

Silencing and counter-silencing of the Lsr2-like protein CgpS in *Corynebacterium glutamicum*

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„Der Weg zu allem Großen geht durch die Stille.“

Paul Keller, Das letzte Märchen – Ein Idyll (1905)

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Abbreviations

Abi	Abortive infection	MTase	Methyltransferase
ALE	Adaptive laboratory evolution	NAP	Nucleoid-associated protein
ATc	Anhydrotetracycline	NMR	Nuclear magnetic resonance spectroscopy
a.u.	arbitrary unit	NTP	Nucleoside triphosphate
BREX	Bacteriophage exclusion	PALM	Photoactivated localization microscopy
BS	Binding site	PAM	Protospacer adjacent motif
CBASS	Cyclic oligonucleotide-based anti-phage signalling system	PBM	Protein binding microarray
cGAMP	Cyclic GMP-AMP	PCx	Pyruvate carboxylase
cGAS	Cyclic GMP-AMP synthase	PDHC	Pyruvate dehydrogenase complex
CRISPR-Cas	Clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins	PEPCx	Phosphoenolpyruvate carboxylase
crRNA	CRISPR RNA	REase	Restriction endonuclease
CgpS	<i>C. glutamicum</i> prophage silencer	R-M system	Restriction modification system
ChAP-seq	Chromatin affinity purification and sequencing	RNA	Ribonucleic acid
ChIP	Chromatin immunoprecipitation	RNA Pol	RNA polymerase
DDFA	Differential DNA footprint analysis	SFM	Scanning force microscopy
DISARM	Defence islands system associated with restriction modification	STORM	Stochastic optical reconstruction microscopy
DNA	Deoxyribonucleic acid	SPI	<i>Salmonella</i> pathogenicity island
DNA BD	DNA binding domain	SPR analysis	Surface plasmon resonance analysis
Dnd defence system	DNA degradation defence system	sptPALM	Single-particle tracking PALM
DRE	Downstream regulatory element	TCA cycle	Tricarboxylic acid cycle
e.g.	<i>exempli gratia</i>	TF	Transcription factor
EHEC	Enterohemorrhagic <i>E. coli</i>	TSS	Transcriptional start site
EPEC	Enteropathogenic <i>E. coli</i>	T3SS	Type III secretion system
et al.	<i>et alii</i>	UPEC	Uropathogenic <i>E. coli</i>
HGT	Horizontal gene transfer	URE	Upstream regulatory element
Ler	Locus of enterocyte effacement (LEE)-encoded regulator	WT	Wild-type
LLPS	Liquid-liquid phase separation	XS	Xenogeneic silencer
MGE	Mobile genetic element		

Further abbreviations not included in this section are according to international standards, as, for example, listed in the author guidelines of the Journal of Cell Biology (<https://rupress.org/jcb/pages/standard-abbreviations>).

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1. Abstracts

1.1. Summary

Horizontal gene transfer (HGT) is a major driving force of microbial evolution as it allows the rapid acquisition of new genetic traits. However, foreign DNA is likely to decrease the fitness of recipient cells by causing detrimental effects and therefore requires stringent control of gene expression. Hence, bacteria evolved a number of mechanisms allowing them to discriminate between self and non-self. Xenogeneic silencer (XS) proteins are nucleoid-associated proteins that preferentially bind to horizontally acquired DNA based on differences in nucleotide composition, in particular a higher AT content. XS proteins are widely distributed in bacteria and belong to one of the four classes comprising the H-NS-like XS, Rok, MvaT/U-like proteins, and Lsr2-like XS proteins. They play a predominant role in the acquisition of novel genetic material and oligomerization of XS proteins to higher-order nucleoprotein complexes tightly inhibits transcription. Binding of a transcription factor (TF) within a silenced region may interfere with the XS-DNA complex leading to counter-silencing and activation of gene expression. Consequently, XS and counter-silencing facilitate the integration of novel genetic material into host regulatory circuits enabling the appropriate expression in response to physiological and environmental stimuli.

The aim of this thesis was to investigate the rules underlying silencing and counter-silencing of the medically and biotechnologically relevant Lsr2-like proteins conserved in actinobacteria by using CgpS from *Corynebacterium glutamicum* as a model. CgpS has previously been identified as an Lsr2-like XS, which is crucial for maintaining the lysogenic state of an AT-rich, cryptic prophage element.

In this thesis, genome-wide bioinformatic analyses showed that CgpS preferentially binds to long and consecutive AT-rich stretches and that CgpS targets typically feature a distinct drop in GC-profile close to the transcriptional start site (TSS). Furthermore, a sequence-specific binding motif containing multiple A/T steps was overrepresented in CgpS bound regions. The importance of the drop in GC-profile and the putative binding motif for CgpS silencing was verified by performing in vivo reporter studies with synthetic variants of native CgpS target promoters, demonstrating that both DNA features cooperatively support CgpS-mediated silencing.

Following a synthetic counter-silencer approach, the operator sequence of the gluconate-responsive TF GntR was inserted within diverse CgpS silenced promoters at different positions. Binding of GntR led to disruptive counter-silencing at various CgpS target promoters demonstrating flexibility in promoter architecture of these horizontally acquired targets. Almost all binding site positions resulted in differential gene expression, but counter-silencing efficiency strongly depended on the binding site position. The most prominent effect was observed at the position of maximal CgpS binding close to the TSS.

Counter-silencer constructs usually showed only low background activity. To verify their potential as expression systems for synthetic biology applications demanding stringent control of gene expression, activities were compared to established expression systems (P_{tac} and P_{tet}). It was demonstrated that counter-silencer constructs have a significantly reduced background activity while showing strong inducibility with temporal tunability.

By combining all synthetic constructs showing differential gene expression, a gluconate-responsive synthetic promoter library was generated (28 activated and 16 repressed promoters). Finally, this overall principle of xenogeneic silencing and counter-silencing was harnessed for the design of a GntR-controlled synthetic toggle switch featuring robust and reversible output in response to gluconate availability. This regulatory circuit was successfully implemented as metabolic toggle in a *C. glutamicum* L-valine production strain and enabled the dynamic redirection of carbon flux between biomass and product formation.

In conclusion, this thesis provides comprehensive insights into the CgpS target recognition mechanism during silencing and shows the first systematic analysis of mechanisms allowing counter-silencing of Lsr2-like XS proteins in actinobacteria. Furthermore, the potential of silencing and counter-silencing for synthetic and biotechnological applications was demonstrated.

