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Dynamic *in silico* assessment of potential gene targets for enhancing clavulanic acid production in *Streptomyces clavuligerus* submerged cultures

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ABSTRACT

Clavulanic acid (CA) is an antibiotic of the β-lactam class with moderate antimicrobial activity but a high inhibitory effect on β-lactamase enzymes. CA is one of the most important products of the secondary metabolism in *Streptomyces clavuligerus*, which is still the main industrial source of CA marketed worldwide. However, CA titers produced in submerged cultures by wild-type *S. clavuligerus* is characteristically low. The application of systems biology and constraint-based approaches such as the flux balance analysis (FBA) allow us to get insights into the metabolism, especially when it is coupled to a validated genome-scale model. In this study, FBA was applied for the *in silico* determination of potential metabolic targets aimed to increase the CA biosynthesis rate. In the metabolic network, the overexpression of N2-(2-carboxyethyl)-arginine synthase (CEAS), knockout of pyruvate kinase (PYK), and dampening of phosphoglycerate kinase showed the best flux ratio of CA biosynthesis. Then, submerged fed-batch cultivations with CEAS and PYK engineered strains were simulated by using a dynamic FBA (dFBA) for analysis of the correspondent metabolic flux distribution. The dFBA provided insights about the carbon redistribution produced by the genetic perturbations potentially increasing CA production up to 1.9 and 1.1-fold for the calculated wild-type strain (2.74 mmol.l^{-1}).

INTRODUCTION

Clavulanic acid (CA) is an antibiotic of the β-lactam class with moderate antimicrobial activity but a high inhibitory effect on β-lactamase enzymes. CA is one of the most important specialized metabolites produced by *Streptomyces clavuligerus*; indeed, this strain is still the only industrial source for CA production (López-Agudelo *et al.*, 2021). The CA's importance lies in its wide use as a medical treatment for infections caused by antibiotic-resistant bacteria, including some of those listed

in the World Health Organization's priority list, such as the case of Streptococcus pneumoniae, *E*scherichia coli, Neisseria gonorrhoeae, Staphylococcus aureus, Enterobacteriaceae, and Klebsiella species (Gómez-Ríos and Ramírez-Malule, 2019).

The nutritional restriction in *S. clavuligerus* activates the secondary metabolism, leading to the secretion of penicillin and clavaminic acid derivatives, with cephamicyn C (CephC) and CA being the most important of them. The low CA productivity in the submerged cultivation of *S. clavuligerus* and the complexity of the downstream process have a direct impact on the production cost of this pharmaceutical compound, which is commonly coformulated with wide-spectrum β-lactam antibiotics. Strategies like nutritional and environmental perturbation appear as feasible alternatives to enhance the strain's productivity (López-Agudelo *et al.*, 2021; Ramirez-Malule, 2018). Metabolic engineering emerges as an alternative

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for the rational enhancement of CA in *S. clavuligerus* cultures through the perturbation of metabolic and regulatory features (Liras and Martín, 2021).

In this sense, the systems biology approaches have contributed to the understanding of *S. clavuligerus* metabolism under different nutritional and environmental conditions (Gómez-Rios *et al.*, 2022; Ramirez-Malule *et al.*, 2021). Nevertheless, the classical steady-state approaches like the flux balance analysis (FBA) present limitations like the prediction of cellular growth and product secretion rates only for fixed values of substrate uptake rates. In addition, it has strict applicability to the balanced growth phase in batch cultures or steady-state growth in continuous cultures. The intrinsic dynamic condition of batch and fed-batch cultivations has repercussions on the prediction of cell phenotypical states, product yield, and bioreactor control. Thus, modeling tools like FBA must be adapted to cover dynamic states that allow us to get a more accurate description of cellular physiology during such nonsteady processes. Indeed, the highest CA titers for wild-type *S. clavuligerus* cultivation have been reported in batch and fed-batch operation; thus, high CA productivity necessarily must consider this operation mode. In this work, steady-state FBA was used for the *in silico* identification of potential gene targets exerting a positive effect on the CA biosynthesis. Afterward, a dynamic FBA (dFBA) framework was used for the *in silico* evaluation of fed-batch production scenarios, potentially leading to CA overproduction considering the identified gene targets as promissory alternatives for further *S. clavuligerus* strain engineering.

