Corynebacterium glutamicum Metabolic Engineering with CRISPR Interference (CRISPRi)

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Ⓢ Supporting Information

ABSTRACT: *Corynebacterium glutamicum* is an important organism for the industrial production of amino acids. Metabolic pathways in this organism are usually engineered by conventional methods such as homologous recombination, which depends on rare double-crossover events. To facilitate the mapping of gene expression levels to metabolic outputs, we applied CRISPR interference (CRISPRi) technology using deactivated Cas9 (dCas9) to repress genes in *C. glutamicum*. We then determined the effects of target repression on amino acid titers. Single-guide RNAs directing dCas9 to specific targets reduced expression of *pgi* and *pck* up to 98%, and of *pyk* up to 97%, resulting in titer enhancement ratios of L-lysine and

L-glutamate production comparable to levels achieved by gene deletion. This approach for *C. glutamicum* metabolic engineering, which only requires 3 days, indicates that CRISPRi can be used for quick and efficient metabolic pathway remodeling without the need for gene deletions or mutations and subsequent selection.

KEYWORDS: *C. glutamicum*, CRISPRi, sgRNA/dCas9, amino acid, metabolic engineering
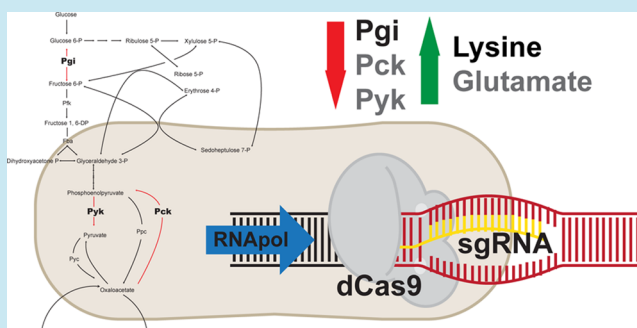
For the past 50 years, the industrialized world has relied on the extraordinary ability of the soil organism *Corynebacterium glutamicum* to synthesize and secrete copious amounts of amino acids.[1−3] Molecules generated *via* *C. glutamicum* fermentation are used as components in animal feed, nutritional supplements, cosmetics, flavor enhancers, and the synthesis of pharmaceuticals.[4−6] These products rank among the most important industrial bioproducts and are projected to reach a market size of US $20.4 billion by 2020.[7]

Early efforts to increase amino acid production by *C. glutamicum* involved random chemical or ultraviolet DNA mutagenesis.[1,8] Despite their stronger producer phenotypes, the mutagenized bacteria, initially genetically uncharacterized, were often genetically unstable or had growth defects.[1,9,10] With the advancement of molecular genetics and whole genome sequencing, methods of rational metabolic engineering could be applied: specific genes could be knocked out or introduced into the genetic makeup of an organism to enhance its production of the desired metabolites.[11] Genetic modifications can be introduced in *C. glutamicum* by transposon mutagenesis, homologous recombination, and recombinase-mediated deletions. Transposable elements can be randomly inserted into the *C. glutamicum* genome,[9] but precise gene modifications rely on the integration of suicide vectors, which can be followed by a second recombination event to remove the plasmid backbone.

However, these genetic engineering tools are not very efficient. Suicide vectors are integrated into the bacterial genome with an efficiency of 10^2 mutants per μg of DNA, of which 98% correspond to Campbell-like integrations and 2% correspond to double-crossover events.[12] SacB counterselection facilitates screening for double-crossover events: for example, out of 2×10^6 cells having a single-crossover event, 200 (0.01%) grew on sucrose, 56 of which had undergone a second recombination that excised the plasmid backbone only.[13] Others have reported integration efficiencies (Campbell-like) of 2.4×10^2 per μg of nonmethylated plasmid DNA.[14] These integrative vectors can be engineered to overexpress genes, by using constitutive or inducible promoters such as P_{180} (constitutive), P_{tac} (inducible if LacI^q present), P_{trc} (inducible if LacI^q present), and P_{prpD2} (inducible).[9,15,16] The recombinase-based Cre/*loxP* system has further enabled the deletion of chromosomal regions.[9,17,18] This efficient two-component system requires the integration of two *loxP* sites in the same orientation by homologous recombination. Once the sites are integrated so as to delimit the locus of interest and Cre recombinase is expressed, the DNA between the two *loxP* sites is efficiently excised.[19]

Systems biology has made considerable contributions to our understanding of the underlying intricacies and interrelationships of metabolic pathways[2,20,21] and may enable the predictive design of strains that meet well-defined specifications.[22−26] If the predicted phenotype resulting from the loss or reduced expression of one or more gene functions could be quickly and easily confirmed, the design-build-test cycle for the metabolic engineering of *C. glutamicum* could be accelerated. For example, it can be useful to construct derivative strains in which the expression of particular genes is reduced rather than knocked-out. The deletion of *gltA* in lysine-producing *C. glutamicum* rendered it unable to grow on glucose; however, this deletion resulted in suppressor mutants producing increased levels of L-lysine.[27] This led to another study where laborious promoter tuning to identify optimal levels of *gltA* expression for L-lysine production by *C. glutamicum* was performed, resulting in the highest reported L-lysine yield at that time.[28] Using existing approaches, it has been technically more difficult to generate mutants with reduced gene expression than those entirely lacking the expression of a particular gene (*i.e.*, knockout mutants).[29]

In other organisms, tools are available for perturbing gene expression in a targeted fashion.[30] For example, small regulatory RNAs (sRNA) have been used to identify and modulate the expression of genes of interest in *Escherichia coli*. Via combinatorial knockdown of several genes, the capacity of *E. coli* to produce tyrosine was approximately doubled,[31] and *E. coli* strains having a 55% increase in cadaverine production were identified by using a library of synthetic sRNAs.[31] This system, which works at the translational level, consists of a scaffold sequence and a target-binding sequence. The scaffold sequence is responsible for recruiting the RNA chaperone Hfq, which is required for the annealing of sRNA to its mRNA target, facilitating the degradation of that specific mRNA.[32]

Although Hfq homologues have been found in more than 50% of sequenced bacteria, *C. glutamicum* does not contain one.[33,34] In fact, knowledge of sRNAs in Actinobacteria is very limited. Only recently have putative sRNA genes been identified in *C. glutamicum* (over 800 were found by using a high-throughput sequencing approach[34]). Despite some advances,[35] it is not fully understood how this sRNA regulatory mechanism functions in *C. glutamicum*. Further studies are thus required to determine the suitability of using synthetic sRNAs for modulating gene expression in this organism, as has been done with *E. coli*.