1.2. Zusammenfassung

Der horizontale Gentransfer (HGT) stellt eine wichtige treibende Kraft der mikrobiellen Evolution dar, da er die schnelle Gewinnung neuer genetischer Merkmale ermöglicht. Fremd-DNA verringert jedoch aufgrund schädlicher Auswirkungen mit hoher Wahrscheinlichkeit die Fitness der Empfängerzellen und erfordert daher eine stringente Kontrolle der Genexpression. Daher entwickelten Bakterien eine Reihe von Mechanismen, die ihnen die Unterscheidung zwischen Selbst und Nicht-Selbst ermöglichen. Xenogene *silencer* (XS) Proteine sind Nukleoid-assoziierte Proteine, die auf Basis von Unterschieden in der Nukleotid-Zusammensetzung, insbesondere einem höheren AT-Gehalt, bevorzugt an horizontal erworbene DNA binden. XS-Proteine sind in Bakterien weit verbreitet und gehören zu einer von vier Klassen. Diese umfassen die H-NS- und MvaT/U-artigen XS, Rok und Lsr2-ähnliche XS-Proteine. XS spielen eine entscheidende Rolle bei dem Gewinn von neuem genetischen Material und durch Oligomerisierung zu Nukleoprotein-Komplexen höherer Ordnung inhibieren sie stark die Transkription. Die Bindung eines Transkriptionsfaktors (TF) innerhalb einer stillgelegten Region kann mit dem XS-DNA-Komplex interferieren. Dies führt zu einer Aufhebung des *silencing* und zur Aktivierung der Genexpression (*counter-silencing*). Demnach erleichtern XS und *counter-silencing* die Integration von neuem genetischen Material in das regulatorische Netzwerk des Wirts und ermöglichen dessen angemessene Expression in Reaktion auf physiologische und umgebungsbedingte Stimuli.

Das Ziel dieser Dissertation war die Untersuchung der Regeln, die dem *silencing* und *counter-silencing* der medizinisch und biotechnologisch relevanten, in Aktinobakterien konservierten Lsr2-ähnlichen Proteinen zugrunde liegen, unter Verwendung von CgpS von *Corynebacterium glutamicum* als Modell. CgpS war zuvor als ein Lsr2-ähnliches XS-Protein identifiziert worden und ist für die Aufrechterhaltung des lysogenen Zustands eines AT-reichen, kryptischen Prophagen-Elements von entscheidender Bedeutung.

In dieser Arbeit zeigten genomweite bioinformatische Analysen, dass CgpS bevorzugt lange und konsekutive AT-reiche Sequenzen bindet und dass CgpS-Ziele typischerweise einen deutlichen Abfall des GC-Profiles in der Nähe des Transkriptionsstarts (TSS) aufweisen. Zusätzlich war ein sequenzspezifisches DNA-Motiv mit mehreren A/T-Schritten in CgpS-gebundenen Regionen überrepräsentiert. Dessen Bedeutung und die des mutmaßlichen DNA-Bindungsmotivs für das CgpS-*silencing* wurde durch in vivo Reporterstudien mit synthetischen Varianten von nativen CgpS-Zielpromotoren verifiziert. Diese zeigten, dass beide DNA-Merkmale kooperativ zum CgpS-vermittelten *silencing* beitragen.

In einem synthetischen *counter-silencing* Ansatz wurde die Operatorsequenz des Glukonat-abhängigen TF GntR in verschiedene CgpS-gebundene Promotoren an unterschiedlichen Positionen eingefügt. Die Bindung von GntR führte zu disruptivem *counter-silencing* von mehreren CgpS-Zielpromotoren, wodurch die Flexibilität in der Promotorenarchitektur dieser horizontal erworbenen Ziele demonstriert wurde. Fast alle Bindestellenpositionen führten zu einer differentiellen Genexpression, jedoch war die *counter-silencing* Effizienz stark von der Positionierung abhängig. Der auffälligste Effekt wurde an der Stelle der maximalen CgpS-Bindung in der Nähe des TSS beobachtet.

Üblicherweise wiesen die *counter-silencing*-Konstrukte nur eine geringe Basalaktivität auf. Zur Verifizierung ihres Potenzials als Expressionssysteme für Anwendungen der Synthetischen Biologie, die eine stringente Kontrolle der Genexpression erfordern, wurden ihre Aktivitäten mit denen von etablierten Expressionssystemen (P_{lac} und P_{tet}) verglichen. Dabei konnte gezeigt werden, dass *counter-silencing*-Konstrukte eine signifikant reduzierte Basalaktivität und gleichzeitig eine starke Induzierbarkeit mit zeitlicher Abstimmbarkeit aufweisen.

Durch die Kombination aller synthetischen Konstrukte, die eine differentielle Genexpression zeigten, wurde eine Glukonat abhängige, synthetische Promotorbibliothek generiert (28 aktivierte und 16 reprimierte Promotoren). Die *silencing* und *counter-silencing* Prinzipien wurden schließlich für die Konstruktion eines synthetischen, GntR-gesteuerten genetischen Schalters genutzt, der robust und reversibel auf die Verfügbarkeit von Glukonat reagierte. Dieser Regelkreis wurde erfolgreich als metabolischer Schalter in einem *C. glutamicum* L-Valin Produktionsstamm implementiert, wo er die dynamische Umlenkung des Kohlenstoffflusses zwischen Biomasse- und Produktbildung ermöglichte.

Zusammenfassend bietet diese Arbeit umfassende Einblicke in den CgpS-Zielerkennungsmechanismus während des *silencing* und zeigt die erste systematische Analyse von Mechanismen, die das *counter-silencing* von Lsr2-ähnlichen XS-Proteinen in Aktinobakterien ermöglichen. Darüber hinaus wurde das Potenzial von *silencing* und *counter-silencing* für synthetische und biotechnologische Anwendungen demonstriert.

2. Scientific context and key results of this thesis

2.1. Horizontal gene transfer – a major driving force of microbial evolution

Horizontal gene transfer (HGT), the lateral transfer of DNA between unicellular or multicellular organisms, represents an important evolutionary driving force and strongly increases genetic diversity (Will et al., 2015). Evidences for horizontally transferred DNA are widespread and detectable in all free-living bacteria (Navarre, 2016). Such events were identified among prokaryotes (Jain et al., 1999) and even between members of different kingdoms, e.g. from animals to bacteria (Wolf et al., 1999), from bacteria to archaea (Nelson et al., 1999), and from bacteria to eukaryotes (Doolittle, 1998; White et al., 1983). In contrast to point mutations and genome rearrangements, horizontally transferred mobile genetic elements (MGEs) like plasmids, gene cassettes, transposons, and bacteriophages allow the dynamic acquisition and deletion of large parts of DNA, thereby contributing to rapid adaptations of bacteria to fluctuating and challenging environments (de la Cruz and Davies, 2000; Ochman et al., 2000). For instance, such beneficial traits may provide new metabolic abilities like the utilization of alternative carbon sources (Chu et al., 2018), provide tolerance towards different stresses (Wang et al., 2010) and mainly contribute to the rapid spread of antibiotic drug resistance within microbial communities (Raz and Tannenbaum, 2010; Walsh, 2000). Furthermore, most bacterial virulence genes were acquired via HGT (Navarre, 2016).