MATERIALS AND METHODS

Genome-scale metabolic model and sensitivity analysis

The validated genome-scale metabolic model iDG1237 of *S. clavuligerus* metabolism was used in both FBA and dFBA methodologies (Gómez-Ríos et al., 2020). The iDG1237 biomodel consists of 2,177 reactions, 543 internal/external metabolites, 1,237 annotated genes, and it has been checked for thermodynamic and topological consistency (Gómez-Ríos et al., 2020). Our experimental studies have previously determined suitable environmental and nutritional conditions favoring CA production in wild-type *S. clavuligerus* (Gómez-Ríos *et al.*, 2019; Gómez-Rios *et al.*, 2022). In this regard, glycerol, glutamate, and oxygen have been identified as key nutrients in the CA biosynthetic pathway, which is activated under phosphate limitation. Previous studies have not considered CA production as a physiological objective in the FBA formulation. In this study, the FBA simulations were performed for exploring the effect of glycerol, glutamate, oxygen, ammonium, phosphate, CephC, and clavam-2-carboxylate (a clavam metabolite) on both physiological objectives, biomass synthesis (Z) and CA secretion flux (v_{CA_ex}). The FBA formulations are described by Equations (1) and (2), respectively.

$$\text{Max } Z = f^T v \tag{1}$$

$$\begin{bmatrix} s.t. \quad S \cdot v = 0 \\ l_b \le v \le u_b \end{bmatrix}$$

$$\text{Max } v_{CA\_ex} \tag{2}$$

$$\begin{bmatrix} s.t. \quad S \cdot v = 0 \\ l_b \le v \le u_b \end{bmatrix}$$

where S is the stoichiometric matrix; v is the reaction fluxes; and l_b and u_b are the lower and upper bounds for the metabolic fluxes, respectively.

After the sensitivity analysis, the genome-scale model was used for the identification of potential metabolic targets for gene knockout, dampening, and/or overexpression, which might favor the carbon flux toward the CA biosynthetic pathway. Initially, a gene essentiality analysis in a synthetic medium according to the usual production conditions was performed. Then, the application of the (robust, overexpression, knockout, and dampening) pipeline allowed the determination of the potential gene candidates for strain engineering (Stanford *et al.*, 2015).

dFBA implementation

The dFBA was formulated according to the static optimization approach, which assumes a pseudo-steady-state condition in each time interval, and the optimization FBA problem is solved. The FBA problem is constrained dynamically for each time interval by the solution of a set of ordinary differential equations (ODE), whose parameters correspond to experimentally measured rates in *S. clavuligerus* wild-type cultivations. The set of ordinary equations [Eqs. (3)–(5)] accounts for the dynamics of growth rate, glycerol, glutamate, phosphate, and ammonia depletions, as well as gas transfer rates for oxygen and carbon dioxide. Based on the simplified stoichiometry of biomass synthesis, a kinetic Monod model was proposed for growth, as shown in Equation (6).

$$\frac{dV}{dt} = F \tag{3}$$

$$\frac{dX}{dt} = X\left(\mu_D - k_d\right) - \left(\frac{F}{V}\right)X \tag{4}$$

$$\frac{dC_i}{dt} = X\left(\frac{\mu_D}{Y_{X/i}}\right) + \left(\frac{F}{V}\right)\left(C_{i,f} - C_i\right) \tag{5}$$

$$\mu_D = \frac{\mu_{max}\, C_s}{K_s\, X + C_s} \tag{6}$$

where C_i is the concentration of i species, X is the biomass concentration, F is the constant feed rate during fed-batch operation (from $t = 34$ hours onwards), and V is the volume. For the Monod growth rate expression [Eq. (6)], μ_D is the specific growth rate for the dynamic model, K_d is the specific death constant, K_s is the affinity constant, and C_s is the concentration of the carbon source. The values of the kinetic used are currently available in the literature for wild-type *S. clavuligerus* (Sanchez *et al.*, 2012). dFBA simulations for the *in silico* engineered strains were conducted under the same kinetic macroscopic parameters validated for wild-type fed-batch cultivation since the carbon flux toward secondary metabolism is very low in comparison with the central metabolism (Gómez-Ríos *et al.*, 2022). The initial conditions are presented in Table 1.

dFBA was constrained by the solutions of the ODE set and steady-state constraints for exchange reactions of low-concentrated nutrients and metabolites. The growth rate estimated is conciliated through the error minimization between the growth rates predicted by solving the FBA and ODE set, as observed in Equation (7). The dFBA definition is presented in Equation (8).

$$min \sum (\mu_D - \mu_F)^2 \qquad (7)$$

$s.t$

$$
\begin{aligned}
&\max \mu_F \\
&s.t \quad S \cdot v_n = 0 \\
&\qquad lb \le v_{n-m} \le ub \\
&\quad v_m = \frac{1}{X}\left(\frac{dC_m}{dt}\right)
\end{aligned} \qquad (8)
$$

where μ_F is the growth rate estimated for the pseudo-steady-state model, v_m is the fluxes calculated by integration of the ODE set, and v_n is the fluxes determined from the optimization of the FBA problem [Eq. (14)]. The FBA was implemented in COBRA Toolbox 3.0 for MATLAB. The optimization steps were solved with Gurobi 7.5 MATLAB API.

