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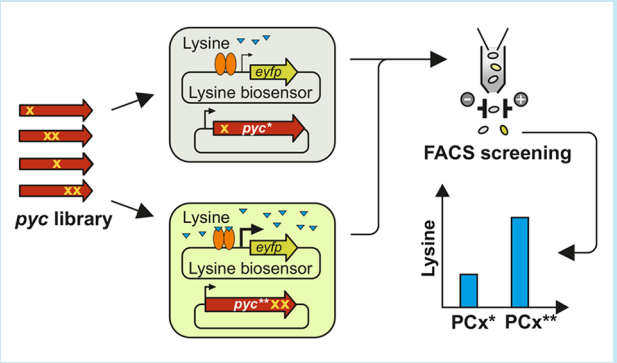
Pyruvate Carboxylase Variants Enabling Improved Lysine Production from Glucose Identified by Biosensor-Based High-Throughput Fluorescence-Activated Cell Sorting Screening

Maike Kortmann, Christina Mack, Meike Baumgart, and Michael Bott*

IBG-1: Biotechnology, Institute of Bio- and Geosciences, Forschungszentrum Jülich, 52425 Jülich, Germany

Supporting Information

ABSTRACT: Pyruvate carboxylase is an anaplerotic carbon dioxide-fixing enzyme replenishing the tricarboxylic acid cycle with oxaloacetate during growth on sugars. In this study, we applied a lysine biosensor to identify pyruvate carboxylase variants in *Corynebacterium glutamicum* that enable improved L-lysine production from glucose. A suitable reporter strain was transformed with a *pyc* gene library created by error-prone PCR and screened by fluorescence-activated cell sorting (FACS) for cells with increased fluorescence triggered by an elevated cytoplasmic lysine concentration. Two pyruvate carboxylase variants, $PCx^{T343A,I1012S}$ and PCx^{T132A} were identified allowing 9% and 19% increased lysine titers upon plasmid-based expression. Chromosomal expression of PCx^{T132A} and PCx^{T343A} variants led to 6% and 14% higher L-lysine levels. The new PCx variants can be useful also for other microbial strains producing TCA cycle-derived metabolites. Our approach indicates that a biosensor such as pSenLys enables directed evolution of many enzymes involved in converting a carbon source into the target metabolite.



KEYWORDS: *pyruvate carboxylase, Corynebacterium glutamicum, FACS-based screening, biosensor, lysine production, transcriptional regulator, LysG, metabolic engineering*

Many chemicals produced industrially with microbial cell factories as biocatalysts are intermediates or derivatives of the tricarboxylic acid (TCA) cycle, such as amino acids, organic acids, or diamines.[1] For continuous operation of the TCA cycle, anaplerotic reactions are required to replenish the deprived intermediates.[2] When sugars serve as carbon sources, the anaplerotic functions are fulfilled by pyruvate carboxylase (PCx)[3] and/or by phosphoenolpyruvate carboxylase (PEPCx).[4] Consequently, these enzymes are crucial for production of TCA cycle-derived metabolites from sugars (Figure 1A).

Corynebacterium glutamicum is the most prominent industrial amino acid producer with L-glutamate (flavor enhancer) and L-lysine (feed additive) being produced in the million tons scale per year.[5] In the past years, strains for production of many other TCA cycle-derived metabolites, such as succinate,[6,7] 1,5-diaminopentane,[8] or putrescine,[9] were constructed. Regarding anaplerosis, *C. glutamicum* is equipped with both PEPCx[10,11] and PCx.[12−14] Whereas PEPCx (encoded by the *ppc* gene) was shown to be dispensable for lysine production,[15,16] the deletion of the *pyc* gene for PCx caused a strong decrease, and its overexpression caused an increase in the synthesis of this amino acid.[17] Tween 60-induced glutamate production was lowered upon *pyc* deletion and increased by *pyc* overexpression, as well.[17] These results emphasize the importance of PCx as an anaplerotic enzyme for the production of amino acids of the glutamate and aspartate families. Overexpression of either the native *pyc* gene or of its pyc^{P458S} variant (see below) has often been used to improve various production strains of *C. glutamicum*, as summarized in Table S1. These examples confirm PCx as an important target to optimize sugar-based production processes for metabolites derived from the TCA cycle.

