0.01	0.27 ± 0.03
FA (4.9 ± 0.1)	0.17 ± 0.01	0.52 ± 0.01	b.d.	0.02 ± 0.01	0.36 ± 0.02
Van (1.0 ± 0.1)	0.14 ± 0.02	0.34 ± 0.03	0.02 ± 0.03	0.03 ± 0.01	0.38 ± 0.01
VA (7.3 ± 0.1)	0.08 ± 0.01	0.38 ± 0.06	0.03 ± 0.05	0.03 ± 0.01	0.35 ± 0.04

b.d., below detection limit; n.d., not determined.

FA (up to 0.5 mM VA) and Van (up to 0.02 mM VA; data not shown).

Dynamically Induced Valine Production With *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ Overexpressing *ilvBNCE*

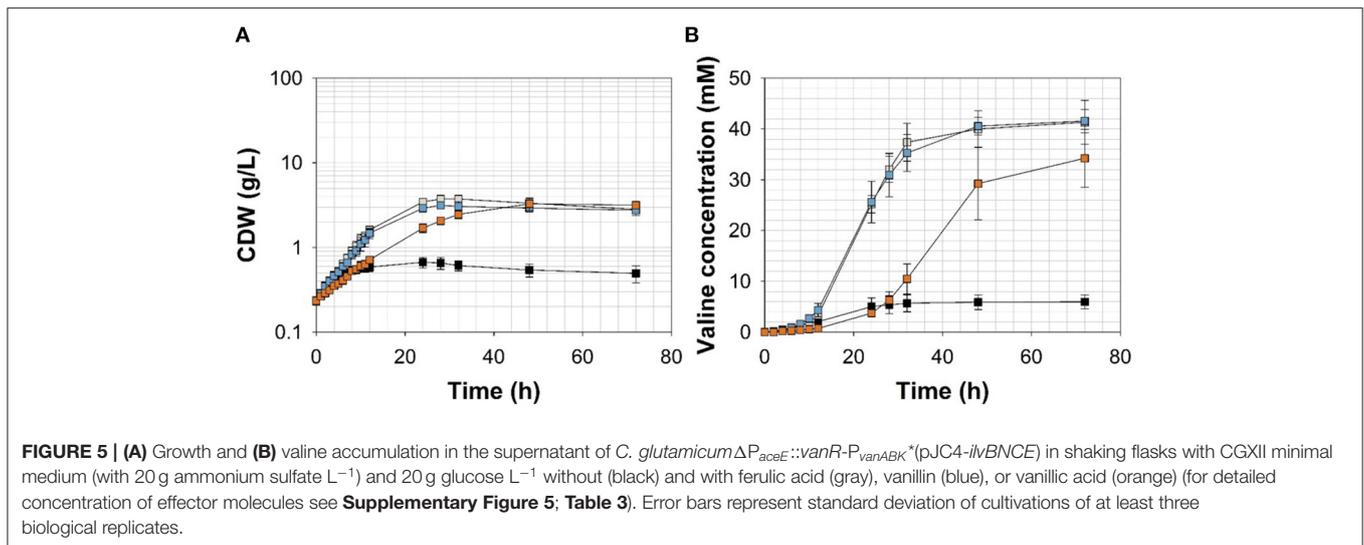
To channel the carbon flux more efficiently from pyruvate toward valine, we transformed *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ with the plasmid pJC4-*ilvBNCE* (Radmacher et al., 2002) to overexpress the valine biosynthesis genes *ilvBNCE* encoding acetoxyacid synthase (*ilvBN*), isomeroreductase (*ilvC*), and transaminase B (*ilvE*). *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (pJC4-*ilvBNCE*) and the control strain *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (pJC4) were cultivated in shaking flasks with CGXII minimal medium and 20 g glucose L⁻¹ supplemented with 5 mM FA, 1 mM Van, and 7.5 mM VA (Figure 5; Table 3). Under the conditions tested, *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ and its plasmid carrying derivatives showed very similar growth and consumption of glucose and the phenolic compounds, and all the strains induced production after consumption of the respective effector molecule (Figures 5A, 4B; Supplementary Figures 5, 6; Table 3). *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ and the derivative with the empty vector secreted similar amounts of pyruvate, alanine, and valine into the medium (Supplementary Figures 3B–D, 6C,E). However, overexpression of the *ilvBNCE* genes in *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ shifted the product spectrum toward valine. In the medium containing FA, Van, or VA *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ (pJC4-*ilvBNCE*) produced 40 ± 3.6, 40.6 ± 1.7, and 29.3 ± 7.1 mM valine after 48 h with a product yield of 0.36 ± 0.02, 0.38 ± 0.01, and 0.35 ± 0.04 mol valine per mol glucose (Figure 5B; Table 3). The concentration of pyruvate and

alanine was, under all conditions, below 5 mM over the whole cultivation time (Supplementary Figures 4B,C).