To understand how modifying the expression of specific genes in *C. glutamicum* affects the titer of a particular bioproduct or alters pathway flux,[36,37] a quick, reliable and easy approach is needed. The deactivated version of the Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 (CRISPR/Cas9)[38,39] system, also known as CRISPR/dCas9 or CRISPR interference, offers a way to manipulate expression levels of specific genes.[40,41] Using the *Streptococcus pyogenes* Cas9 protein, a single guide RNA (sgRNA) sequence is sufficient to direct Cas9 binding to specific DNA targets with a protospacer adjacent motif containing a GG dinucleotide.[42] As opposed to the active Cas9 nuclease, the deactivated Cas9 protein (dCas9) used in CRISPRi lacks nuclease activity. dCas9 nonetheless retains the capacity to complex with the sgRNA and bind to the homologous locus. Instead of cleaving the DNA, this complex sterically blocks the progression of RNA polymerase and inhibits transcription. This process does not introduce permanent DNA-encoded mutations and thus

phenotypes induced by CRISPRi can be reversed by shutting the CRISPRi system off.[40,43]

Here, we report the application of CRISPRi to *C. glutamicum*, and its usefulness in modulating the production of the amino acids L-lysine and L-glutamate via metabolic pathway regulation. CRISPRi provides a way to bypass the traditional process of creating genetic mutants, which can be time-consuming and cumbersome. Ultimately, CRISPRi should enable the scalable and combinatorial targeting of multiple genes, the mapping of gene expression pathways to biosynthesis, and the enhanced productivity of *C. glutamicum*.

■ RESULTS AND DISCUSSION

CRISPRi-based *rfp* Repression. To analyze the utility of CRISPRi for gene repression in *C. glutamicum*, we first integrated the gene coding for a red fluorescent protein (*rfp*) into its chromosome. We chose to target both the template (T) and nontemplate (NT) strands of *rfp* as there are contradictory reports regarding the efficiency of T-targeting at repressing transcription. Some authors have reported that T-targeting is inefficient,[40,42] whereas others have found T-targeting to lead to effective transcriptional repression.[41,44] Inducing the expression of a plasmid-borne *dcas9* with sodium propionate (using the P_{prpD2} promoter) in cells expressing sgRNAs targeted at the template (T) or nontemplate (NT) strand of the *rfp* gene resulted in reduced RFP production (Supplemental Figure S1). Surprisingly, adding sodium propionate to the strain expressing *dcas9* alone (*i.e.*, no sgRNA) resulted in higher levels of *rfp* expression.

CRISPRi-Based Regulation of Amino Acid Production by *C. glutamicum*. Having seen that a heterologous gene (*rfp*) could be repressed by CRISPRi in *C. glutamicum*, we used this tool to target three endogenous genes of commercial relevance: *pgi*, *pck* and *pyk*. The deletion of *pgi* leads to NADPH overproduction through the pentose-phosphate pathway and this results in increased L-lysine titers.[23,45] The deletion of *pck* or *pyk* indirectly increases L-glutamate production.[24,46] The disruption of *pyk* is thought to result in increased L-glutamate production through an enhanced anaplerotic flux.[24,47] Disrupting *pck* also results in an accumulation of L-glutamate, via an enhanced flux toward oxaloacetate in the TCA cycle, due to the disruption of backward flux from oxaloacetate to phosphoenolpyruvate.[46]

To repress these genes, we built several sgRNAs that together with dCas9 would sterically block transcription. In initial experiments, we found that producing dCas9 from an unrepressed P_{tac} promoter resulted in no clones after transformation (data not shown). Previous work showed that other bacterial hosts in which dCas9 was expressed grew poorly or not at all.[44,48] To overcome this issue, we repressed sgRNA and *dcas9* expression from P_{tac} via the LacI transcription factor, which can be induced by the addition of IPTG. These constructs were located on independent replicative plasmids: sgRNAs from pAL374 (Table 1), and dCas9 from plasmid pZ8−1 (Table 1). The following experiments were performed in CgXII minimal medium with 2% (w/v) glucose as carbon source. In this section, sampling for amino acid quantification was performed immediately after glucose depletion with cells in stationary phase (the standard for quantification of maximal amino acid production[49,50]).

When the CRISPRi system targeted the NT strand of *pgi*, the titer of L-lysine ($p = 4.65 \times 10^{-13}$) increased by a factor of 2.1 over its sgRNA-less counterpart (Table 2; Figure 1), suggesting

Table 1. Strains and Plasmids Used in This Study

strain	properties	source
C. glutamicum ATCC 13032	Wild type, biotin auxotroph	ATCC
C. glutamicum DM1729 (DSM17576)	DM1729 is an aminoethylcysteine-resistant mutant of ATCC 13032; pyc(P458S) hom(V59A) lysC(T311I)-L-Lysine overproducer	Evonik Degussa GmbH
E. coli DH5α	Cloning strain	Lab stock

plasmids	properties	source
pDSW204	*E. coli* IPTG inducible expression vector, AmpR	[59]
pZ8−1	*E. coli−C. glutamicum* P_{tac} constitutive expression shuttle vector, KanR	Evonik Degussa GmbH
pAL374	*E. coli−Corynebacterineae* expression shuttle vector, SpecR	[60]
pZ8-T_dCas9	pZ8−1 plasmid carrying *dcas9*, driven by the IPTG-inducible P_{tac} promoter, KanR	This study
pZ8-P_dCas9	pZ8−1 plasmid carrying *dcas9* driven by the propionate-inducible *prpD2* promoter (P_{prpD2}), KanR	This study
pZ8-Ptac	IPTG inducible version of pZ8−1 plasmid, to which *lacI^q* was added, KanR	This study
pZ8-Prp	pZ8−1 with the P_{prpD2} propionate-inducible promoter instead of P_{tac}, KanR	This study
pPP208	Donor of *dcas9*	[54]
pAL-pgi_T	pAL374 plasmid carrying the *pgi* (T) sgRNA, targeting the template strand of *pgi*, SpecR	This study
pAL-pgi_NT	pAL374 plasmid carrying the *pgi* (NT) sgRNA, targeting the nontemplate strand of *pgi*, SpecR	This study
pAL-pck_T	pAL374 plasmid carrying the *pck* (T) sgRNA targeting, the template strand of *pck*, SpecR	This study
pAL-pck_NT	pAL374 plasmid carrying the *pck* (NT) sgRNA, targeting the nontemplate strand of *pck*, SpecR	This study
pAL-pyk_T	pAL374 plasmid carrying the *pyk* (T) sgRNA targeting the template strand of *pyk*, SpecR	This study
pAL-pyk_NT	pAL374 plasmid carrying the *pyk* (NT) sgRNA targeting the nontemplate strand of *pyk*, SpecR	This study
pRIM2	*E.coli−C. glutamicum* shuttle vector, integrative in *C. glutamicum* downstream of *ppc*, KanR	[61]
pRIM3	GentR version of pRIM2	This study
pRIM3-rfp	pRIM3 carrying *rfp* GentR	This study
pBTK30	Donor of GentR	[62]
pZ8−1_rfp	Donor of *rfp*, KanR	Sinskey Lab (MIT)
pAL-rfp_NT	pAL374 plasmid carrying the *rfp* (NT) sgRNA targeting the nontemplate strand of *rfp*, SpecR	This study
pAL-rfp_T	pAL374 plasmid carrying the *rfp* (T) sgRNA targeting the template strand of *rfp*, SpecR	This study