Only two studies have been reported on PCx variants of *C. glutamicum*. In the lysine-producing strain *C. glutamicum* B6, which was obtained from the type strain ATCC13032 by several rounds of random mutagenesis and selection, the amino acid exchange PCx^{P458S} was identified.[18] Introduction of the P458S mutation into the genomic *pyc* gene of strain AHD-2 (hom^{V59A}, $lysC^{T311I}$) caused an increase in lysine production from about 75 g/L to about 80 g/L.[18] The influence of the P458S mutation on PCx properties is unknown. In the patent US 7,300,777 B2,[19] a PCx variant was identified by genome sequence analysis of the lysine-producing *C. glutamicum* strain NRRL-B11474, which contained six amino acid exchanges (E153D, A182S, A206S, H227R, A455G, D1120E) compared to the reference sequence from *C. glutamicum* strain ATCC21253. Whereas PCx activity from ATCC21253 was

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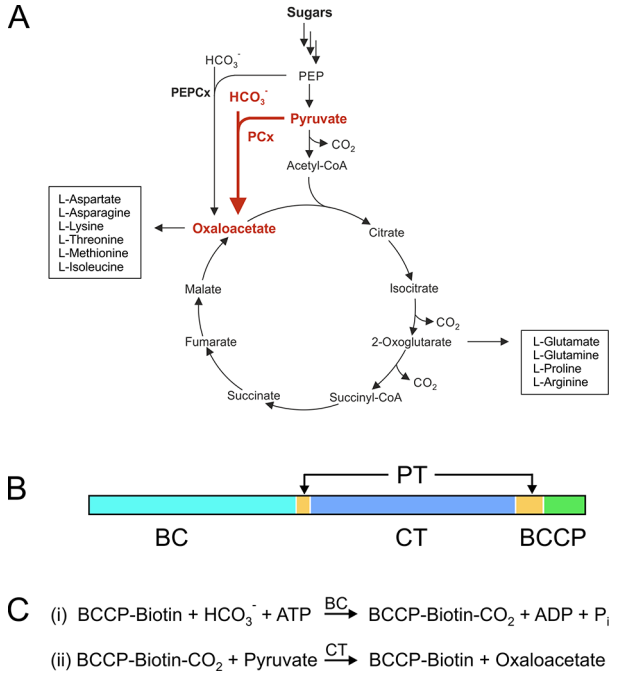

Figure 1. Function, domain organization, and partial reactions of pyruvate carboxylase. (A) Diagram highlighting the function of PCx as anaplerotic enzyme during growth on sugars and showing amino acids derived from the TCA cycle intermediates oxaloacetate and 2-oxoglutarate. (B) Domain organization of PCx. (C) Partial reactions catalyzed by PCx: BC, biotin carboxylase domains; CT, carboxyltransferase domain; BCCP, biotin carboxyl carrier protein; PT, tetramerization or allosteric domain.

strongly inhibited by low aspartate concentrations, PCx from NRRL-B11474 was activated by aspartate concentrations up to 30 mM. Higher concentrations reduced PCx activity, but even at 100 mM aspartate 50% of the original activity was retained. It is unknown how this PCx variant affects lysine production and which of the six amino acid substitutions are responsible for the altered aspartate sensitivity.

Besides DNA sequence analysis of producer strains generated by random mutagenesis, several other strategies to identify or generate improved enzyme variants are available. If structures or structural models of the target protein are available, amino acid residues involved, for example, in allosteric regulation can be identified and exchanged by site-directed mutagenesis. This abolishes or alters regulation, as exemplified by reducing aspartate inhibition of PEPCx.[20] We recently developed a strategy to identify "productive" mutations based on genetically encoded single-cell biosensors and high-throughput (HT) screening by fluorescence-activated cell sorting (FACS).[21] The key elements are selected transcriptional regulators that serve as metabolite sensors, since their activity is dependent on the concentration of a certain metabolite within the cell that acts as coactivator, corepressor, inhibitor, or inducer. By placing a suitable target promoter in front of a reporter gene encoding a fluorescent protein, the metabolite concentration can be converted into a fluorescent output.

A prominent example for this type of biosensor is the plasmid pSenLys, which harbors the transcriptional regulator LysG of *C. glutamicum*.[21] LysG senses increased intracellular concentrations of the basic amino acids L-lysine, L-arginine, and L-histidine and activates expression of its target gene *lysE* in complex with these coactivators.[22] In pSenLys, the *lysE* gene, encoding an exporter for L-lysine and L-arginine,[23] was replaced by *eyfp* to generate an optical output. pSenLys has successfully been employed for ultrafast screening of genome-wide, gene-specific, and site-specific mutant libraries by FACS for variants with improved L-lysine, L-arginine, or L-histidine production.[21,24,25] In this study, we tested whether pSenLys-Spc can be applied to search for novel PCx variants that enable improved production of L-lysine.