DISCUSSION

In this study, the VanR/*P_{vanABK}*^{*} regulatory system was utilized to dynamically control gene expression in *C. glutamicum*. We applied this circuit to switch off the *aceE* expression in a timed manner when the lignin-derived effectors FA, Van, and VA were depleted from the medium. Finally, we showed the feasibility of this approach to adjust biomass formation and to induce the production of valine.

The ability of *C. glutamicum* to tolerate higher concentrations of aromatics than other bacteria was already described (Shen et al., 2012; Morabbi Heravi et al., 2015; Becker and Wittmann, 2019) as well as to utilize the phenolic compounds FA, Van, and VA as sole carbon sources (Merkens et al., 2005; Brinkrolf et al., 2006; Shen et al., 2012; Kallscheuer et al., 2016). Here, we show that these molecules are also co-utilized with glucose (Figure 4C; Supplementary Figure 3A; Table 3), which is a promising feature of *C. glutamicum* with regard to the application of lignocellulosic hydrolysates as feedstock for industrial biotechnology. While minimum inhibitory concentrations (MIC) for the phenolic compounds tested in this study range for *E. coli* between 0.5 and 15 mM (Fitzgerald et al., 2004; Mourtzinos et al., 2009; Borges et al., 2013), *C. glutamicum* still shows, in the presence of up to 20 mM of each molecule, about 67–90% growth rate compared with reference conditions (Figure 2). Remarkably, low concentrations (up to 2 mM) of FA and Van significantly (*p*-value ≤ 0.01) increased the growth rate of *C. glutamicum* (Figure 2). It is known that diphenolic compounds, such as catechol or PCA,



facilitate the growth of *C. glutamicum* (Liebl et al., 1989). Recently, it has been shown that this effect is based on the potential of diphenols, with two adjacent hydroxyl groups or a mix of amino and hydroxyl groups, to chelate iron and/or reduce Fe₃⁺, thus improving the overall iron availability (Müller et al., 2020). If the positive effect of FA and Van is directly mediated by these molecules or if it is the effect of the degradation intermediate PCA (Brinkrolf et al., 2006; Shen et al., 2012; Kallscheuer et al., 2016) remains to be investigated. For VA, no positive effect on growth was observed at the applied concentrations (**Figure 2**). In general, VA has a much higher antimicrobial potential, especially in comparison with Van (Mourtzinou et al., 2009). Thus, it has to be tested if lower concentrations (<1 mM) of VA have a growth-promoting effect.

All the three phenolic compounds proved suitability to induce the VanR/P_{vanABK}^{*} regulatory circuit, providing flexibility with regard to the intended application. However, since 2 mM VA already reduced the growth rate of the *C. glutamicum* wild type significantly (**Figure 2**; *p*-value ≤ 0.01), Van and FA are the preferred inducer molecules. VA, FA, and Van, compared with common inducers such as IPTG or rhamnose, are cheaper and abundant, since these molecules can be derived from lignocellulosic material. Therefore, VA, FA, and Van are, in principle, well-suited for industrial application if the main substrate of the process is not a lignocellulosic hydrolysate containing significant amounts of effector molecules. Future studies have to evaluate if the VanR/P_{vanABK}^{*} regulatory circuit can be applied in large scale as well as in other hosts. As a prerequisite, the microbial system of choice should show considerable tolerance against the aromatic inducer molecules such as *Pseudomonas* (Becker and Wittmann, 2019). Moreover, if applied as synthetic off-switch, the organism should be able to metabolize the inducer molecules and its metabolism should not be under control of catabolite repression, which might result in inducer exclusion.

The final biomass of *C. glutamicum* $\Delta P_{aceE}::vanR-P_{vanABK}^*$ correlated directly with the applied amount of effector molecule.