Table 2. Ratio of Changes in Amino Acid Production, mRNA Levels and Enzymatic Activity in Cultures of *C. glutamicum* when Targeting Genes with CRISPRi[a]

gene targeted (ID)	strand targeted	amino acid ratio (after glucose depletion in stationary phase)		mRNA ratio (exponential phase)	amino acid ratio (exponential phase)	enzyme activity ratio (exponential phase)
		CRISPRi	gene deletion (reference values)			
pgi	T	1.05	1.7[45]	1.12	1.01	1.01
	NT	2.14		0.02	1.31	0.05
pck	T	2.18	4.5[46 b]	0.31	1.28	0.51
	NT	2.24		0.02	1.46	0.17
pyk	T	1.92	1.25[24 c]	0.16	1.97	0.74
	NT	3.25		0.03	3.04	0.48

[a]Results are expressed as the ratio between (dCas9+sgRNA strain)/(dCas9+no sgRNA strain). [b]In the study referenced, Tween 60 was used to induce glutamate production, whereas ethambutol was used in our paper. [c]In the study referenced, biotin was used to induce glutamate production, whereas ethambutol was used in our paper. T = template; NT = nontemplate.

strong repression of Pgi production. A milder effect (a 1.05-fold increase over the control strain with dCas9 but without the sgRNA) was observed when the sgRNA targeted the T strand of the same gene ($p = 0.048$). The increase in L-lysine production was much stronger when targeting the NT *versus* the T strand ($p = 2.31 \times 10^{-12}$). In the absence of dCas9, expression of the sgRNAs alone did not interfere with the production of L-lysine (Figure 1c).

For *pck*, targeting either the T or NT strands resulted in increased glutamate production by ~2.2-fold compared with a similar strain that only lacked the sgRNA (Figure 2; $p_T = 0.02$; $p_{NT} = 0.006$). No significant difference was observed between targeting the T or NT strand (Figure 2; $p = 0.90$). For *pyk*, targeting the T strand increased L-glutamate production by ~1.9 fold ($p = 0.03$) and targeting the NT strand increased it 3.2-fold compared with the control strain carrying the dCas9 but no sgRNA (Figure 3; $p = 0.002$). Similar to *pgi*, there was a statistically significant difference between the fold-induction

achieved by targeting the T *versus* the NT strand of *pyk*, but the magnitude of the difference was smaller (Figure 3; $p = 0.05$). In the absence of dCas9, expression of the sgRNAs alone did not interfere with the production of L-glutamate (Figure 2c and 3c).

The increased L-lysine production we observed by repressing *pgi* with CRISPRi is similar to that observed when *pgi* is deleted (Table 2).[45] The repression of *pck* and *pyk* expression *via* CRISPRi achieved L-glutamate production ratios (targeted/nontargeted) that exceed published results for the deletion of those genes (Table 2).[24,45,46] However, the absolute amounts of amino acids produced using the CRISPRi system were not as high as when the genes were deleted,[24,45,46] which could be due to the use of different strains or conditions, or because of residual enzyme expression due to incomplete repression by CRISPRi.

Quantification of the mRNA Levels of Genes Targeted by CRISPRi. We next evaluated the levels of transcription of each gene targeted by CRISPRi (Figure 4). Here, we sampled
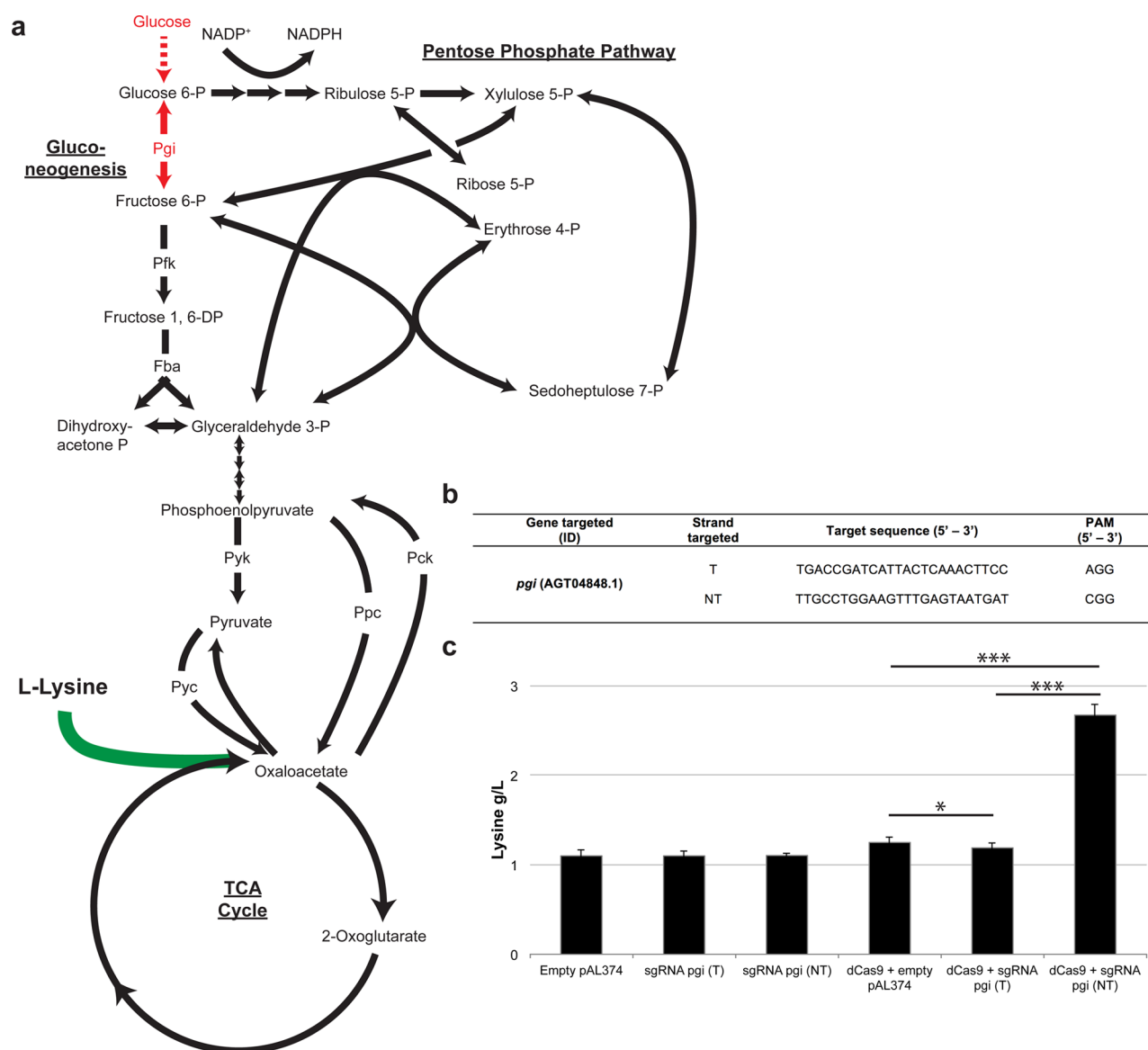
Figure 1. CRISPRi efficiently represses *pgi* transcription, increasing L-lysine production. (a) Central metabolic pathway. The notation in red represents the reduced transcription of *pgi* due to CRISPRi-mediated repression. (b) Gene and sequences targeted by dCas9. (c) Amino acid titers (g/L) of the control and test strains ($N = 9$ per strain) were determined upon glucose depletion. The error bars represent the standard deviation of samples. $*p \leq 0.05$ and $***p \leq 0.001$.