RESULTS AND DISCUSSION

Sensitivity analysis of nutrients uptake and antibiotics secretion

The effects of key nutrients variation on biomass synthesis and CA secretion were assessed via FBA. Additionally, the effects of CephC and clavam-2-carboxylate biosynthesis as the respective products of cephalosporins and clavams 5S pathways were also considered since the synthesis of these compounds were expected to compete with CA biosynthesis. The results of the effect of the abovementioned variables on the specific growth rate are shown in Figure 1.

The increase in the uptake of carbon and nitrogen, i.e., glycerol, glutamate, and ammonium, entails a positive effect on the growth rate since those are the main nutrients during the cultures. The increase in glycerol influx supports biomass synthesis up to 0.095 hour⁻¹, as shown in Figure 1a. Moreover, glutamate may further enhance the growth rate (up to 0.25 hour⁻¹)

due to its content of carbon and nitrogen and its direct incorporation into the urea cycle as an amino acid precursor. Ammonium as the main nitrogen source is essential for growth and promotes growth as long as enough carbon source, either glycerol or glutamate, is provided (Fig. 1b). In the case of phosphate, there exists a threshold in the phosphate influx to support the biomass accumulation and CA biosynthesis (Saudagar and Singhal, 2007). As shown in Figure 1b, phosphate acts as a biomass precursor but uptake rates over 0.2 mmol.gram dry cell weight (gDCW.hour)⁻¹ might inhibit growth. Interestingly, the phosphate limitation slightly decreases the growth rate but does not cause inhibition, suggesting that metabolic mechanisms of phosphate compensation and energetic regulation might compensate for the phosphate scarcity, as indeed occurs in other *Streptomyces* species, in which this correlates with antibiotics production (Esnault *et al.*, 2017; Millan-Oropeza *et al.*, 2020).

In *S. clavuligerus*, secondary metabolism demands for substrates that are simultaneously required for growth as in the case of amino acids from the urea cycle. Specifically, CA biosynthesis requires glyceraldehyde-3-phosphate (GAP), arginine, and tricarboxylic acid (TCA) cycle intermediates. As shown in Figure 1c, the increase in CA synthesis beyond 0.067 mmol. (gDCW.hour)⁻¹ in wild-type *S. clavuligerus* is expected to be detrimental to biomass synthesis. The CephC biosynthesis requires valine and cysteine, also biomass precursors. Similarly, CephC secretion affects biomass production but to a lesser extent. These results suggest that feasible phenotypes producing either CA or CephC can be attained at specific growth rates higher than 0.04 hour⁻¹ and lower than 0.9 hour⁻¹.

According to previous experimental observations in batch and fed-batch cultures of wild-type *S. clavuligerus*, a minimum specific growth rate of 0.05 hour⁻¹ was defined for FBA with CA secretion flux as the objective function (Gómez-Rios *et al.*, 2022; Gómez-Ríos *et al.*, 2019). CA production is not possible without providing glycerol due to its incorporation as GAP into the glycolytic pathway; thus, an increase in glycerol uptake would favor the CA secretion up to a maximum of 0.09 mmol. (gDCW.hour)⁻¹, as shown in Figure 1d. The simultaneous availability of glutamate enhances CA production (Fig. 1d) by increasing the flux of arginine from

Table 1. Initial conditions for the kinetic model of *S. clavuligerus*.