FA and VA showed almost identical slopes of the linear regression, while Van yielded an about 7-fold higher slope, i.e., seven times less Van has to be added to the culture in order to achieve the same biomass as with VA or FA (**Figures 3A–C**). The reason for this effect remains unclear, especially since in cultivations with FA and Van small amounts of the VanR effector VA were secreted into the medium, indicating a limitation in the conversion of VA to PCA catalyzed by vanillate demethylase VanAB. While biochemical data for VanAB are not available yet specifically for *C. glutamicum*, it is known that this enzyme complex shares significant similarity to VanABs of other microorganisms such as *Pseudomonas* species (Merkens et al., 2005; Brinkrolf et al., 2006), where it is described to convert VA to PCA, demanding NAD(P)H₂ plus molecular oxygen and releasing NAD(P), water, and formaldehyde, which has to be further detoxified (Priefert et al., 1997; Hibi et al., 2005). This energy- and oxygen-demanding reaction, along with the release of formaldehyde, is a good indicator for the strict regulation of VanABK in *C. glutamicum* (Merkens et al., 2005; Brinkrolf et al., 2006; Morabbi Heravi et al., 2015).

The PDHC of *C. glutamicum* is a promising target for metabolic engineering (Eikmanns and Blombach, 2014), and it has been shown that the PDHC-deficient strain *C. glutamicum* $\Delta aceE$ is unable to grow on glucose unless supplemented with acetate (Schreiner et al., 2005). This feature has been utilized to adjust biomass formation over the provided amount of acetate. After its depletion, the growth of *C. glutamicum* $\Delta aceE$ stopped, but the cells remained metabolically active and started to produce pyruvate, alanine, and valine from glucose (Blombach et al., 2007). More recently, Wiechert et al. (2020) designed an inducible metabolic toggle switch, which efficiently controls the *aceE* expression by the effector molecule gluconate. However, compared with acetate as well as gluconate as inducers, at least 50 times lower molar amounts of Van have to be introduced into the process to achieve similar biomass concentrations (Blombach et al., 2007; Wiechert et al., 2020).

Compared with *C. glutamicum* $P_{gntK-aceE}$ (Wiechert et al., 2020), *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ showed an around 50% slower growth rate with Van and FA, indicating further potential to optimize the VanR/ P_{vanABK}^* system. Data relating the promoter strength of P_{aceE} , P_{vanABK}^* , and P_{gntK} under comparable conditions are not available. However, while the ribosomal binding site “AGGAG” was utilized in the promoter regions of P_{gntK} and P_{vanABK}^* (Wiechert et al., 2020), the spacer regions are quite different, potentially impacting gene expression (Schneider et al., 2012).

In microliter cultivations, the product pattern of *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ (Figures 3D–F) shifted from pyruvate over alanine to valine with increasing effector and final biomass concentrations. This effect was observed for all three phenolic compounds, indicating biomass-dependent metabolic changes, which promote valine synthesis. However, to channel the carbon flux toward valine, plasmid-based overexpression of the valine biosynthesis genes *ilvBNCE* was more efficient. *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ (pJC4-*ilvBNCE*) showed product yields of about 0.36 mol valine per mol glucose, which are about 6-fold higher compared with the yields of *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ at optimal effector concentrations (Figures 3D–F; Table 3). Although the glucose-based product yield of *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ (pJC4-*ilvBNCE*) is considerably lower in comparison with 0.47 mol valine per mol glucose of *C. glutamicum* $\Delta aceE$ (pJC4-*ilvBNCE*) and 0.52 mol valine per mol glucose of *C. glutamicum* $P_{gntK-aceE}$ (pJC4-*ilvB-ilvBNC-P_{ilvE-ilvE}*), the overall yield (including the amount of effector) of 0.36 C-mol per C-mol of *C. glutamicum* $\Delta P_{aceE::vanR-P_{vanABK}^*}$ (pJC4-*ilvBNCE*) with Van is at least identical to the latter two strains with values of 0.32 and 0.36 C-mol per C-mol, respectively (Blombach et al., 2007; Wiechert et al., 2020).

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Concluding, this study shows the suitability of the VanR/ P_{vanABK}^* system as a tool to control gene expression in *C. glutamicum*. The regulatory circuit can be toggled by the cheap and abundant lignin-derived phenolic compounds FA, Van, and VA. Since these three molecules are consumed in parallel to the main substrate glucose, the regulatory circuit represents a valuable genetic control element to adjust gene expression on demand.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DS and BB conceived and supervised the study and designed the experiments. DS performed the experiments and analyzed the data. DS, JA, and BB wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fbioe.2021.704681/full#supplementary-material>

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