C. glutamicum cultures in midexponential phase (maximal growth rate) to estimate mRNA levels and concomitant amino acid production. We normalized mRNA levels of the targeted genes to those of 16S rRNA and calculated grams of amino acid produced per gram of cell dry weight, to account for the slight variations between the ODs of the strains and independent experiments, at the time of sampling.

No CRISPRi-mediated repression of transcription was observed during the exponential phase when the T strand of *pgi* was targeted; however, targeting the NT strand strongly repressed mRNA levels (Figure 4a, Table 2). The relative levels of mRNA were reduced by nearly 98% ($p = 1.02 \times 10^{-13}$), resulting in a 1.31-fold increase in L-lysine/gCDW, *versus* the control strain, which expressed dCas9 but lacked the sgRNA ($p = 3.40 \times 10^{-8}$). When the T strand of *pck* was targeted, a 69% ($p = 2.04 \times 10^{-7}$) reduction in its mRNA levels and a 1.28-fold increase in the levels of secreted L-glutamate/gCDW ($p = 0.02$) were observed over the control levels of the strain lacking the

sgRNA only (Figure 4, Table 2). In comparison, transcriptional repression was much higher when dCas9 was directed against the NT strand of *pck* (98% reduction in mRNA *versus* the sgRNA-less counterpart, $p = 1.13 \times 10^{-14}$); this repression appeared to result in 1.46-fold more L-glutamate/gCDW compared to the control strain. Despite the strong transcriptional repression (Figure 4b), the variability of the experimental data indicated that the increase in L-glutamate/gCDW in the exponential phase was nonsignificant at the $p = 0.08$ level, which contrasts with the data obtained in stationary phase shown in Figure 2c. Finally, targeting the T and NT strands of *pyk* with CRISPRi resulted in mRNA reductions of 84% ($p_T = 4.35 \times 10^{-5}$) and 97% ($p_{NT} = 0.0002$) and 1.97 ($p_T = 0.016$) and 3.04 ($p_{NT} = 0.0005$) fold increases in secreted L-glutamate/gCDW, respectively (Figure 4, Table 2), *versus* the control strain. In general, greater mRNA repression correlated with higher levels of secreted amino acids per gram of cell dry weight.
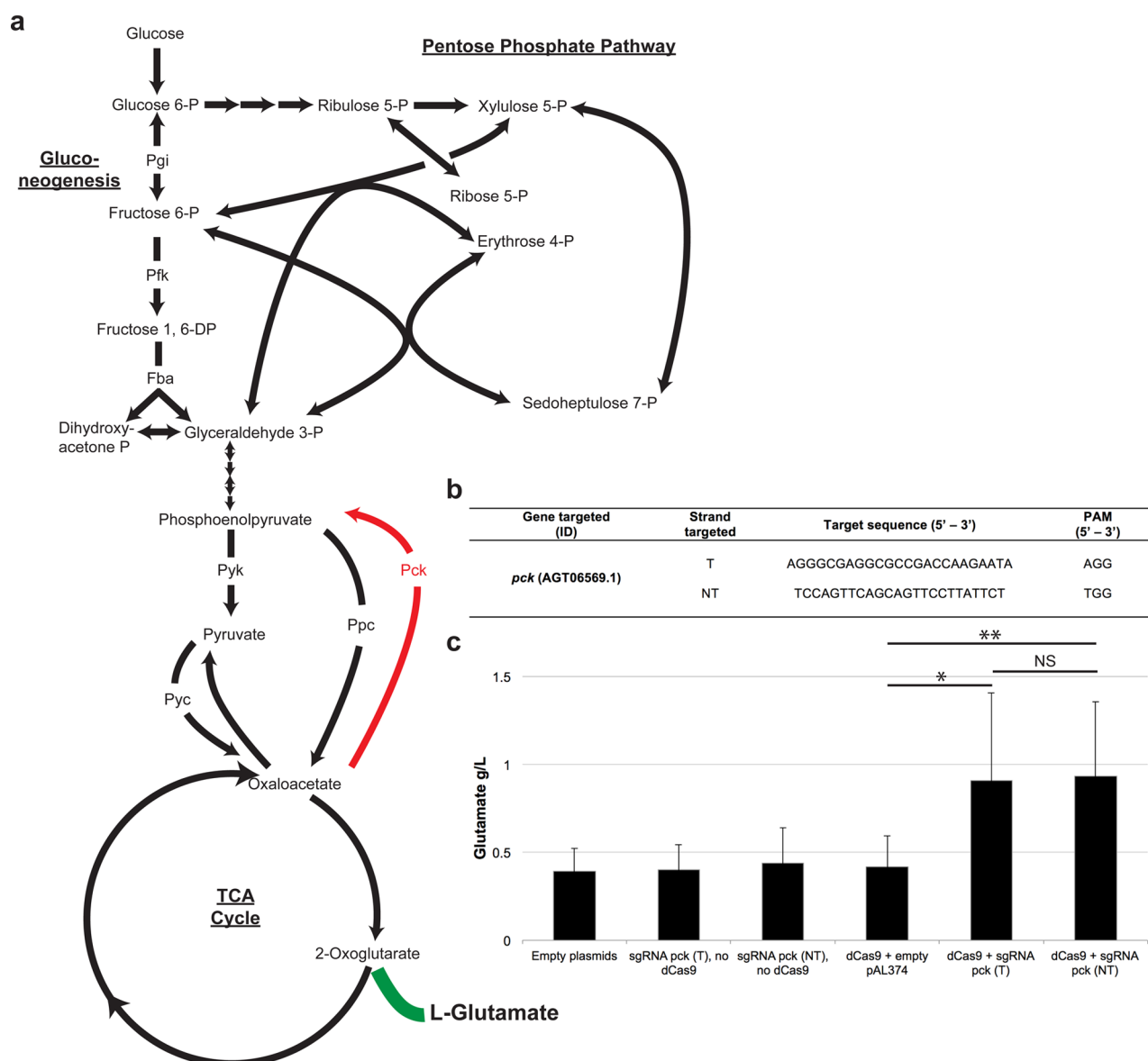
Figure 2. CRISPRi efficiently represses *pck* transcription, increasing L-glutamate production. (a) Central metabolic pathway. The notation in red represents the reduced transcription of *pck* due to CRISPRi-mediated repression (b) Gene and sequences targeted by dCas9. (c) Amino acid titers (g/L) of the control and test strains ($N = 9$ per strain) were determined upon glucose depletion. The error bars represent the standard deviation of samples. "NS" represents nonsignificant ($p > 0.05$), $**p \leq 0.01$ and $***p \leq 0.001$.