The high evolutionary impact of HGT becomes especially remarkable in the light of bacterial speciation and pathogenicity. One example is the contribution of HGT to the divergent evolution of *Salmonella* and *Escherichia coli* from their last common ancestor during the last 140 million years. While housekeeping genes of both species are still very similar (90% identity) (de la Cruz and Davies, 2000), horizontally transferred DNA contributed to evolution of *Salmonella*'s unique pathogenic lifestyle comprising the utilization of various carbon sources, the use of alternative electron acceptors in the absence of oxygen and the induction of inflammation (Desai et al., 2013; Navarre, 2016).

HGT can occur via three different mechanisms: Natural transformation, conjugation, and transduction (Figure 1). While transformation describes the active import of free naked DNA fragments (ssDNA or dsDNA) by a natively competent cell from its surrounding environment (e.g. after cell lysis of a neighbouring cell) (Sun, 2018; Vogan and Higgs, 2011) (Figure 1A), plasmids are typically transferred via conjugation. Conjugation depends on direct cell-cell contacts which are formed by a pilus bridge allowing conjugative plasmids and transposons to autonomously transport one copy of the MGE into another cell (Villa et al., 2019; Vogan and Higgs, 2011) (Figure 1B). Viruses that prey on bacteria, so-called bacteriophages, are present in all ecological niches and represent the most abundant biological entity on earth with an estimated number of 10^{31} – which is ten-times higher than the estimated sum of all bacteria cells (Hendrix et al., 1999). By mediating the transfer of bacterial DNA between a donor

and a recipient cell, bacteriophages represent the key players of the generalized and specialized transduction mechanisms (Touchon et al., 2017; Villa et al., 2019) (Figure 1C).

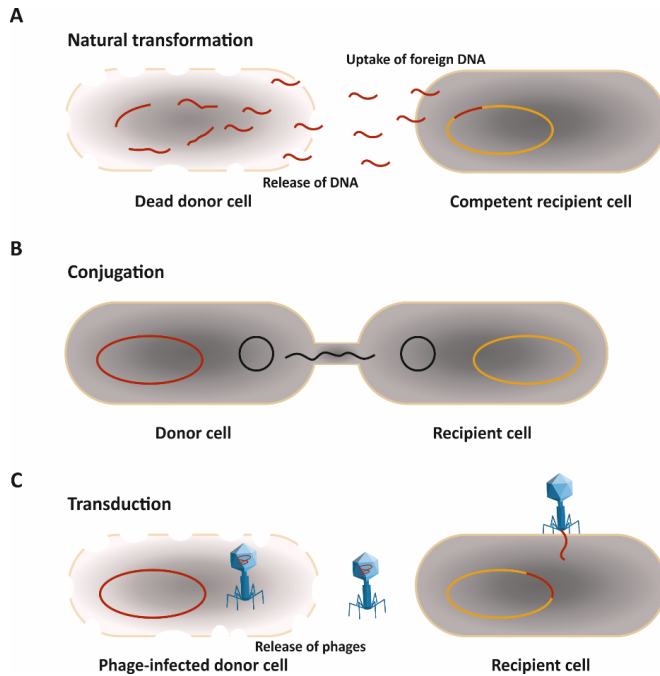


Figure 1: Mechanisms of horizontal gene transfer. A) Natural transformation: Uptake, integration, and expression of extracellular DNA, which has been released upon lysis of the donor cell. **B)** Conjugation: DNA is transferred from the donor to the recipient cell via adhesins or pili by direct cell-cell contact. **C)** Transduction: Bacteriophage-mediated DNA transfer from a previously infected donor cell to a recipient cell. Two transduction processes are known. Shown is the generalized transduction mechanism. Instead of phage DNA, random fragments of bacterial DNA (red) are accidentally loaded into the phage head. The second variant, described as specialized transduction, means that fragments of genomic DNA neighbouring the integrated phage DNA are co-excised and additionally loaded into the phage head (not shown). Adapted from Furuya and Lowy (2006).

Depending on their lifestyles, phages are grouped into lytic and temperate phages. The infection with a lytic phage is indispensably connected with the reprogramming of the bacterial host towards the synthesis of new virions, followed by cell lysis and the release of infectious particles. In contrast, temperate phages have two options: the lytic cycle or the stable maintenance as a prophage (lysogenic cycle). During the lysogenic cycle, the viral DNA is replicated in concert with the bacterial DNA, e.g. upon integration into the host genome or by existing as extrachromosomal elements (Casjens, 2003). Functional temperate phages can be induced either SOS-independently, e.g. as response to quorum-sensing signalling molecules, or by the cellular SOS response. DNA damage as a result of antibiotics or physiological stresses can trigger the SOS response which subsequently induces the lytic-lysogenic switch (Ghosh et al., 2009; Janion, 2008; Melechen and Go, 1980). Since the induction of functional prophages usually results in lysis of the host, these viral elements represent a ubiquitous threat for the

cellular fitness. However, whole-genome sequencing approaches revealed that DNA of viral origin is highly abundant in bacterial chromosomes. In some extreme cases, up to 20% of the bacterial genome is comprised by viral DNA (Casjens, 2003). Most of this DNA is not expressed in the prophage state. However, several temperate phage genes can be expressed in the lysogenic state, which may have effects on the host phenotype, causing the lysogenic conversion of the bacterial cell. For instance, prophages often provide pathogenicity determinants such as toxin encoding genes, as reported for the cholera toxin of *Vibrio cholerae*, the diphtheria toxin of *Corynebacterium diphtheriae* as well as for different toxins of *Pseudomonas aeruginosa* and *E. coli* (Hacker et al., 1997). Interestingly, temperate phages can confer resistance to secondary phage infections by different mechanisms, e.g. by surface modifications to block phage binding or DNA injection as well as by repressing the replication of phage DNA (Bondy-Denomy et al., 2016; van Houte et al., 2016).

The high amounts of cryptic, degenerated phage DNA within bacterial genomes reveal the gradual decay which prophage DNA may undergo during the course of evolution. The results are phage elements with defects in specific functions related to cell lysis, viral replication, the formation of phage particles or infectivity (Bobay et al., 2014). These cryptic, grounded prophages have a lower probability to kill the cell, while beneficial traits such as immunity to secondary phage attack may be maintained. Furthermore, they represent hotspots for HGT and are important drivers of bacterial evolution (Ramisetty and Sudhakari, 2019).