Parameter	Description	Value
μ_{max}	Maximum specific growth rate	0.070 hour⁻¹
F	Feed rate	0.035 l.hour⁻¹
$C_{Glyc,f}$	Concentration of glycerol in the feeding medium	1,304 mmol.l⁻¹
$C_{Pi,f}$	Concentration of phosphate in the feeding medium	11.48 mmol.l⁻¹
$C_{NH4,f}$	Concentration of ammonium in the feeding medium	121.08 mmol.l⁻¹
$C_{Glu,f}$	Concentration of glutamate in the feeding medium	100 mmol.l⁻¹
$X(t=0)$	Initial biomass concentration	1.89 gDCW.l⁻¹
$C_{Glyc}\,(t=0)$	Initial glycerol concentration in the medium	100.5 mmol.l⁻¹
$C_{Glu,}\,(t=0)$	Initial glutamate concentration in the medium	50 mmol.l⁻¹
$C_{Pi,}\,(t=0)$	Initial phosphate concentration in the medium	8 mmol.l⁻¹
$C_{NH4}\,(t=0)$	Initial ammonium concentration in the medium	40 mmol.l⁻¹
$V(t=0)$	Initial operation volume	5 l
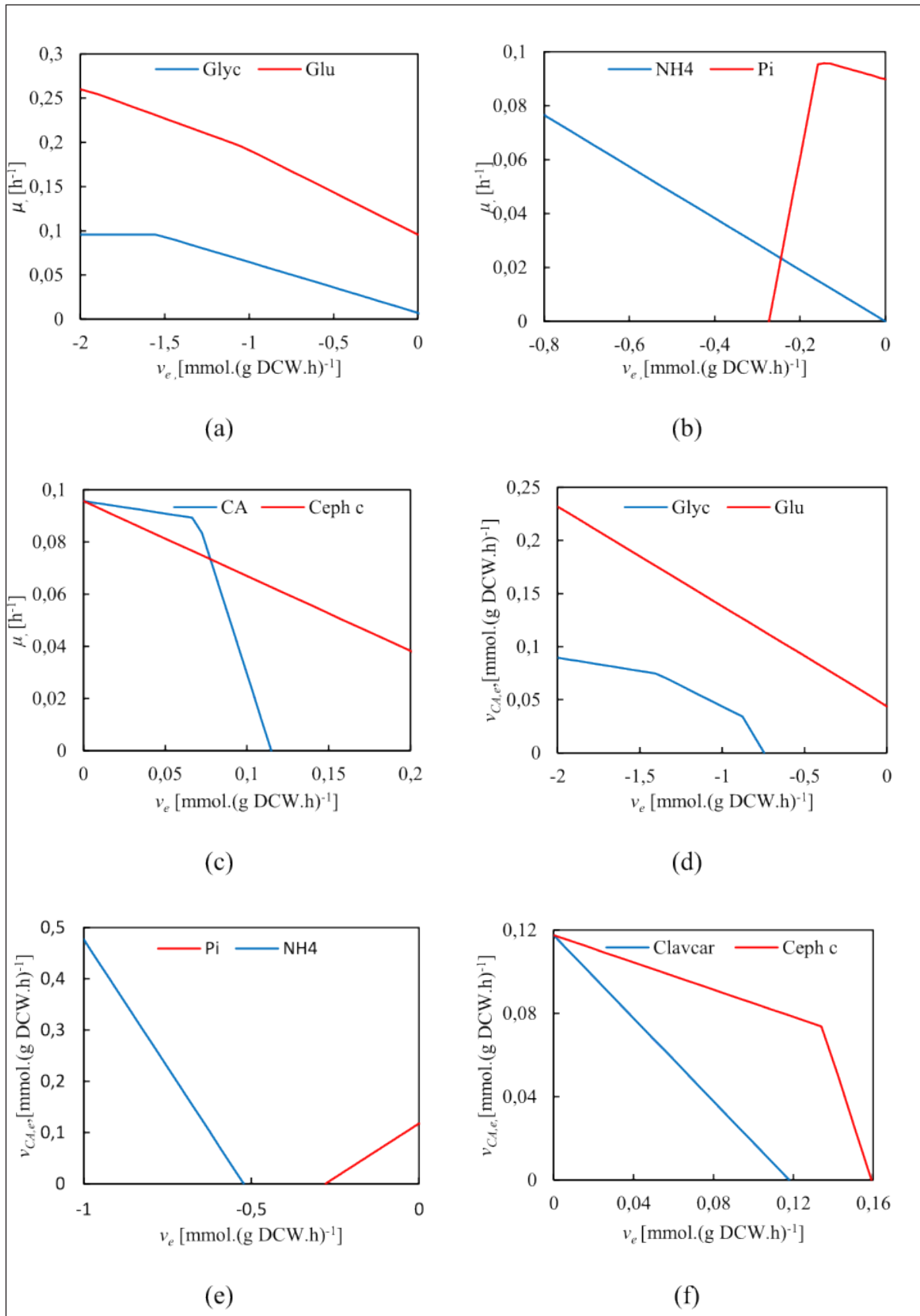
Figure 1. Effect of nutrient uptake and secondary metabolites on the specific growth rate (μ) and CA secretion ($v_{CA,e}$).

the urea cycle, which is an early precursor of CA. Similarly, the assimilation of ammonium is essential for secondary metabolism; as can be seen in Figure 1e, an increase in the ammonium uptake is expected to impact positively the CA production. Figure 1e also shows that the maximum CA production is attained when the phosphate limitation is reached, and no production occurs at

phosphate uptake higher than 0.28 mmol. (gDCW.hour)$^{-1}$. Indeed, the phosphate limitation has been reported as a condition triggering the biosynthesis of antibiotics in several species of *Streptomyces* (Barreiro and Martínez-Castro, 2019).