Quantification of Enzymatic Activity. Given that sgRNA/dCas9 did not fully repress transcription, we decided to investigate enzyme activity levels in midexponential cultures of *C. glutamicum* in which CRISPRi was present or not. These samples were identical to those taken for mRNA quantification in Figure 4. The specific enzymatic activities of Pgi, Pck, and Pyk reflect the amount of active enzyme available in the cell at the time of sampling. Pgi activity levels decreased by 95% in comparison with a control with no sgRNA when the NT strand was targeted ($p = 1.25 \times 10^{-38}$, Figure 5a, Table 2). Targeting the T strand of *pgi* had no impact on the enzyme activity levels observed ($p = 0.80$, Figure 5a, Table 2), *versus* a control with no sgRNA. Targeting the NT strand in the absence of dCas9 had no effect on Pgi activity (0.49 U/mg; $p = 0.20$ *versus* strain with the same sgRNA and dCas9). Pck activity levels decreased by 49% ($p = 0.005$) and 83% ($p = 9.08 \times 10^{-7}$) when the T and NT strands were targeted by specific sgRNAs, respectively,

compared with a control with no sgRNA (Figure 5b, Table 2). For Pyk, targeting the T or NT strands resulted in a 26% ($p = 5.38 \times 10^{-11}$) and 52% ($p = 1.85 \times 10^{-24}$) decrease in specific activity, respectively, compared with the no-sgRNA control (Figure 5c, Table 2). As expected, there was a correlation between reductions in mRNA levels (Figure 4) and the corresponding enzyme activity (Figure 5), with sgRNAs directed against the NT strand leading to lower mRNA and enzyme activity levels compared with those directed against the T strand.

Conclusion. Given its simplicity of design and ease of deployment, the CRISPR/Cas system has enabled researchers to edit DNA or gene transcription levels (sgRNA/dCas9 or CRISPRi) across an extremely diversified range of organisms.[51−53] The goal of our study was to test the performance and suitability of CRISPRi for pathway engineering in *C. glutamicum*. Our approach, involving the use of sgRNAs[42] and
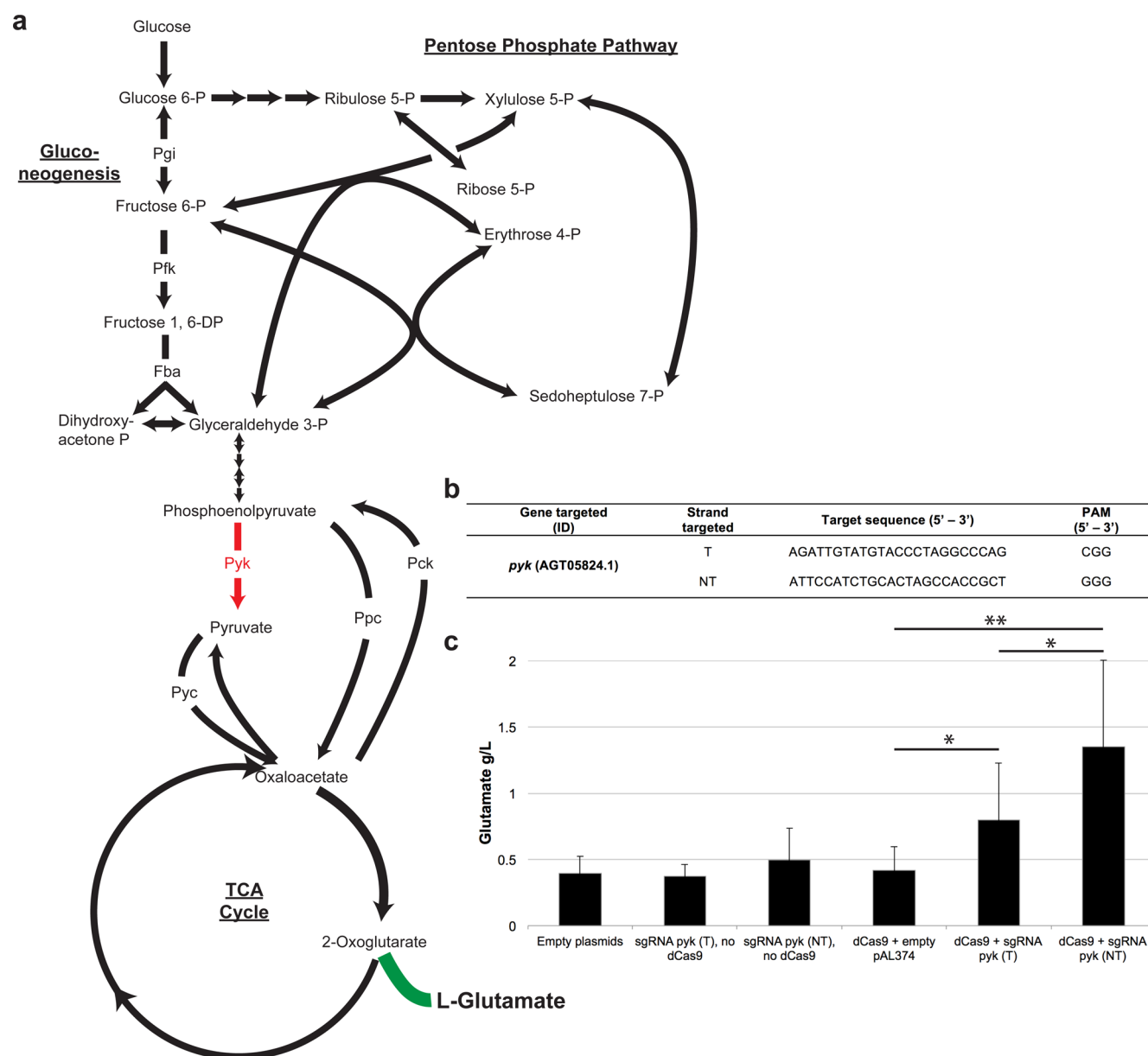
Figure 3. CRISPRi efficiently represses *pyk* transcription, increasing L-glutamate production. (a) Central metabolic pathway. The notation in red represents the reduced transcription of *pgi* due to CRISPRi-mediated repression. (b) Gene and sequences targeted by dCas9. (c) Amino acid titers (g/L) of the control and test strains ($N = 9$ per strain) were determined upon glucose depletion. The error bars represent the standard deviation of samples. $*p \leq 0.05$, $**p \leq 0.01$ and $***p \leq 0.001$.

the dCas9 enzyme to repress the expression of targeted genes,[54] allowed us to go from the initial cloning in *E. coli* to the final engineered *C. glutamicum* strains ready for testing in as little as 3 days. The use of dCas9 instead of Cas9 omits the need to select for rare Cas9-mutated strains[55] or single- or double-crossover mutants obtained *via* suicide vectors.