2.2. How to discriminate between self and foreign?

Although HGT and phage-host interactions strongly shaped bacterial evolution and provided countless beneficial traits, invading foreign DNA still represents a constant threat for the recipient cell forcing microbes to develop defence and control mechanisms. HGT mediated by phages is a double-edged sword for the microbial cell (Faure et al., 2019). It allows the acquisition of beneficial DNA elements. On the other hand, the probability that invading DNA results in harmful effects on the fitness of the recipient cell is high. Foreign DNA elements can interfere with the host regulatory network, disturb the structural integrity of the host chromosome, cause high transcriptional and translational costs or may lead to the production of cytotoxic gene products and sequestration of cellular machineries (Baltrus, 2013; Navarre, 2016; Park and Zhang, 2012; Pfeifer et al., 2016; Vogan and Higgs, 2011). Even the acquisition of potentially useful genes can be a disadvantage if they are not properly controlled in terms of expression timing and strength (Navarre, 2016). Consequently, bacteria evolved various defence mechanisms allowing them to deal with foreign DNA, e.g. DNA of viral origin (Doron et al., 2018). Phage-specific immune systems target different stages of the phage lifecycle. Early systems aim at blocking phage binding and DNA injection, e.g. by surface modification leading to masking, mutating

or loss of host receptor proteins (van Houte et al., 2016). Later mechanisms are based on the recognition of phage nucleic acids or proteins leading to targeted cleavage of the phage genome, to inhibition of phage replication or to the programmed cell death of the host cell (Figure 2). While some defence mechanisms mainly provide immunity for a single cell, others can be considered as multicellular strategy acting at population level. It is important to note that the targeted recognition and degradation of xenogeneic DNA is not limited to phages and can be considered as general mechanisms allowing cells to deal with foreign DNA elements. One important prerequisite of suitable defence systems are mechanisms allowing the recognition and discrimination between self (host) and non-self (horizontally acquired) DNA elements (Goldberg and Marraffini, 2015). Known mechanisms and their molecular basis to meet this challenge will be discussed in the following sections.

2.2.1. Destructive defence systems

Destructive defence mechanisms targeting nucleic acids allow the specific recognition and degradation of foreign genetic elements and thereby limit the risk of HGT and phage infection. During evolution, microorganisms developed two general strategies of cellular immunity: the innate immunity comprising genetically encoded mechanisms that recognize general features of foreign nucleic acids and the adaptive immune system that can be considered as cellular memory to previous infections (Amitai and Sorek, 2016).

In the 1950s, Bertani and Luria firstly described the phenomenon of restriction modification after observing that bacteriophage production in newly infected bacterial strains differs depending on the original host strain (Bertani and Weigle, 1953; Luria and Human, 1952). Restriction modification (R-M) systems are considered as innate immune systems providing resistance against foreign DNA independently of previous adaptation steps (Vasu and Nagaraja, 2013). Nowadays, R-M systems summarize widespread defence mechanisms (present in about 74% of prokaryotic genomes (Oliveira et al., 2014)) which cleave and degrade foreign DNA (Figure 2A1). R-M systems were found in a variety of organisms including bacteria and archaea (Pingoud et al., 2005) and often several different systems co-exist in one genome (Oliveira et al., 2014).

The classical R-M systems combine two enzymatic mechanisms, either separated or combined in one protein complex: a restriction endonuclease (REase, restriction enzyme) recognizing and cleaving specific DNA sequences and a methyltransferase (MTase) which epigenetically methylates these sequences in the host chromosome to protect the own genome from self-cleavage (Vasu and Nagaraja, 2013). Even if the invading DNA is methylated, based on the high diversity of systems (almost 4,000 identified restriction enzymes with more than 300 different specificities), in most cases, the methylation pattern will differ from the one of the host cell (Roberts et al., 2010; Vasu and Nagaraja, 2013). R-M systems are currently categorized into four types (I-IV), differing in the mechanisms of

target recognition and cleavage, subunit compositions, required cofactors and substrate specificity (Roberts et al., 2003; Vasu and Nagaraja, 2013).

Interestingly, host-specific DNA modifications allowing to distinguish DNA in terms of self and non-self are not restricted to methylation. The innate Dnd (DNA degradation) defence system is based on phosphorothioate modification at the sugar phosphate backbone of the DNA. It confers nuclease resistance in bacteria (Wang et al., 2007) and prevents replication of viral DNA in archaea (Xiong et al., 2019). Dpd, a further mechanism of DNA modification with the potential to discriminate between self and non-self was previously identified by Thiaville and colleagues. The authors reported the occurrence of inserted 7-deazaguanine derivatives within the DNA which influenced restriction efficiencies during plasmid transformation (Thiaville et al., 2016). Remarkably, a system derivate was identified in different phages, e.g. in *E. coli* phage 9g, and the authors suggested that this modification might act as anti-restriction system by conferring resistance to restriction enzymes of the host (Thiaville et al., 2016).

Further R-M related anti-phage mechanisms are the BREX (Bacteriophage Exclusion) and the DISARM (Defence Islands System Associated with Restriction Modification) system, both using methylation as basis for self/non-self-discrimination (Goldfarb et al., 2015; Ofir et al., 2018). While DISARM results in restriction of phage DNA and act as a multi-gene restriction-modification module (Ofir et al., 2018), the BREX system leads to a blockage of phage DNA replication (Goldfarb et al., 2015).

In contrast to the abovementioned innate R-M systems, the highly diverse and complex CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and CRISPR-associated proteins) systems represent the adaptive bacterial and archaeal immunity with a memory function (Figure 2AII) (Amitai and Sorek, 2016; Faure et al., 2019). CRISPR-Cas systems were identified in almost all archaeal genomes and in about one third of all bacteria (Faure et al., 2019; Koonin et al., 2017). The CRISPR-Cas immune response can be divided into three stages: adaptation, processing, and interference. During adaptation, a complex of different Cas proteins recognizes and binds a two to four base pair short protospacer adjacent motif (PAM) within the foreign DNA, followed by the excision of a short DNA fragment (protospacer), which is inserted as spacer into the host CRISPR array. Upon transcription of the CRISPR array, the precursor CRISPR RNA (crRNA) is processed into small matured, one spacer crRNAs by Cas proteins or non-Cas ribonucleases. During interference, crRNAs function as guide for Cas nucleases by specifically recognizing sequence complementarities to protospacers in an invading plasmid or viral genome. Finally, Cas nucleases cleave and inactivate the complementary foreign DNA, thereby protecting the cell against potentially detrimental fitness effects (Amitai and Sorek, 2016; Faure et al., 2019). Remarkably, the authors of a recent review summarized observations hinting that CRISPR-Cas might have functions going beyond immunity, e.g. inhibiting or stimulating regulatory

effects on HGT, signal transduction, programmed cell death, DNA repair systems, plasmid maintenance and transposon integration (Faure et al., 2019).