Figure 1f shows that the higher the CephC biosynthesis flux, the lower the CA production. CephC biosynthesis has been reported as a competitor pathway of CA biosynthesis, although both can be secreted under appropriate nutritional conditions (Bellão *et al.*, 2013). This is probably linked to the consumption of 2-oxoglutarate and oxygen in the oxidation steps of the pathway which also occurs in CA biosynthesis. Moreover, the synthesis of CephC amino acid precursors (valine, cysteine, and lysine) requires the flux of glutamate as also occurs with arginine in the urea cycle and further CA synthesis. The clavam-2-carboxylate is a product of the 5S clavam pathway. Figure 1f also suggests that the higher the clavam 2-carboxylate production, the lower the CA secretion. The 5S clavams pathway is a direct competitor of CA biosynthesis since both pathways use the same precursor, clavaminic acid, whose synthesis acts as a bifurcation point between both pathways (Tahlan *et al.*, 2007).

Potential gene targets for enhancing CA production

The reaction essentiality analysis performed with the iDG1237 biomodel showed that 216 reactions (9.7%) are essential for growth in the synthetic medium; these reactions are associated with 109 genes, which is 8.8% of the genes annotated. In the context of the pseudo-steady-state condition, 96 reactions were identified initially as potential targets for a single or double knockout and 72 for overexpression or dampening linked to the objective of increasing CA production. The ratio of growth rate-CA flux to glycerol uptake ($\mu.v_{CA,e}/v_{Glyc,e}$) was used as an indicator for selecting the best potential candidates for the strain design. For the case of wild-type *S. clavuligerus*, this ratio was 0.0024; on the contrary, for the best candidate, i.e., overexpression of genes encoding (CEAS) enzyme N2-(2-carboxyethyl)-arginine synthase, this ratio was 0.028. A summary of the best potential target candidates for strain engineering is presented in Table 2.

Some studies have focused on the genetic engineering of expression levels of genes directly associated with the biosynthetic pathway of CA or its regulation. Consistent with what has been reported by other authors, the overexpression of the regulatory genes associated with the CA synthesis would generate significant increases in its production (Jnawali *et al.*, 2011; Kurt-Kizildoğan *et al.*, 2017; Song *et al.*, 2010). In our study, the best ranking in increasing the CA secretion was obtained for the overexpression of the enzymes in the early steps of the CA biosynthesis pathway. Particularly, the overexpression of CEAS leads to an increase in the carbon flux along the complete CA pathway, leading to an increase in the CA secretion. In our FBA simulations, no significant fluxes through the 5S clavams pathway were observed under phosphate limitation and adequate carbon, nitrogen, and oxygen supply. Therefore, all the carbon fluxes directed to CEAS might directly impact the CA biosynthesis. As suggested by the results shown in Table 2, the dampening of phosphoglycerate kinase (PGK) would improve the flux of GAP as the C-3 precursor of CA, while the flux of aspartate synthesized from glutamate would provide the flux of the C-5 precursor required for CA production under such condition.

The analysis of potential strain modifications indicates that a lower increase in CA production could be achieved by overexpressing the pyridoxal phosphate-dependent aminotransferase catalyzing the production of clavulanate-1-aldehyde, either by single overexpression or in combination with other modifications. Nevertheless, the synthesis of (3R, 5R) clavulanate-1-aldehyde has not been completely elucidated. However, this step might be related to the orf17 cluster encoding the N-glycyl-clavaminate synthetase, which has been proposed as one of the enzymes catalyzing the reactions leading to (3R, 5R) clavaldehyde (Gómez *et al.*, 2020; Ramirez-Malule *et al.*, 2016). Moreover, the clavaminic acid synthesis is the bifurcation point between 5S clavams and CA biosynthesis; therefore, the overexpression of regulatory or encoding genes linked to clavulanate-1-aldehyde synthesis would drive the carbon flux preferably toward CA instead of 5S clavams. Similarly, the single overex-

Table 2. Potential reaction targets for the enhancement of CA production by strain engineering of *S. clavuligerus.*