RESULTS AND DISCUSSION

Construction and Initial Characterization of a Reporter Strain for Screening of a *pyc* Mutant Library. The aim of the current study was to identify novel PCx variants that allow improved pyruvate carboxylation, measured as an increase in L-lysine production. For this purpose, we used the lysine biosensor pSenLys-Spc as a tool for FACS-based HT-screening of a plasmid-encoded PCx mutant library in *C. glutamicum*. This approach required a *C. glutamicum* strain already producing moderate levels of L-lysine, as PCx mutations alone are not sufficient to trigger lysine overproduction in a wild-type background due to feedback inhibition of aspartate kinase by lysine and threonine.[26] Therefore, we used strain DM1868, which is an ATCC13032 derivative encoding the feedback-resistant aspartate kinase variant LysC^{T311I} (Table S2). The T311I mutation is known to be sufficient to trigger lysine overproduction.[18] To avoid interference with the plasmid-encoded PCx variants, we deleted the chromosomal *pyc* gene in strain DM1868, resulting in strain DM1868Δ*pyc*. The deletion was confirmed by PCR using the oligonucleotides listed in Table S3 and by the growth defect of the strain in lactate minimal medium (Figure S1, see supplementary data for details).

To study the influence of *pyc* deletion and overexpression on L-lysine production in DM1868, the relevant strains were cultivated in CGXII minimal medium with 4% (w/v) glucose in a BioLector microcultivation system for 24 h. As shown in Table S4, the lysine titer of strain DM1868Δ*pyc*/pAN6 was reduced by 14% compared to the lysine titer of the parental strain DM1868/pAN6. In a previous study with the *C. glutamicum* strain DG52-5, deletion of *pyc* had a much stronger effect, as lysine production dropped by about 60%.[17] The reasons for this discrepancy could be the different strain background (strain DG52-5 was generated by random mutagenesis) and differences in the cultivation conditions. IPTG-induced overexpression of *pyc* via plasmid pAN6-*pyc* in strains DM1868 and DM1868Δ*pyc* caused an increase of the lysine titer by 63% compared to strain DM1868/pAN6. These results confirm that PCx activity based on chromosomal expression of *pyc* from its native promoter is a bottleneck for lysine overproduction. Similar results were obtained previously with strain DG52-5, where IPTG-induced *pyc* overexpression increased the lysine titer by 47%.[17] The results described above for strain DM1868Δ*pyc* suggested that this strain is suitable for screening a mutant library for improved PCx variants.

Testing the Reporter Strain by Comparison of Wild-Type PCx and the PCx^{P458S} Variant. To test whether the biosensor pSenLys-Spc[24] allows to distinguish cells with small differences in lysine production, strain DM1868Δ*pyc* was cotransformed with pSenLys-Spc and either pAN6, pAN6-*pyc*, or pAN6-*pyc*^{P458S}. The *pyc*^{P458S} variant was reported to enable a 7% increase in lysine production compared to wild-type *pyc* in a strain carrying additionally the *lysC*^{T311I} mutation.[18] After

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cultivation, the strains were analyzed by FACS and the median fluorescence output of the cells was determined. As shown in Figure 2, cells of the strain with the PCx^{P458S} variant showed a

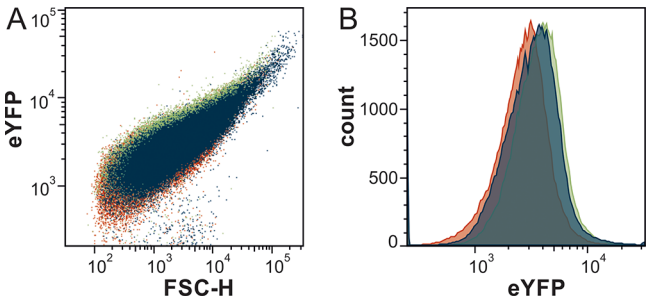

Figure 2. FACS analysis of *C. glutamicum* DM1868Δ*pyc* carrying pSenLys-Spc and different plasmid-encoded *pyc* variants. An overlay of DM1868Δ*pyc*/pSenLys-Spc transformed with either pAN6 (red), pAN6-*pyc* (blue), or pAN6-*pyc*^{P458S} (green) is shown as dot plot (A) and histogram (B). In the dot plot, the fluorescence intensity (eYFP) is plotted against cell size (FSC-H), whereas the histogram shows the cell count against the fluorescence intensity (eYFP).

median fluorescence of 3690, which is 13% higher than that of the strain with wild-type PCx and 36% higher than that of a PCx-negative strain. On the basis of these results, our reporter strain DM1868Δ*pyc*/pSenLys-Spc can be employed to distinguish between wild-type PCx and improved variants. However, due to the rather small difference in fluorescence output between the cells with wild-type PCx and optimized PCx variants, a high number of false positive cells was expected after FACS.

Construction and Screening of a *pyc* Mutant Library. A *pyc* mutant library was generated via error-prone PCR and cloned into pAN6, resulting in the library L-pAN6-*pyc*^{Mut}, which was subsequently used to transform *E. coli* NEB5α. The plasmids were isolated from about 9.6×10^4 colonies and used to transform the screening strain *C. glutamicum* DM1868Δ*pyc*/pSenLys-Spc. The resulting 1.1×10^6 transformants were pooled for further analysis. The mutant library and control strains were cultivated in glucose minimal medium until they reached on OD_{600} of 2 at which *pyc* expression was induced with 1 mM IPTG, and the cultures were incubated for another 4−5 h. After suitable dilution the cultures were used for FACS analysis (Figure 3).