R-M and CRISPR-Cas systems were intensively studied in the past and were demonstrated to efficiently provide resistance against incoming foreign DNA. However, recent studies revealed that we have still underestimated the degree of complexity of bacterial defence systems. While the above described mechanisms mainly act at the cellular level, the secretion of small secondary molecules were recently described by Kronheim and colleagues as putative innate chemical anti-phage defence strategy at population level (Figure 2B) (Kronheim et al., 2018). By systematically screening secondary molecules naturally produced by *Streptomyces*, they could show that sub-inhibitory concentrations of several DNA-intercalating molecules (e.g. daunorubicin and doxorubicin) inhibit replication of phage dsDNA but not bacterial cell growth. The authors suggested that the self/non-self-discrimination was based on differences in the DNA states of the host and the phage genome: the invading phage genome is linear, non-supercoiled and unbound by protecting DNA-binding proteins (Kronheim et al., 2018).

Further anti-phage defence strategies are collectively referred to as abortive infection (Abi) systems (Bernheim and Sorek, 2020) (Figure 2C). In general, these post infection resistance mechanisms are part of the bacterial innate immunity and act at population level (Dy et al., 2014). In cases when earlier immune responses failed to prevent phage infection (e.g. R-M systems), Abi systems prevent the spreading of phages by enabling the host cell to kill itself (programmed cell death) or to arrest in a dormant state before functional phage particles are formed (Bernheim and Sorek, 2020). These strategies are diverse and found in a variety of microorganism, e.g. *E. coli* (Bingham et al., 2000), *Lactococcus spp.* (Durmaz and Klaenhammer, 2007), and *Staphylococcus spp.* (Depardieu et al., 2016). Their common objective is the protection of the bacterial community from a viral epidemic by sacrificing the infected host cell. Therefore, Abi systems are considered as altruistic cell death systems (Bernheim and Sorek, 2020; Dy et al., 2014; van Houte et al., 2016). Typical triggers of these mechanisms are phage proteins, phage nucleic acids or the state of a cell after phage infection (Bernheim and Sorek, 2020).

In addition to the discussed strategies, a variety of further systems exist which are suggested to provide defence against foreign DNA elements (reviewed in (Bernheim and Sorek, 2020; van Houte et al., 2016)), highlighting the multiplicity of defence mechanisms. At present, we are only just beginning to understand the complexity of prokaryotic immune systems, including their molecular mechanisms, not to mention the far-reaching interplay of these mechanisms in living cells.