C. glutamicum plays a multibillion dollar role in the production of two amino acids with the largest market sizes, L-lysine for animal feed and L-glutamate for food additives. The genes chosen for the present study, *pgi*, *pck* and *pyk*, indirectly impact the production of these two amino acids. For *pck* and *pyk*, we found that the repression level caused by the steric blockage of the progression of the RNA polymerase to be equally efficient whether dCas9 annealed onto the T or NT strand of the coding region. For all three genes, the amino acid production measured after glucose depletion in early stationary

phase increased in a statistically significant manner when gene repression was mediated by any of the sgRNAs in the presence of dCas9 (Figure 1−3). In most cases, targeting the NT strand with the CRISPRi system resulted in greater transcriptional repression and amino acid production than targeting the T strand. This observation is consistent with earlier publications that reported that template-strand targeting can be inefficient,[40,42] even though other groups have shown that template-strand repression can be achieved.[41,44]

Because of its programmability and ease of implementation, we envision that CRISPRi-based gene repression could be used to identify putative genes that should be targeted for enhancing bioproduction. Unlike knockout-based approaches to metabolic engineering, the likelihood of completely inhibiting gene expression with CRISPRi is low. One could take advantage of this property to map how intermediate levels of gene
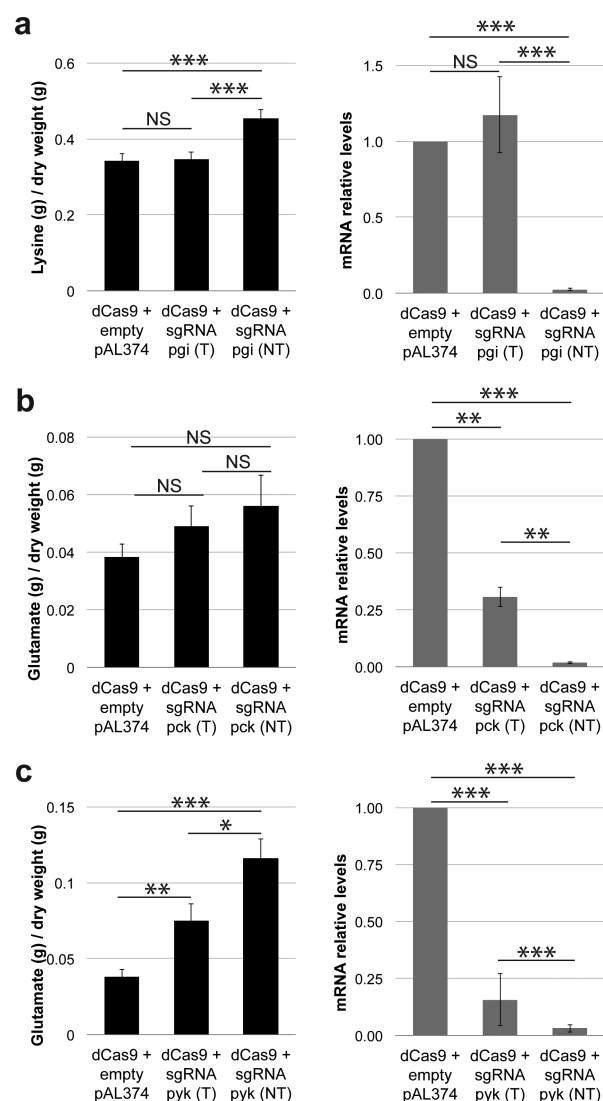
Figure 4. mRNA levels decrease with CRISPRi targeting, resulting in increases in amino acid levels. In order to measure the mRNA levels of *pgi*, *pck* and *pyk* in the cells, samples were collected in midexponential phase. Upon total RNA extraction, qRT-PCR was performed on each of the samples (three biological replicates per strain, each with three technical replicates) and using 16S rRNA for data normalization. Amino acid titers were measured by HPLC and are reported as grams of amino acid per cell dry weight. (a) sgRNA/dCas9-mediated *pgi* targeting, (b) sgRNA/dCas9-mediated *pck* targeting and (c) sgRNA/dCas9-mediated *pyk* targeting. "NS" represents nonsignificant ($p > 0.05$), *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

Figure 5. The specific Pgi, Pck and Pyk activities in crude extracts decrease when the corresponding genes are targeted by the CRISPRi system. Crude extracts from midexponential growing strains (9 biological replicates per strain, each with three technical replicates) were obtained upon cell lysis and used for the quantification of the Pgi, Pck or Pyk activity, as seen by a decrease in the NADH available. The specific activity (U/mg) of (a) Pgi, (b) Pck and (c) Pyk when CRISPRi targets the template (T) or nontemplate (NT) DNA strands is shown. "NS" represents nonsignificant ($p > 0.05$), ***$p \leq 0.001$.

expression translate to metabolic production. Furthermore, multiplexed gene perturbation *via* the expression of multiple sgRNAs from barcoded constructs (*e.g.*, those built using CombiGEM assembly[56]) coupled with high-throughput characterization *via* liquid chromatography coupled to mass spectrometry could be used for combinatorial metabolic pathway engineering. However, in other cases, even a low expression level of certain metabolic genes could potentially contribute enough enzyme activity to maintain flux at a near wild-type level and thus mask the effect of targeting such genes. In the future, dynamic metabolic pathways could be engineered by coupling the expression of CRISPRi system to promoters that are responsive to metabolite concentration. The ability to tune metabolic pathways in response to intracellular or
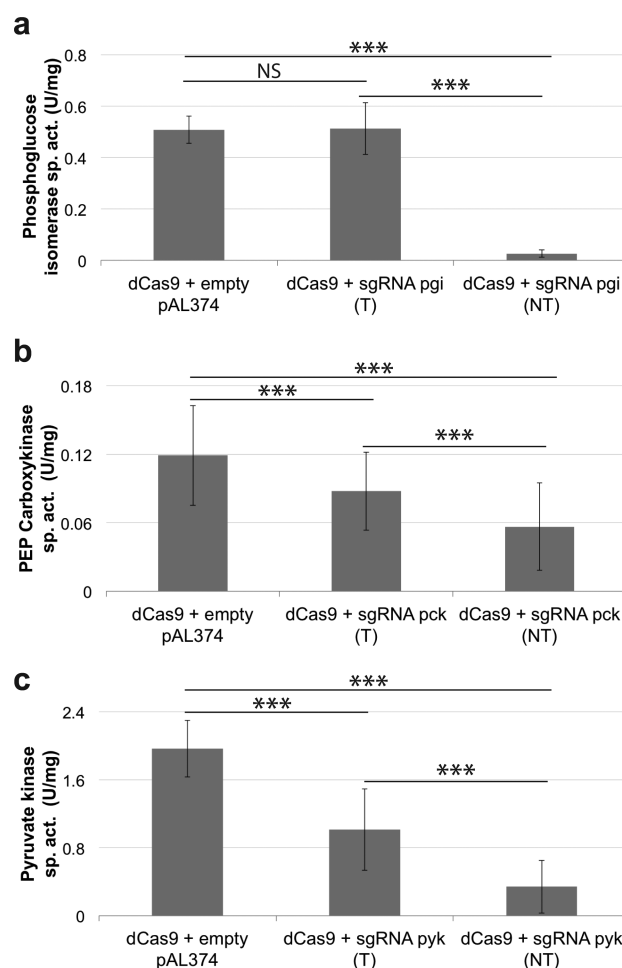
extracellular conditions may help to maximize production titers for the molecules of interest.[57,58]

■ MATERIALS AND METHODS

Microorganisms, Plasmids and Growth Conditions. Bacterial strains and plasmids are listed in Table 1.