Gate 0 was set for all subsequent FACS experiments to exclude cell doublets and debris from screening, which made up approximately 7% of all analyzed events of each culture (Table 1). Sorting of cells with increased fluorescence compared to the reference strain with wild-type PCx was accomplished using two different approaches: gate 1 was set in a dot plot of eYFP fluorescence against FSC (forward scatter, a parameter for cell size) in such a way that both the number of cells of the positive control strain with pAN6-*pyc*^{P458S} falling within the gate and the number of cells containing either pAN6-*pyc* or only pAN6 being excluded from the gate were maximized (Figure 3). Of 100 000 analyzed cells each, 272 cells of the positive control with pAN6-*pyc*^{P458S}, 185 cells of the reference strain expressing wild-type *pyc*, and only 9 cells of the negative control with pAN6 fell within this gate (Table 1). The overall fluorescence of the *pyc* mutant library was lower compared to cells harboring exclusively pAN6-*pyc* or pAN6-*pyc*^{P458S}, presumably because random mutagenesis resulted in a large fraction of PCx variants with lowered or lost activity.

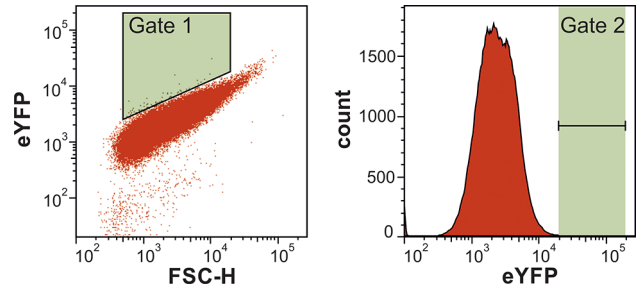

Figure 3. Sorting strategy for screening of the *pyc* mutant library in *C. glutamicum* DM1868Δ*pyc*/pSenLys-Spc. Cells were inoculated to an OD_{600} of 1 in CGXII minimal medium with 4% (w/v) glucose and cultivated at 30 °C. At an OD_{600} of 2, *pyc* expression was induced with 1 mM IPTG. After 5 h incubation, the cells were diluted to an OD_{600} < 0.1 in sterile PBS and directly analyzed by FACS. Selection of single cells with increased fluorescence was performed by setting either gate 1 in the dot plot or gate 2 in the histogram. Cells outside the gates were not analyzed further.

Nevertheless, 80 out of 100 000 analyzed cells of the mutant library fell into gate 1 (Figure 3; Table 1).

Gate 2 for sorting highly fluorescent cells was set in the histogram showing the cell count with respect to fluorescence intensity. In this approach, only one cell out of 100 000 cells of the negative control, 63 cells of the reference strain with pAN6-*pyc*, and 115 cells of the positive control with pAN6-*pyc*^{P458S} fell into this gate. With gate 2, cells were sorted independent of their cell size and hence only bigger cells were collected (data not shown). In contrast to the approach used for gate 1, only six out of 100 000 cells of the library were sorted using gate 2 (Figure 3; Table 1).

Using the gates described above, more than 1.7×10^6 cells of the mutant library were screened and 96 clones each of gate 1 and gate 2 were sorted onto BHI agar plates with kanamycin and spectinomycin for further analysis. After cultivation for 24 h, 74 cells from gate 1 and 71 cells from gate 2 had formed colonies.

Testing of Clones Isolated from the *pyc* Mutant Library Regarding Growth and L-Lysine Production. A total of 40 clones of each gate were evaluated regarding growth and specific fluorescence by cultivation in a BioLector and measurement of the L-lysine concentration in the supernatant after 28 h (Table 2). Three clones of gate 2 did not grow and were therefore not analyzed further. Moreover, 35 clones of gate 1 and 31 clones of gate 2 were excluded from further analysis because of lower or equal lysine titers and specific fluorescences compared to the reference strain DM1828Δ*pyc*/pSenLys-Spc/pAN6-*pyc*. As mentioned before, the high number of false positive clones was expected due to the small differences in fluorescence between wild-type PCx and the improved variants. Nevertheless, five clones isolated from the *pyc* library with gate 1 and six clones isolated with gate 2 were chosen for further analysis due to their increased L-lysine titers. Sequence analysis of the 11 *pyc* variants revealed two which did not contain any mutation and were therefore not analyzed further. In the other nine *pyc* variants, 21 mutations were identified, three of which were silent, whereas 18 resulted in amino acid exchanges (Table 2). These nine PCx variants (PCx^{C1}, PCx^{C2}, etc.) contained 1−4 amino acid exchanges each.