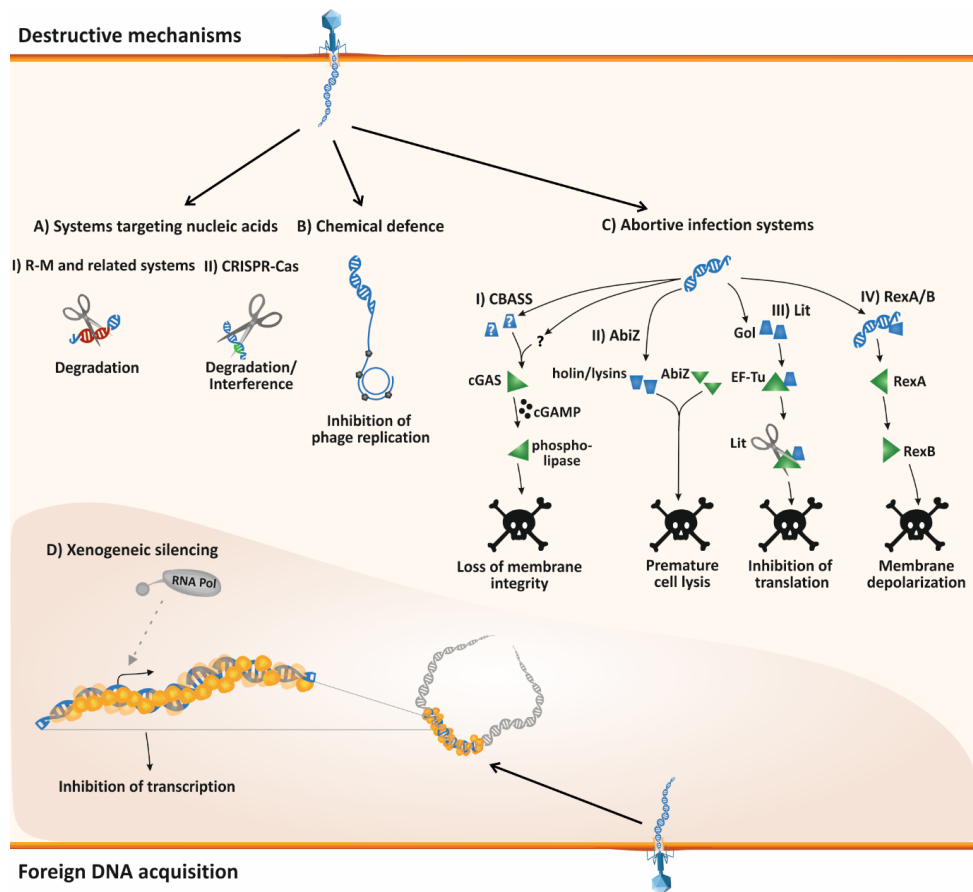


Figure 2: Defence systems protecting cells against invading foreign DNA. A) Systems targeting nucleic acids. I) Restriction modification (R-M) and related systems. II) CRISPR-Cas systems. B) Chemical defence systems. C) Abortive infection (Abi) systems. Different examples for Abi systems are given. I) Cyclic oligonucleotide-based anti-phage signalling systems (CBASSs) of *E. coli* and *V. cholerae*. Cyclic GMP-AMP (cGAMP) is produced by cyclic GMP-AMP synthase (cGAS) as response to phage infection. The molecular mechanism has yet not been identified. cGAMP signalling activates downstream effector proteins, e.g. a phospholipase which degrades the inner cell membrane, which finally leads to cell death (Cohen et al., 2019). II) The AbiZ protein of *Lactococcus spp.* acts together with phage lysins and holins and triggers cell lysis before functional phage particles are formed (Durmaz and Klaenhammer, 2007). III) *E. coli* Lit protein senses a complex composed of the T4 phage peptide Gol and the ribosomal elongation factor EF-Tu. Consequently, Lit cleaves EF-Tu resulting in translation inhibition and cell death (Bingham et al., 2000). IV) The Rex system of lysogenic *E. coli* strains which is involved in maintaining the lysogenic state of phage λ . Upon sensing of phage protein-DNA complexes, RexA becomes active and activates the membrane-anchored ion channel protein RexB. RexB activation leads to membrane depolarization and dropping ATP levels. Consequently, ATP-dependent replication of phage DNA is aborted (Parma et al., 1992; van Houte et al., 2016) D) Foreign DNA acquisition mediated by xenogenic silencing. Xenogenic silencer proteins recognize AT-rich foreign DNA. DNA binding of these proteins leads to the formation of a higher-order nucleoprotein complex which inhibits transcription, thereby leading to silencing of the horizontally acquired DNA (Pfeifer et al., 2019). Although XS proteins preferentially bind to these foreign genetic elements and play an important role in maintaining the lysogenic state of prophages (Pfeifer et al., 2016), the function of XS proteins during phage infection is still unclear. Adapted from Bernheim and Sorek (2020), Pfeifer et al. (2019), van Houte et al. (2016).

Interestingly, different defence systems or derivatives of the same mechanism often co-exist in one single genome with the potential to act synergistically (Bernheim and Sorek, 2020). In general, defence systems are often physically clustered in certain genome regions, so-called defence islands. These

regions are often enriched with genes encoding recombinases and transposases which might facilitate HGT by conferring mobility of the islands (Makarova et al., 2011). Furthermore, these islands can provide hotspots for the integration of horizontally transferred elements (Bernheim and Sorek, 2020). Recently, Doron and colleagues performed an elegant analysis based on the clustering of immune systems in defence islands (Doron et al., 2018). Following the idea that unknown defence systems might be located close to known systems in bacterial genomes and that gene orders could be conserved, they systematically mapped microbial genome regions to identify putative candidates. By performing comprehensive characterization, they were able to identify nine new anti-phage defence systems and one system involved in defence against plasmids (Doron et al., 2018), highlighting the abovementioned complexity of defence strategies.

2.2.2. Xenogeneic silencers

In contrast to destructive defence mechanisms, xenogeneic silencer (XS) proteins promote tolerance towards foreign (xenogeneic) DNA (Figure 2D) (Navarre et al., 2007; Pfeifer et al., 2019). XS proteins are specialized nucleoid-associated proteins (NAPs) and represent the molecular basis of the xenogeneic silencing mechanism. They recognize and bind AT-rich, horizontally acquired DNA and their subsequent oligomerization inhibits transcription of these genetic elements (Fang and Rimsky, 2008; Gordon et al., 2011; Navarre, 2016; Navarre et al., 2007). The discrimination between self and non-self is mainly based on differences in nucleotide composition of the DNA, in particular the proportion of adenines and thymines (AT content), between the foreign element and the genome of the recipient cell (Navarre et al., 2007). Since XS proteins prevent the uncontrolled expression of foreign DNA and consequently protect the cells from potential detrimental effects, they were recently considered as part of the bacterial immune system (Navarre, 2016; Pfeifer et al., 2019). Xenogeneic silencing prevents the production of putative toxic compounds. Furthermore, it also represents an important fitness trait to avoid sequestering of the RNA polymerase and spurious transcription of AT-rich DNA sequences from adventitious promoters, explaining XS binding sites in intragenic regions. In particular, XS prevents the titration of the RNA polymerase away from GC-rich promoters, antisense transcript expression as well as transcription of neighbouring genes (Lamberte et al., 2017; Singh et al., 2014). The importance of XS proteins is reflected in the consequences of their loss for bacterial fitness, ranging from subtle to extreme and even to cell death depending on the bacterial species (Castang and Dove, 2012; Navarre, 2016; Navarre et al., 2006; Pfeifer et al., 2016).

2.2.3. Base composition as discrimination factor between self and non-self

Bacterial genomes show strong variance in overall GC content and differ between less than 20% in bacterial endosymbionts to actinobacterial genomes of more than 70% (Hildebrand et al., 2010;

Zamenhof et al., 1952). Extreme examples are the genomes of the proteobacterial symbiont *Carsonella ruddii* strain Pv with a low GC content of about 16.5% (Nakabachi et al., 2006) and the actinobacterium *Frankia alni* strain ACN14a having a high GC content of 72.8% (Normand et al., 2007). Nevertheless, as mentioned before, all known XS preferentially target DNA which has a higher AT content than the host genome, meaning they use base composition as discrimination factor between self and non-self.

Theoretically, different molecular reasons would prefer the accumulation of adenines and thymines in bacterial genomes in the course of evolution (Figure 3). For instance, energetic costs for guanine and cytosine are higher compared to adenine and thymine and high amounts of adenosine triphosphate (ATP) are present in bacterial cells (Rocha and Danchin, 2002). Furthermore, reactive oxygen species can trigger the oxidative deamination of cytosine to uracil-like products, resulting in C-T transitions, or can induce the oxidation of guanine, leading to G-T transversions (Wang et al., 1998) (Figure 3). In theory, in the absence of appropriate DNA repair systems, these factors would favour the accumulation of A and T within a bacterial genome over time. Indeed, in bacterial endosymbionts lacking important DNA repair systems due to drastic genome reductions, mutation rates from G/C to A/T are three times higher than from A/T to G/C, resulting in highly AT-rich genomes of these host-restricted bacteria (Moran et al., 2009).

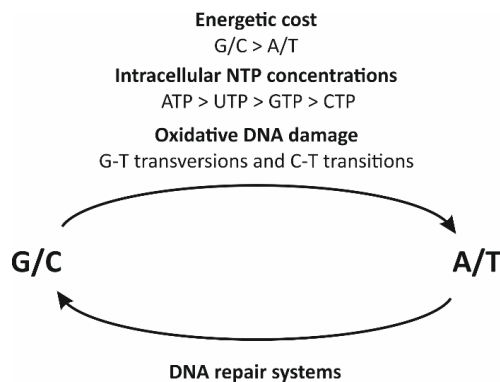


Figure 3: Molecular reasons for the shift towards high AT content in the absence of appropriate DNA repair systems. Different molecular reasons would favor the accumulation of A and T in DNA molecules, including the lower energetic costs of A and T in comparison to G and C, the intracellular ratios of nucleoside triphosphates (NTPs), as well as G-T transversions and C-T transitions caused by oxidative DNA damages. Appropriate DNA repair systems counteract this base shift allowing bacteria to keep a certain GC content (Moran et al., 2009; Rocha and Danchin, 2002; Wang et al., 1998). [Abbreviations: NTP, nucleoside triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate; GTP, guanosine triphosphate; CTP, cytidine triphosphate].