Modifications	Strategy	Enzyme	NCBI protein ID	$\mu.v_{CA,e}/v_{Glyc,e}$
Single	Overexpression	CEAS	ANW18,135	0.02756
Single	Knockout	PYK	ANW20,351.1	0.02041
Single	Dampening	PGK	ANW20,748.1	0.01821
Double	Overexpression	Claviminate aminotransferase (CV1)		0.00905
	Dampening	Ferredoxin (NADP)	ANW18,690.1	
Double	Overexpression	CV1		0.00507
	Dampening	PYRDC	ANW18,804.1	
Double	Overexpression	CV1		0.00448
	Dampening	ORNDC	ANW19,435.1	
Single	Overexpression	CAR	ANW18,142	0.00435
Double	Knockout	PGM	ANW17,043.1	0.00300
	Knockout	GGGABADr	ANW18,767.1	
Double	Knockout	ENO	ANW19,850.1	0.00255
	Knockout	Cystathionine beta synthase	ANW19,434.1	
Single	Dampening	Succinyl-CoA synthetase	ANW17,690.1	0.00245

pression of clavulanate-1-aldehyde dehydrogenase (CAR) would also potentially increase the CA secretion as the last step in biosynthesis. Since the demand for reducing cofactors increases when enhancing the CA production, these modifications might be accompanied by attenuations that would reduce the cost of these cofactors in other reactions without compromising cell growth. Likewise, to preserve the intracellular pool of intermediates required for the biosynthesis of CA, some decarboxylases can also be attenuated, such as pyruvate decarboxylase (PYRDC) and ornithine decarboxylase (ORNDC).

Previous experimental reports have shown that a significant increase in the production of CA can be achieved by disrupting the glyceraldehyde 3-phosphate dehydrogenase (GAPD), directing part of the flux of GAP to CA biosynthesis (Jnawali *et al.*, 2010; Li and Townsend, 2006). Our *in silico* analysis showed that the single deletion of the gene encoding the reaction catalyzed by pyruvate kinase (PYK) would also improve the flux of GAP toward CA biosynthesis. This modification implies the regulation of the flux passing from glycolysis to TCA cycle through the phosphoenolpyruvate carboxylase (PPC) to maintain the oxidative metabolism in the TCA cycle. Nevertheless, the kinase regulation in the *Streptomyces* genus is complex and it is still not completely elucidated, given its direct relationship with phosphate utilization and the response to environmental conditions. Phosphate limitation affects the adenosine triphosphate (ATP) availability and abundant generation of reduced cofactors and ATP may favor antibiotics synthesis to adjust the ATP generation to low phosphate availability (Gómez-Ríos *et al*., 2020). In the genus, more than 80 sensor kinases and more than 60 response regulators have been identified in the genome (Paradkar, 2013). Although the model shows insights into the stoichiometric effect of phosphate depletion on CA biosynthesis, the regulatory features are not captured by the FBA and dFBA approaches. Some reports have indicated that the expression levels of genes downstream from the CA gene cluster (orf-22 and orf-23) with sensor kinase activity affect the CA production levels (Paradkar, 2013). This suggests that the expression of kinases would play a role in the carbon flux regulation toward the CA biosynthetic pathway and those conditions might arise during the cultivation under specific conditions and not only via gene manipulation.

The potential knockout of phosphoglycerate mutase (PGM) would cut the conversion of 3-phosphoglycerate to 2-phosphoglycerate by alternatively activating the alcohol dehydrogenase for the use of glycerol as the sole source of carbon. This pathway is conducive to the formation of 2-phosphoglycerate via glycerate in three reactions, which is also a shorter route than glycolysis itself, so the flow of carbon to the TCA cycle is not compromised. The consequent accumulation of 2-phosphoglycerate and 3-phosphoglycerate favors the synthesis of amino acids and 2-oxoglutatarate, both widely required by secondary metabolism. Similarly, the formation of 3-phosphoglycerate is favored by the deletion of the cystathionine beta-synthase, avoiding the supply of 3-phosphoglycerate as a precursor in the serine–cysteine pathway. The gamma-glutamyl-gamma aminobutyric acid dehydrogenase (GGGABADr) connects succinate from the TCA cycle with glutamate metabolism; its dampening would reduce the carbon flux from glutamate to the TCA cycle, favoring the flux toward the urea

cycle. The deletion of enolase would cause the repression of PPC from 2-phosphoglycerate, generating the accumulation of this intermediate, then increasing the production of CA. The *in silico* modifications with the highest ratio of CA to glycerol usage were further assessed under a dFBA approach.

dFBA assessment of potential targets for strain engineering

Considering that fed-batch operation using a medium with glycerol, ammonium, and glutamate is suitable for the enhancement of CA production, an *in silico* analysis of cultivation was performed considering the most promissory gene modifications exerting the highest CA flux. *In silico* single overexpression of *ceas*2 and single deletion of *pyk* genes showed the best ratio of glycerol usage and CA secretion. Therefore, fed-batch cultivation of overexpressed *ceas*2 (twofold) and deleted *pyk* were simulated to assess the potential CA yield. As shown in Figure 2, the time course of CA secretion was different in all cases indicating a flux redistribution that positively impacts the CA biosynthesis. The highest *in silico* CA concentration was observed for *S. clavuligerus* Δ*ceas2*, whose maximum concentration was 1.9-fold calculated for the wild-type strain. The knockout of the *pyk* would also increase the CA production but to a lesser extent, showing a maximum increase of 1.1-fold for the concentration of the wild-type cultivation.