Analysis of Isolated Clones after Reconstruction. Potential beneficial mutations identified by the screening

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Table 1. FACS Analysis of *C. glutamicum* DM1868Δ*pyc*/pSenLys-Spc Carrying Either the *pyc* Mutant Library or Control Plasmids[a]

DM1868Δ*pyc*/pSenLys-Spc transformed with	Gate 0		Gate 1		Gate 2	
	events	median fluoresc.	events	median fluoresc.	events	median fluoresc.
pAN6	93 044	1 723	9	10 782	1	18 091
pAN6-*pyc*^{WT}	93 145	3 287	185	7 287	63	18 737
pAN6-*pyc*^{P458S}	93 031	3 504	272	7 861	115	18 862
L-pAN6-*pyc*^{Mut}	92 581	2 397	80	6 861	6	18 767

[a]100 000 cells of each strain were analyzed. For all sortings, electronic gating was set in a dot plot of FSC-H against FSC-W to exclude cell doublets and cell debris (gate 0). Selection of single cells with increased fluorescence was performed by setting either a gate in a dot plot (gate 1) or in a histogram (gate 2). The number of cells falling into the respective gates as well as the median fluorescence of the population within each gate is shown.

Table 2. Genotypic and Phenotypic Characterization of *C. glutamicum* Strains Isolated by FACS from the *pyc* Mutant Library and of Control Strains

gate or control strains	clones	mutation in *pyc*[a]	amino acid exchange in PCx[a]	specific fluorescence (AU)[b]	L-lysine (mM)[b]	L-lysine (mM) of reconstructed clones (RC)[c]
1	(R)C1	A205G	K069E	0.92	59.8	36.2 ± 2.9
		A2708G	D903G			
		T3001C	F1001L			
	(R)C2	C882T	H294H	0.94	57.7	32.6 ± 1.1
		A1171G	I391V			
		C1318A	R440R			
		G1957A	D653N			
		T3254C	V1085A			
		T3302C	I1101T			
	(R)C3	G1452C	K484N	0.86	61.9	17.5 ± 2.0
		G1564A	A522N			
	(R)C4	A1027G	T343A	1.02	53.9	41.8 ± 2.5*
		T3035G	I1012S			
	(R)C5	T3245C	V1082A	0.83	50.4	37.2 ± 3.0
2	(R)C7	A394G	T132A	0.46	47.2	45.7 ± 1.7**
	(R)C8			0.41	42.9	
	(R)C9	A2104G	I702V	1.38	42.7	40.8 ± 1.0*
	(R)C11	A3355G	I1119V	0.38	47.8	38.5 ± 1.7
	(R)C12			0.39	46.4	
	(R)C13	T2422C	Y808H	0.41	55.5	38.2 ± 3.9
		A2584G	N862D			
control strains	pAN6					20.9 ± 2.3
	pAN6-*pyc*			Gate 1:0.9	Gate 1:53.0	38.4 ± 2.2
				Gate 2:0.7	Gate 2:42.1	
	pAN6-*pyc*^{P458S}	C1372T	P458S			39.3 ± 3.0

[a]Positions of point mutations in *pyc* genes and the resulting amino acid exchanges in PCx are listed for 13 clones isolated by FACS (C1−C13). RC indicates the clones in which the *pyc* variants were reconstructed and transferred into *C. glutamicum* DM1868Δ*pyc*. [b]The specific fluoresence and the L-lysine titers of clones C1−C13 were determined after 28 h cultivation in a BioLector from a single experiment. [c]The *pyc* genes from clones C1−C13 were recloned into pAN6 and the resulting plasmids pAN6-*pyc*$^{RC1\text{-}RC13}$ were transferred into *C. glutamicum* DM1828Δ*pyc* without pSenLys-Spc. After cultivation for 24 h in a BioLector, the lysine concentration was determined. In this case, data represent average values and standard deviations of at least six biological replicates. Single and double asterisks indicate *p*-values of ≤0.05 and ≤0.01, respectively (*t* test).

procedure described above had to be re-evaluated in an independent strain and plasmid background in order to exclude secondary mutations on the plasmids or within the genome as causes of the improved L-lysine production. Hence, the mutated *pyc* genes of the clones C1, C2, C3, C4, C5, C7, C9, C11, and C13 were amplified by high-fidelity PCR and recloned into pAN6. *C. glutamicum* DM1868Δ*pyc* without pSenLys-Spc was transformed with the different pAN6-*pyc*^{Cx} plasmids and the resulting strains RC1, RC2, etc., as well as the reference strain DM1868Δ*pyc*/pAN6-*pyc* and the negative control DM1868Δ*pyc*/pAN6, were analyzed with respect to growth and L-lysine production as described above. As shown in Table 2, three strains showed a higher lysine titer than the reference strain after reconstruction. The best strain was RC7, which carried a single amino acid exchange (PCx^{T132A}) and formed 19% more lysine (45.7 ± 1.7 mM) than the reference strain (38.4 ± 2.2 mM). Second best was strain RC4 carrying two amino acid exchanges (PCx$^{T343A,\ I1012S}$) with a 9% higher lysine titer (41.8 ± 1.0 mM). The mentioned strains grew like the reference strain (data not shown).