The plasmid pRIM3_rfp was assembled from pRIM2 by the isothermal assembly method.[63] The original kanamycin resistance cassette (same as in pZ8-Prp) was replaced with a gentamycin cassette and the *rfp* gene was cloned from pZ8−1_rfp, together with the P_{tac} promoter and T1 terminator.

The plasmid pZ8-Ptac, which is an inducible version of plasmid pZ8−1, was obtained by cloning $lacI^q$ from pDSW405 upstream of the P_{tac} promoter by the isothermal assembly method.

The plasmid pZ8-Prp is pZ8−1 with P_{tac} replaced by the propionate-inducible promoter P_{prpD2} (Table 1). The promoter was ordered as a gBlock from Integrated DNA Technologies (IDT, USA) and cloned into pZ8−1 lacking P_{tac} by the isothermal assembly method.

The gene coding for a nuclease-inactivated version of Cas9—*dcas9*—from *Streptococcus pyogenes* was cloned from plasmid pPP208 into the *C. glutamicum* replicative plasmids pZ8-Ptac and pZ8-Prp, under the control of an IPTG- or a propionate-inducible promoter, respectively.

The short, synthetic version of the sgRNA was designed to contain a 24-bp region of homology to the transcriptional template (T) or nontemplate (NT) strands of the target DNA including the seed sequence, followed by the dCas9 handle and the *S. pyogenes* terminator. The expression of this sgRNA was driven by a P$_{tac}$, ordered as a gBlock from IDT and cloned into the replicative plasmid pAL374. The base-pairing regions, handle and terminators used were the same as those previously described for the sgRNA.[40] The target sequences are listed in Figures 1, 2 and 3. All sgRNAs were cloned into pAL374 by the isothermal assembly method, the plasmid backbone having been amplified using the primers listed in Table 2, which also removed P$_{trc}$.

All plasmids were introduced by transformation into *E. coli* DH5α and maintained in that strain, grown in Lysogeny (Miller, LabExpress, USA) broth (for liquid cultures) or agar (for plates, with 15% agar, Apex) with the appropriate antibiotics (Table 2), and incubated at 37 °C.

C. glutamicum strains were also maintained on LB agar plates, supplemented with the appropriate antibiotic when necessary (Table 1), but incubated at 30 °C. Liquid cultures were prepared in Brain−Heart Infusion (BHI, Sigma-Aldrich, Germany) medium. Electrocompetent cells were prepared as described elsewhere.[64]

All strains were kept as glycerol stocks at −80 °C, in 30% glycerol LB broth.

Integration of pRIM3_RFP in *C. glutamicum*. pRIM3_RFP is a suicide vector that integrates into the host chromosome by a single crossover event downstream of *ppc*, which has been previously described to be a safe harbor.[61] After electroporation and integration of this plasmid, the insertion can be phenotypically screened, as in addition to the acquired gentamicin resistance, the colonies having the insertion exhibit a red color.

CRISPRi-Based Regulation of RFP Expression in *C. glutamicum*. sgRNAs were designed to target the T or NT strands of *rfp* within its first 150 bp (Supplemental Figure S1a). For the *rfp* repression assay, the expression of *dcas9* was driven by a propionate-inducible promoter. Overnight cultures without inducer were used as inocula for new cultures with and without inducer. This experiment was performed three times with two biological replicates.

All strains were grown in LB supplemented with 2 g/L of sodium propionate, at 30 °C for 48 h. Aliquots of all cultures were diluted 1:100 in Phosphate Buffered Saline (pH = 7) and run in a BD-FACS LSR Fortessa cell analyzer (BD Biosciences, CA). The fluorescence was measured using a Texas-Red filter with a 561 nm excitation laser and a 610/20 nm emission filter. For each sample, 50,000 cells were analyzed and gated using forward scatter and side scatter.

CRISPRi-Based Regulation of Amino Acid Production by *C. glutamicum*. Genes *pgi* (AGT04848.1), *pck* (AGT06569.1) and *pyk* (AGT05824.1), coding for enzymes whose absence leads to an increased production of L-lysine (*pgi*) and L-glutamate (*pck* and *pyk*), were selected as individual targets for repression by the sgRNA/dCas9 complex. The sgRNAs were designed to anneal onto the first 150 bp of the T

and NT strands of genes *pgi*, *pck* or *pyk* (Supplemental Figure S2).

Prior to every amino acid production experiment (Figure 1−3), *C. glutamicum* cells from glycerol stocks were streaked onto LB agar plates with the appropriate antibiotics and incubated at 30 °C overnight. These cells were subsequently used to inoculate BHI broth. Upon overnight growth, the uninduced precultures were harvested by centrifugation (4000 rpm, 5 min, 4 °C) and washed with 0.9% NaCl. Ten mL of 2% (w/v) glucose CgXII minimal medium,[65] supplemented with 30 µg/L protocatechuic acid and 1 mM of the *dcas9* inducer IPTG were inoculated to an optical density (OD) of 1 at 610 nm. When appropriate, kanamycin and/or spectinomycin were added to a final concentration of 25 µg/mL and 100 µg/mL, respectively.

Amino acids are typically quantified in the early stationary phase, when glucose is depleted and the concentration of amino acids peaks due to their accumulation over time.[49,50] The cultures were grown in 125 mL flasks at 30 °C, with a shaking frequency of 200 rpm, until glucose was depleted, as measured by Quantofix Glucose strips (Machery-Nagel, Germany).

C. glutamicum ATCC 13032 (Table 1) was used for L-glutamate production. It required supplementing CgXII with 20 mg/L ethambutol, which stimulates L-glutamate secretion.[66]

C. glutamicum DM1729 (Table 1) is an *S*-aminoethyl-L-cysteine-resistant mutant of *C. glutamicum* ATCC 13032, previously developed for the specific purpose of L-lysine overproduction. It did not require any additional supplements.

Growth of all cultures was monitored over time by measuring their OD with an Infinite 200 Pro microplate reader (Tecan, Switzerland). Three independent experiments were performed in triplicate. The OD$_{610nm}$ conversion to cell dry weight (CDW) followed the ratio previously described of OD$_{610nm}$1 = 0.25 g CDW.[67]

Quantification of the mRNA Levels of Genes Targeted by CRISPRi. In order to determine the level of CRISPRi-based transcriptional repression, quantitative reverse transcription PCR (qRT-PCR) was performed on total RNA samples (Figure 4). This analysis was performed with cells in midexponential phase. One-mL aliquots of each culture were taken at midexponential phase for total RNA extraction and amino acid quantification.