Figure 2 shows a summary of the dynamic fluxes distribution along primary metabolism and CA biosynthesis. The implementation of a fed-batch operation at 34 hours before the carbon sources starvation promotes higher and more stable metabolic activity in most of the enzymes, contributing to maintaining higher fluxes along the CA biosynthesis (Gómez-Ríos *et al*., 2022). The simulation of cultivation with Δ*ceas2* strain shows less carbon flux along the glycolysis in comparison with the Δ*pyk* and wild-type strains, shown in the lower reaction fluxes of GAPD and pyruvate dehydrogenase (PDH) in Figure 2. This is due to considerable demand for GAP directly from CEAS enzyme. Additionally, this modification would also cause an increase in the demand for TCA cycle products 2-oxoglutarate and arginine, leading to an increase in the reaction fluxes of most of the enzymes in the TCA cycle, and downstream in the urea cycle. Indeed, transcriptomic evidence has shown upregulation of CA pathway enzymes when synthesized under extensive amino acid supply (Pinilla *et al*., 2019). The demand for carbon from central metabolism and CA biosynthesis also reduces the fluxes considerably toward the pentose–phosphate pathway. Therefore, the highest fluxes for the whole TCA cycle and CA biosynthesis are expected for the Δ*ceas2* strain that showed the highest *in silico* CA production.

The deletion of *pyk* causes a slight redistribution of the metabolic fluxes by cutting the pyruvate synthesis from phosphoenolpyruvate. Thus, the carbon flux is redistributed toward the pentose–phosphate pathway, CA biosynthesis, and PPC. The flux through the Glucose 6-phosphate dehydrogenase as the first reaction on the pentose–phosphate pathway was the highest observed among all the simulations, as shown in Figure 2. In case of glycolysis, the PPC must act as the auxiliary connection point between the glycolysis and TCA cycle maintaining the carbon flux to the oxidative metabolism and the intermediates supply to the urea cycle and CA biosynthesis. Additionally, the demand for pyruvate from PDH and TCA cycle is compensated
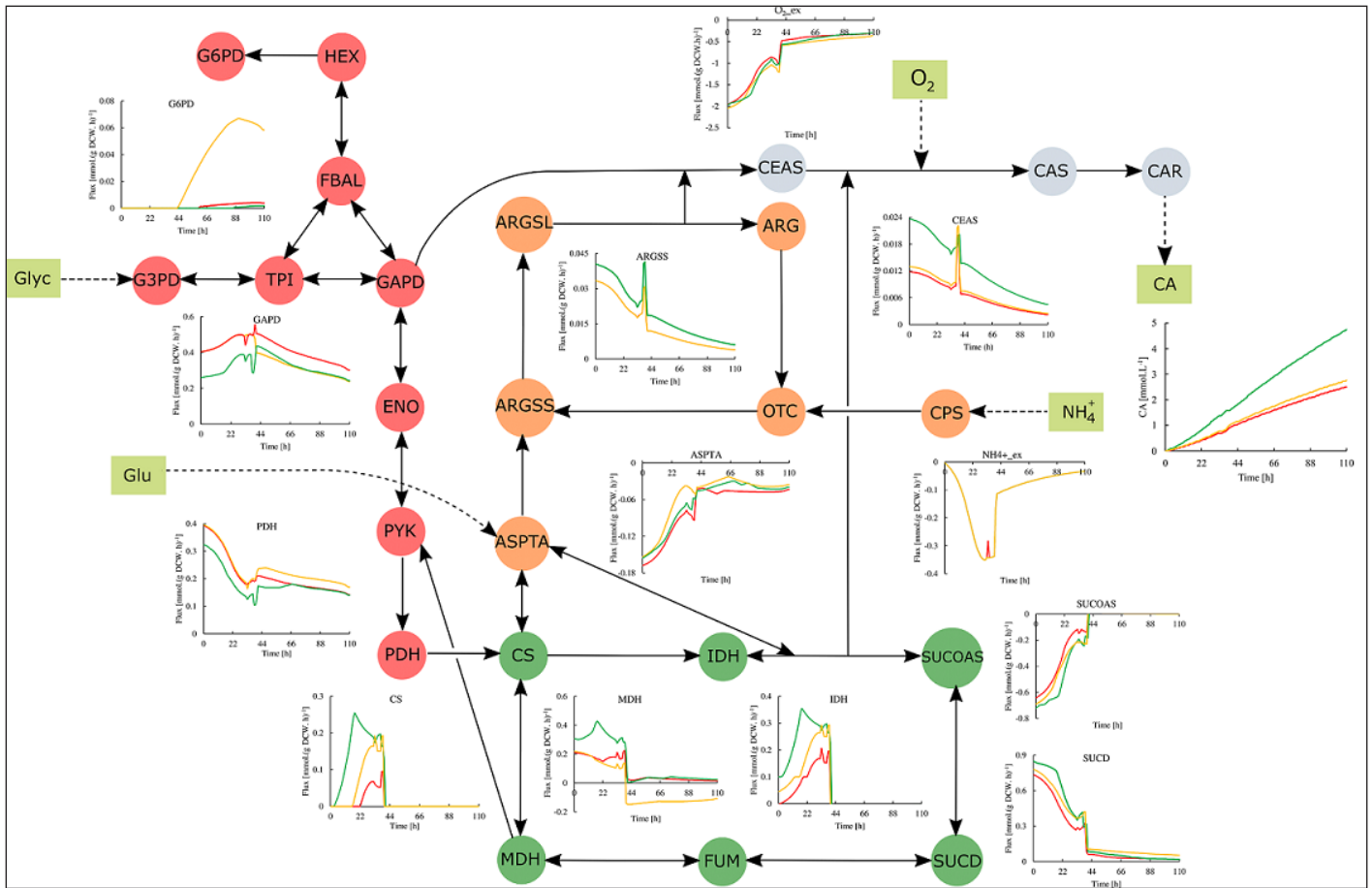
Figure 2. *In silico* dynamic flux distributions in *S. clavuligerus* for fed-batch cultures with wild-type (red line), Δ*ceas2* (green line), and Δ*pyk* (orange line) strains. The color in the metabolic network indicates enzymes related to central carbon pathways; red: glycolysis; green: TCA cycle; orange: urea cycle; and blue: CA biosynthesis.