Chromosomal Integration of Two *pyc* Mutations and Analysis of Their Impact on Growth and L-Lysine Production. For biotechnological production processes, chromosomally encoded variants of improved enzymes are often desirable. To exemplarily analyze the effects of chromosomal PCx mutations, we constructed the strains

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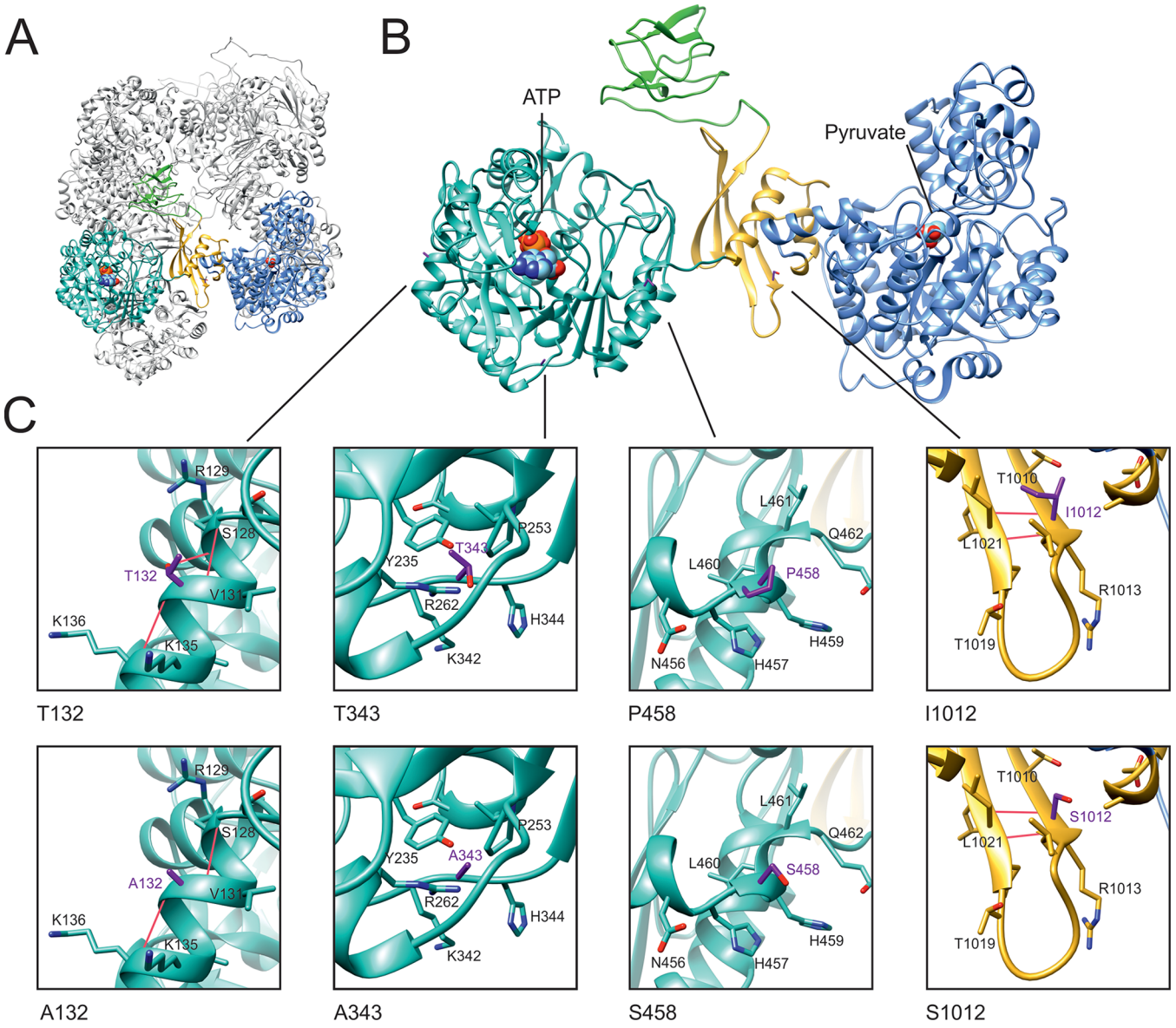