These reasons might also explain why HGT elements such as plasmids and phages often tend to be AT-rich when they enter a host cell (Rocha and Danchin, 2002). Consistently, genes which are unique for single or few species and therefore considered as being horizontally acquired typically show lower, even decreasing GC contents over time. In contrast, many bacteria species strictly keep a certain GC

content in their core genome, e.g. by using efficient DNA repair mechanisms (Desai et al., 2013; Navarre, 2016). These stable differences in GC content between ancestral core genes and horizontally acquired DNA elements suggest an evolutionary selection pressure which prevents the adaptation of GC contents (Desai et al., 2013; Navarre, 2016). In the light of evolution, a potential reason for this phenomenon might be that XS proteins use base composition as discrimination factor between self and non-self (Navarre et al., 2007). XS proteins preferentially bind to AT-rich DNA and therefore enable silencing of previously acquired AT-rich foreign DNA elements as well as of those which prospectively enter the cell. In this way, they protect the cell from uncontrolled expression of potentially harmful genes. In contrast, GC-rich xenogeneic DNA cannot be silenced by most XS proteins due to their mechanism of target recognition (will be discussed in chapter 2.3.3. "How do xenogeneic silencers recognize their targets?"). Therefore, XS proteins cannot buffer against harmful effects of GC-rich elements. These fitness consequences might lead to cell death of the recipient cell and prevent the stable manifestation of GC-rich elements in bacterial genomes (Navarre, 2016). Consistently, XS do not target all DNA elements which are horizontally acquired. For instance, Smits and colleagues demonstrated that the XS protein Rok does not bind the defective prophage PBSX which has a nucleotide composition similar to that of the *Bacillus subtilis* host genome (Smits and Grossman, 2010).

2.3. Xenogeneic silencing

The following sections will provide an overview on the four currently known classes of XS proteins, their main targets, the molecular basis of target recognition and formation of the nucleoprotein complex as well as involved accessory factors.

2.3.1. Four classes of xenogeneic silencers

XS proteins are present in a variety of different bacterial species including Gram-positive and -negative bacteria (Suzuki-Minakuchi and Navarre, 2019). Currently known XS proteins are grouped into four previously described classes based on their amino acid sequence and structure: H-NS and its paralogs were found in different α -, β -, and λ -proteobacteria like *Salmonella*, *Yersinia* and *E. coli* (Heroven et al., 2004; Navarre et al., 2006; Oshima et al., 2006), MvaT/U-like XS proteins have been identified in λ -proteobacteria of the order Pseudomonadales, e.g. *P. aeruginosa* (Castang et al., 2008; Tendeng et al., 2003), while Lsr2-like proteins function as XS in Actinomycetes (Gordon et al., 2008; Gordon et al., 2010; Pfeifer et al., 2016). Finally, Smits and colleagues proposed in 2010 that Rok, found in different *bacilli* species such as *B. subtilis*, is a functional analogue of H-NS and Lsr2 and represents the fourth class of XS proteins (Smits and Grossman, 2010).

Members of the different XS families display no or very low sequence similarities (<20%) but share a similar overall domain architecture comprising an N-terminal oligomerization domain and a C-terminal DNA binding domain connected by a flexible linker (Duan et al., 2018; Gordon et al., 2010; Navarre, 2016; Smits and Grossman, 2010; Tendeng et al., 2003). Interestingly, recent studies reported that the linker of H-NS has multiple functions: it provides flexibility between both domains and contributes to filament formation as well as to DNA binding affinity (Gao et al., 2017; Gulvady et al., 2018). Although they differ in terms of their molecular binding mechanism, they all show a preference for DNA sequences which are AT-rich in comparison to their host genomes and these elements are often of horizontal origin (Navarre, 2016). Upon binding to the AT-rich DNA, XS proteins oligomerize to form a dense nucleoprotein complex which finally leads to gene silencing. These convergently evolved functional similarities allowed in several cross-complementation studies with unrelated XS the full or partial recovery of wild type-like phenotypes of XS-deficient strains (Gordon et al., 2008; Pfeifer et al., 2016; Tendeng et al., 2003). Interestingly, Gordon and colleagues demonstrated even an interchangeability of the single domains between H-NS and Lsr2 resulting in functional chimeric XS proteins (Gordon et al., 2010), highlighting the functional similarities of the single domains.

The best characterized XS, the histone-like nucleoid structuring protein H-NS, is with about 20,000 copies per cell highly abundant in exponentially growing proteobacterial cells and represents one of the major NAPs in these species (Amit et al., 2003). The family of H-NS-like XS is highly diverse and one main function of these proteins is the silencing of AT-rich, horizontally acquired foreign DNA elements (Lucchini et al., 2006; Navarre et al., 2006). However, disruption of H-NS affected over 5% of all genes in *E. coli* which appear to be unlinked (Amit et al., 2003; Arold et al., 2010; Hommais et al., 2001). Furthermore, H-NS mediates gene expression in response to changes in osmolarity, pH, and temperature (Amit et al., 2003; Arold et al., 2010), demonstrating its function as global regulator of gene expression. As a NAP, H-NS inherits also an important role in the organization of the chromosome structure (Noom et al., 2007; Ueguchi et al., 1996; Wang et al., 2011)

In 1998, MvaT was initially identified as regulator of mevalonate catabolism in *Pseudomonas mevalonii* (Rosenthal and Rodwell, 1998). Based on complementation assays with *hns* deficient *E. coli* mutants and sequence alignments, Tendeng and colleagues showed that MvaT from *Pseudomonas Y1000* is functionally related to H-NS (Tendeng et al., 2003). MvaT shares structural similarity with H-NS (Ding et al., 2015). However, in comparison to H-NS from *E. coli*, MvaT displays an amino acid sequence similarity of less than 20% and has a different DNA binding mechanism (Tendeng et al., 2003). Remarkably, while MvaT was only found in λ -proteobacteria, all *Pseudomonas* species harbour at least one MvaT homologous; *Pseudomonas putida* has even five (Tendeng et al., 2003). By performing

chromatin immunoprecipitation (ChIP) coupled with DNA microarrays (ChIP-on-chip) experiments, Castang and colleagues demonstrated strongly overlapping binding patterns for MvaT and its homolog MvaU in the opportunistic pathogen *P. aeruginosa*, suggesting that both proteins function coordinately and even form heteromeric silencer complexes. Based on the binding preference of these proteins for AT-rich DNA regions which are often horizontally acquired, the authors confirmed that MvaT and MvaU function as XS in *Pseudomonas* (Castang et al., 2008).