by the malic enzyme, increasing the demand for malate, and then causing the flux inversion of the malate dehydrogenase. Thus, the flux throughout the TCA cycle does not decrease, despite the deletion of the last reaction of the glycolysis. The lower CA biosynthesis in comparison with the Δ*ceas2* strain is concordant with the lower activity of Argininosuccinate synthase, Aspartate dehydrogenase, and CEAS in connection with a lower demand for C-5 precursors.

CONCLUSION

Pseudo-steady-state simulations allowed us to analyze the sensitivity of the genome-scale metabolic model to constraints associated with the environmental cultivation conditions with biomass and CA as physiological objectives. Potential metabolic targets were determined via FBA based on the CA flux ratio. The single perturbations of CEAS and PYK reactions would cause a redistribution of metabolic fluxes during the culture, potentially favoring the CA accumulation. The dynamic simulations indicated that an increase in final antibiotic titers up to 1.9-fold for Δ*ceas2* and 1.1-fold for Δ*pyk* might be possible in fed-batch submerged cultivation. Our approach applied to the genome-scale under dynamic conditions provided complementary perspectives for genetic perturbation of *S. clavuligerus* metabolism, which aimed at improving antibiotic production levels. The proposed framework

constitutes a valuable tool for the exploration of production scenarios under different environmental conditions, genetic perturbations, and bioreactor operations (for instance, cell retention or product separation during the culture) as feasible strategies for redirecting the metabolism toward products of interest.

AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit it to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

LIST OF ABBREVIATIONS

ATP: Adenosine triphosphate; CA: Clavulanic acid; CAR: clavulanate-1-aldehyde dehydrogenase; CEAS: N2-(2-carboxyethyl)-arginine synthase; CephC: cephamycin C; CV1: Claviminate aminotransferase; dFBA: dynamic flux balance analysis; FBA: flux balance analysis; GAP: Glyceraldehyde 3-phosphate; GAPD: Glyceraldehyde 3-phosphate dehydrogenase; gDCW grams dry cell weight; GGGABADr: Gamma-glutamyl-gamma aminobutyric acid dehydrogenase; ODE: ordinary differential equation; ORNDC: Ornithine decarboxylase; PDH: Pyruvate dehydrogenase; PGK: Phosphoglycerate kinase; PGM:

Phosphoglycerate mutase; PYK: Pyruvate kinase; PYRDC: Pyruvate decarboxylase; TCA cycle: Tricarboxylic acid cycle.

CONFLICTS OF INTEREST

The authors report no financial or other conflicts of interest in this work.

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All data generated and analyzed are included within this research article.

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