Figure 4. Structural model of PCx of *C. glutamicum* generated with SWISS-MODEL. Structure modeling was performed with SWISS-Model[28] using the crystal structure of *Staphylococcus aureus* PCx (PDB ID: 3BG5)[29] which shows 47% amino acid sequence identity to *C. glutamicum* PCx. In graphic A, the homotetrameric form of PCx is shown with one subunit colored. In graphic B, the colored monomer is shown in more detail with the biotin carboxylation domain (BC) in cyan, the PCx tetramerization domain (PT) in yellow, the biotin carboxyl carrier domain in green, and the carboxyltransferase domain (CT) in blue. Pyruvate is shown in the binding pocket of the CT domain and ATP in the binding pocket of the BC domain. In panels C, the vicinity of the four amino residues whose exchange was found to stimulate lysine synthesis is enlarged. The relevant regions with the wild-type amino acid residues (purple) and with the mutated residues (purple) are shown below each other. Oxygen atoms are marked in red, nitrogen atoms in blue, and hydrogen bonds are shown as red solid lines. The image was edited by using UCSF Chimera 1.10.2 (Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco).

DM1868 *pyc*^{T132A} and DM1868 *pyc*^{T343A} (Figure S2). Strain DM1800 was used as positive control, since it encodes the feedback-resistant aspartate kinase LysC^{T311I} as well as PCx^{P458S}. All strains were cultivated in a BioLector and the L-lysine concentration was measured in the supernatants after 24 h. The strains showed a comparable growth behavior and reached the stationary phase after 12 h (data not shown). Strain DM1800 produced 27.5 ± 3.1 mM lysine, which corresponds to an increase of 1.2% compared to strain DM1868 (27.2 ± 1.1 mM) and was not significant in a *t* test. The newly constructed chromosomal variants PCx^{T132A} and PCx^{T343A} led to an increase of the lysine titer by 7% (29.1 ± 3.0 mM) and by 15% (31.4 ± 1.7 mM), respectively.

Alignment and Structural Modeling of PCx. A structure-based alignment of different biotin-dependent carboxylases was performed using a 3DM database[27] to check the conservation of the mutated residues (Table S5). A sequence-based alignment of several PCx proteins is shown in Figure S3. The proline residue of the previously identified PCx^{P458S} variant is highly abundant among biotin-dependent carboxylases with 21.7% of all 9901 aligned sequences showing proline at position 458. Conversely, a serine residue at this position was found in only about 1% of the aligned sequences. The threonine residues changed in variants PCx^{T132A} and PCx^{T343A} show a low degree of conservation, and alanine residues are also found at these positions. Also, the isoleucine

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residue changed in the PCx^{I1012S} variant has a low degree of conservation and is often replaced by other branched-chain amino acids, but rarely by serine.

A homology model of *C. glutamicum* PCx was generated to analyze the amino acid exchanges T132A, T343A, I1012S, and P458S on the structural level (Figure 4). Residues T132, T343, and P458 are located on the surface of the BC domain, whereas I1012 is present in the PT domain. None of the residues is located near the active sites of PCx and the consequences of the mutations cannot be predicted from the model. Higher affinities for pyruvate or bicarbonate or reduced inhibition by aspartate could be possible reasons for the positive effects of the identified mutations on lysine production.

Concluding Remarks. The genetically encoded single-cell biosensor pSenLys enables the detection of increased cytoplasmic lysine concentrations as increased fluorescence at the single-cell level and allows FACS-based HT-screening.[21] In a previous study, pSenLys was employed to identify feedback-resistant variants of aspartate kinase.[24] In that case, wild-type *C. glutamicum* could be used as background for the *lysC* mutant library, as amino acid exchanges causing feedback-resistance of LysC are sufficient to trigger strong overproduction of lysine and consequently a strong increase in fluorescence. Cells carrying such LysC variants could be easily distinguished by FACS from cells carrying wild-type LysC. In the present study, the prerequisites for successful screening of a PCx mutant library with pSenLys were much more intricate, as PCx mutations alone are not sufficient to trigger lysine overproduction. Therefore, a strain that already overproduced lysine had to be used as a parent strain and the improved PCx variants enabled only small increases in lysine production and thus fluorescence. Nevertheless, our results demonstrate that these small differences were sufficient to identify new beneficial mutations in PCx, while also leading to a high fraction of false positive clones that had to be eliminated. For this purpose, efficient medium-throughput analysis of the originally sorted clones by microscale cultivation devices and rapid product analysis methods is inevitable.

The fact that, despite the difficult experimental setup, PCx variants enabling increased lysine production could be isolated from a relatively small library (ca. 10^5 clones) underlines the potential of the directed evolution approach used here. Particularly, it suggests that biosensors such as pSenLys can be used to screen for optimized variants of many enzymes involved in transforming the substrate, for example, glucose, into the analyzed product, for example, lysine. Thus, the approach might enable the identification of improved variants of enzymes of central metabolic pathways such as glycolysis or the pentose phosphate pathway, which in turn are expected to be useful for metabolic engineering of a large spectrum of producer strains for diverse products, all of which involve these enzymes in their biosynthesis.