Aliquots were centrifuged at 17 000 rpm for 15 s, and the pellets were immediately flash frozen and stored at −80 °C until further processing. The cells were lysed by resuspension of pellets in RA1 buffer and bead beating for 2 × 15 s (Mini-Beadbeater-16, Biospec Products, USA) with intermittent cooling on ice. The steel beads used were RNase-free and 0.2 mm in diameter (KSE Scientific, USA). Total RNA was extracted using the Illustra RNAspin Mini Kit according to the recommendations of the manufacturer (GE Life Sciences, U.K.). To remove residual coextracted DNA, the purified RNA was treated with DNase (Turbo DNA-free kit, Life Technologies, USA).

qRT-PCR was performed using the Kapa SYBRFast One-Step qRT-PCR kit (Kapa Biosystems, USA), the LightCycler 96 System (Roche, USA), 50 ng of total RNA extracted and the primers indicated in Table 3. Results were analyzed following the Livak method,[68] using the threshold cycle of 16S rRNA for normalization.[69]

Enzyme Activity Assays. To directly investigate the actual amounts of enzyme being made (Figure 5), 9 mL aliquots of midexponential *C. glutamicum* cultures were collected con-

Table 3. Primers Used for the Quantification of the mRNA in the Samples by qRT-PCR

template	strand	primer sequence (5′−3′)
pgi	F	TCATTGGTTTCGCTCGTCCA
	R	AGCGTTCTTACCGAAAGCCA
pck	F	ATTGGCTACAACGCTGGTGA
	R	CCACTTCAGAACGCGAGAGT
pyk	F	GATACCGCAAAGCGTGTGG
	R	GACAGGTGGACACAGGAAGG
16S rRNA	F	TTACCTGGGCTTGACATGGAC
	R	GCTGGCAACATAAGACAAGGG

comitantly with those for mRNA measurements (*i.e.*, at midexponential phase). They were then centrifuged at 4000 rpm for 5 min at 4 °C, and the pellets were immediately flash frozen and stored at −80 °C until they underwent further processing.

Cell pellets used for the Pgi enzyme assay were resuspended in 50 mM triethanolamine (TEA) at pH 7.2. Cell pellets used for the Pck and Pyk enzyme assays were resuspended in a solution consisting of 20 mM KCl, 5 mM MnSO$_4$, 2 mM DTT, 0.1 mM EDTA, 30% glycerol and 100 mM Tris-HCl at pH 7.5. The cells were then lysed by a 6 min, 0.5 cycle and 55 amplitude sonication (Sonopuls GM 200, Bandelin electronic GmbH & Co KG, Germany) and centrifuged for 60 min at 4 °C and 14 600 rpm. Supernatants were collected as crude extracts to measure the glucose-6-phosphate isomerase, pyruvate kinase, and PEP carboxykinase activities.

Glucose-6-phosphate isomerase activity was assayed as described by Lindner *et al.*[23] The formation of NADPH (ε(340 nm) = 6.3 mM^{-1} cm^{-1}) was monitored at 340 nm.

Pyruvate kinase activity was assayed essentially as described by Netzer *et al.*[47] The assay mixture contained 100 mM TEA buffer (pH 7), 15 mM MgCl$_2$, 1 mM ADP, 0.25 mM NADH, 12 mM PEP, 5 U L-lactate dehydrogenase, and between 5 and 10 μL of crude extract (corresponding to 0.075 and 0.15 mg of protein, respectively). The decrease of NADH (ε(340 nm) = 6.3 mM^{-1} cm^{-1}) was monitored at 340 nm.

The activity of PEP carboxykinase was assayed as described by Klaffl *et al.*[70] with the following modifications: the assay mixture contained 100 mM HEPES buffer, (pH 6.8), 0.4 mM NADH, 1 mM GDP, 50 mM NaHCO$_3$, 2.5 mM PEP, 0.5 mM MnCl$_2$, 10 mM MgCl$_2$, 4 U malate dehydrogenase, and between 5 and 10 μL of crude extract (corresponding to 0.075 and 0.15 mg of protein, respectively). The decrease of NADH (ε(340 nm) = 6.3 mM^{-1} cm^{-1}) was monitored at 340 nm.

One unit of enzymatic activity was defined as 1 mmol of NADPH formed per minute (U/min) or 1 mmol of NADH consumed per minute (U/min), and the calculation of the specific activity took into account the enzyme concentration in each of the samples (U/mg).

Amino Acid Quantification. Samples for amino acid quantification were taken in midexponential phase (mRNA *vs* amino acid analysis, Figure 4) or directly after glucose depletion (effect of CRISPRi on amino acid production, Figure 1-3).

The samples were centrifuged at 17 000 rpm for 15 s and automatically derivatized using the Fluoraldehyde *o*-Phthaldial-dehyde Reagent Solution (Thermo Scientific, USA), prior to entering the chromatographic separation column. Compounds were separated by high-pressure liquid chromatography (HPLC, 1200 series, Agilent) with a RP18 column (Eclipse

XDB-C18, 4.6 × 150 mm, Agilent) and a fluorescence detector (FLD G1321A, 1200 series, Agilent).

The mobile phases were 0.1 M sodium acetate at pH 7.2 (A) and 100% methanol (B). The gradient used was as follows: 0 min 20% B, 0.5 min 38% B, 2.5 min 46% B, 3.7 min 65% B, 5.5 min 70% B, 6 min 75% B, 6.2 min 85% B, 9.7 min 20% B and 11.9 min 20%, at a flow rate of 1.2 mL/min.

Ornithine was used as the internal standard, and standard curves for determining the amino acid concentrations in the supernatants were drawn by using solutions of L-glutamate and L-lysine at known concentrations.

Statistical Treatment of Data. All results were tested for significance using the unpaired heteroscedastic Student's *t*-test. The level of significance of the differences observed between the control (strain carrying plasmids pZ8-T_dCas9 and pAL374) and test samples (strains carrying plasmids pZ8-T_dCas9 and pAL374 with an sgRNA) was expressed as one, two or three stars, for *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$, respectively. "NS" stands for *nonsignificant*, when $p > 0.05$.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.5b00216.

Figure S1: The CRISPRi system efficiently represses chromosomally inserted *rfp*. (a) Gene and sequences targeted by dCas9. (b) Schematic of the loci targeted by the T and NT sgRNAs. The leading and lagging strands of the *rfp* are shown, and the first base of its start codon is numbered as 1. The target sequences (template and nontemplate strands) are highlighted in gray and the PAM sequence in light gray. Their loci are denoted by the numbering. Mean RFP levels (AU) measured by FACS of the control and test strains determined in stationary phase ($N = 6$ per strain). The error bars represent the standard deviation of samples. "NS" represents nonsignificant ($p > 0.05$), *$p \leq 0.05$ and ***$p \leq 0.001$. (PDF)

Figure S2: Loci for CRISPRi annealing on *pgi, pck* and *pyk*. Schematic of the (a) *pgi*, (b) *pck* and (c) *pyk* loci targeted by the T and NT sgRNAs. The leading and lagging strands of the gene are shown, and the first base of its start codon is numbered as 1. The target sequences (template and nontemplate strands) are highlighted in gray and the PAM sequence in light gray. Their location is denoted by the numbering. (PDF)

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

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■ NOTE ADDED AFTER ASAP PUBLICATION

Due to a production error, Figures 1 and 2 and the Supporting Information Figures 1 and 2 were incorrect in the version published ASAP on February 16, 2016; the correct versions reposted on March 15, 2106.