METHODS

Bacterial Strains, Plasmids, Growth Conditions, Recombinant DNA Techniques, and Lysine Quantification. Strains, plasmids, growth conditions, recombinant DNA techniques, and lysine quantification by HPLC are described in the Supporting Information.

Library Construction. For the construction of a *pyc* mutant library, the *C. glutamicum pyc* open reading frame (cg0791) with additionally ~50 bp upstream and downstream regions was amplified from plasmid pUC18-*pyc* with the GeneMorph II Random Mutagenesis Kit (Agilent Technology, Santa Clara, CA, USA) and oligonucleotides EP-*pyc*-for and EP-*pyc*-rev. Conditions were adjusted in such a way that about four mutations/gene were introduced. Error prone-PCR products were purified, digested with NdeI and MfeI, and ligated with plasmid pAN6 that had been cut with NdeI and EcoRI. The ligation mixture was used to transform chemically competent *E. coli* NEB5α cells. The transformation mixture was spread onto two agar plates (Ø 260 mm) with 50 mg L^{-1} kanamycin and incubated for 24 h at 30 °C. Additional control plates were prepared for the determination of the library size. Afterward, all colonies were washed off the plate and pooled in LB medium. The L-pAN6-pyc^{Mut} library was isolated with the QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany) and used to transform *C. glutamicum* DM1868Δ*pyc* already harboring plasmid pSenLys-Spc. The cells were spread onto BHI agar plates (Ø 260 mm) with 12.5 mg L^{-1} kanamycin and 125 mg L^{-1} spectinomycin. After 24 h of incubation at 30 °C all colonies were pooled in 4 mL of BHI medium with appropriate antibiotics. Aliquots were supplemented with glycerol to a final concentration of 12.5% (v/v) and stored at −80 °C.

FACS Analysis. FACS analysis was performed using a FACS ARIA II high-speed cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). *C. glutamicum* strains were first cultivated as 10 mL BHI cultures in 100 mL baffled shaking flasks at 130 rpm and 30 °C for 16 h. After these cells were washed with 0.9% (w/v) NaCl, they were used to inoculate the main cultures of 50 mL of CGXII medium with 4% (w/v) glucose in 500 mL baffled shake flasks to an OD_{600} of 1. Expression of *pyc* was induced by the addition of 1 mM IPTG at an OD_{600} of 2. After further cultivation for 4−5 h, the cells were diluted to an OD_{600} below 0.1 in sterile phosphate-buffered saline (PBS, 37 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and used immediately for FACS analysis. A 488 nm blue solid state laser was used to detect eYFP fluorescence with a 502 nm long-pass and a 530/30 nm band-pass filter set. Forward-scatter (FSC) and side-scatter (SSC) were detected as a voltage pulse of height (H), weight (W), and area (A). Cells were analyzed with a threshold rate up to 6000 events s^{-1} and a sample pressure of 70 psi. For cell sorting, a preselection of cells was performed to exclude doublets and debris by electronic gating using FSC-W against FSC-H. Gates in the eYFP channel were set to exclude low-fluorescent cells harboring pAN6 or pAN6-*pyc*. Clones of each gate showing an enhanced eYFP signal were sorted on BHI agar plates with 12.5 mg L^{-1} kanamycin and 125 mg L^{-1} spectinomycin, which were subsequently incubated for 24 h at 30 °C. Afterward, each colony was used to inoculate 5 mL of BHI medium supplemented with 12.5 mg L^{-1} kanamycin and 125 mg L^{-1} spectinomycin, and cells were grown overnight at 30 °C and 170 rpm. From these cultures glycerol stocks were prepared with a final glycerol concentration of 12.5% (v/v) in BHI media and stored at −80 °C until the clones were analyzed further. All FACS data were analyzed using BD DIVA 6.1.3 and FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA).

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.8b00510.

Bacterial strains, plasmids, and growth conditions; recombinant DNA techniques; construction of plasmid pUC18-*pyc*, strain DM1868Δ*pyc*, and strains with chromosomally encoded PCx variants; Western blot analysis; lysine quantification; relevance of PCx for growth of *C. glutamicum* DM1868 on lactate as carbon source; additional tables and figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: +49 2461 613294. Fax: +49 2461 612710. E-mail: m.bott@fz-juelich.de.

ORCID

Maike Kortmann: 0000-0001-9733-9569

Meike Baumgart: 0000-0002-9874-1151

Michael Bott: 0000-0002-4701-8254

Author Contributions

M.K. performed the experiments, analyzed the data and participated in the design of the research. C.M. supported some of the experimental work. M.Bo. and M.Ba. analyzed the data and designed the research. All authors participated in writing of the manuscript.

Notes

The authors declare the following competing financial interest(s): M.Bo., M.Ba. and M.K. have filed patent applications covering results described in this study.

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