Rok was initially identified as negative regulator of natural competence in *B. subtilis* since it represses the expression of the *comK* gene, coding for the master regulator of this pathway (Hoa et al., 2002). However, Rok controls the acquisition of horizontal gene elements on a second layer. ChIP-chip analyses revealed that Rok binds to DNA elements which are characterized to be AT-rich and many of its targets are thought to be horizontally acquired (Smits and Grossman, 2010). Comparably to H-NS and MvaT, the N-terminal domain of Rok mediates multimerization into a higher-order nucleoprotein complex leading to silencing of target genes (Duan et al., 2018; Smits and Grossman, 2010). Interestingly, Rok itself is suggested to be the result of a HGT event since it is only present in some *bacilli* species including *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* where it is inserted into an otherwise conserved chromosome region (Albano et al., 2005; Duan et al., 2018). Contrastingly, no Rok orthologs could be identified in other *Bacillus* species such as *B. anthracis*, *B. cereus*, and *B. halodurans* (Albano et al., 2005; Duan et al., 2018).

Lsr2 is a strongly conserved XS protein found in Gram-positive Actinomycetes, e.g. in mycobacterial and *Streptomyces* species (Gehrke et al., 2019; Gordon et al., 2008). Although it displays no significant sequence similarity with H-NS and both differ strongly in their secondary structure, complementation assays with *E. coli* H-NS-deficient mutants revealed that Lsr2 acts as a functional analogue of H-NS (Gordon et al., 2008). Comparably, its targets appear to have higher AT contents and are often horizontally acquired (Gordon et al., 2010).

Recently, Pfeifer and colleagues identified CgpS (*C. glutamicum* prophage silencer) as the first example of a prophage-encoded Lsr2-like XS protein in the actinobacterium *Corynebacterium glutamicum* (Pfeifer et al., 2016). It is homologous to mycobacterial Lsr2. While both proteins share considerable low amino acid sequence identity (~23%), they display striking similarities at the structural level. Genome-wide binding profiles of chromatin affinity purification and sequencing (ChAP-seq) experiments revealed an association of CgpS to AT-rich DNA elements, most of which are located within cryptic prophage regions (about 65% of the 90 targets). CgpS is not essential in the absence of the cryptic prophage CGP3, but interference with CgpS oligomerization resulted in prophage induction in wild-type cells. Altogether, these findings demonstrated that its major function is the silencing of

the prophage element CGP3 to control its lysogenic state (Pfeifer et al., 2016). Remarkably, CgpS was the first characterized example of a prophage-encoded XS and further bioinformatic analysis revealed that this feature is unique for Lsr2-like XS (Pfeifer et al., 2019). The rules underlying CgpS mediated xenogeneic silencing and mechanisms allowing the implementation of foreign DNA elements into host regulatory networks of *C. glutamicum* were the main topics of this thesis (Wiechert et al., 2020a).

2.3.2. Main targets of xenogeneic silencer proteins

A huge amount of transcriptome and binding assays revealed that the scopes of targets of individual XS proteins are highly diverse and most of them are thought to be horizontally acquired and significantly AT-rich (Castang et al., 2008; Gehrke et al., 2019; Gordon et al., 2010; Navarre et al., 2006; Pfeifer et al., 2016; Smits and Grossman, 2010). Remarkably, binding assays identified an association of XS-silencer proteins of all families with their own promoter region, indicating autoregulation as important point in controlling silencing activity (Castang et al., 2008; Gordon et al., 2010; Pfeifer et al., 2016; Smits and Grossman, 2010; Ueguchi et al., 1993).

Furthermore, proteins from all XS classes are involved in the repression of prophage-like elements (Castang and Dove, 2012; Gordon et al., 2010; Hong et al., 2010; Pfeifer et al., 2016; Smits and Grossman, 2010), highlighting their ancient function to protect the host cells from detrimental effects caused by viral gene expression. However, in contrast to the Lsr2-like XS protein CgpS from *C. glutamicum* which has a specialized function as regulator of prophage elements (Pfeifer et al., 2016), several other XS proteins act as central regulators of virulence, secondary metabolic pathways, and drug resistance. For instance, ChIP-on-chip experiments with H-NS from *Salmonella* revealed that H-NS binds to more than 17% of all chromosomal genes comprising several virulence elements such as *Salmonella* pathogenicity island (SPI) -1 and SPI-2, encoding two type III secretion systems (T3SSs) (Navarre et al., 2006). Comparably, H-NS is an important regulator of virulence in *Yersinia* (Heroven et al., 2004), in enterohemorrhagic and uropathogenic *E. coli* (Müller et al., 2006; Wan et al., 2016), in *Shigella flexneri* (Turner and Dorman, 2007) as well as in *V. cholerae* (Ghosh et al., 2006).

ChIP-on-chip experiments with MvaT and its homolog MvaU from *P. aeruginosa* PAO1 revealed a large set of targets (almost 400 genes) including genes coding for toxic secondary metabolites, regulators of virulence and quorum sensing, the filamentous prophage Pf4 as well as many putative and known virulence factors (Castang and Dove, 2012; Castang et al., 2008). ChIP-seq analysis with Rok from *B. subtilis* led to the identification of more than 250 target regions, which were often associated with cooperative DnaA binding, e.g. the *oriC* region (Seid et al., 2017). Targets of Rok are the conjugative transposon *ICEBs1* and prophage elements, demonstrating its function as XS. Further important targets of Rok are the aforementioned *comK* gene as well as genes encoding secreted antibiotics and signalling molecules or for proteins involved in transport and secretion (Albano et al., 2005; Smits and Grossman,

2010). By performing ChIP-chip analyses with Lsr2 from *Mycobacterium tuberculosis*, Gordon and colleagues identified binding to 21% of all coding genes. Lsr2 targets are involved in virulence, cell wall and cell processes as well as PE/PEE genes coding for surface-exposed antigens which are important for interactions with the host (Gordon et al., 2010). In contrast, Lsr2 from *Streptomyces venezuelae* has evolved as a central regulator of specialized metabolic clusters comprising horizontally acquired and well-conserved clusters (Gehrke et al., 2019).

These findings that Lsr2 is involved in the regulation of medically relevant mycobacterial virulence determinants (Gordon et al., 2010) and biotechnologically interesting cryptic metabolite clusters in *Streptomyces* (Gehrke et al., 2019) motivated us to understand Lsr2-mediated silencing as well as the mechanisms allowing the reactivation of silenced genes. In the here presented work we used CgpS of *C. glutamicum* as a model system to decipher the rules underlying silencing and counter-silencing of Lsr2-like XS proteins (Wiechert et al., 2020a).

Loss of the silencing mechanism, e.g. by deleting the encoding gene or by inducing interference effects, can, but do not need to cause lethality. This strongly depends on how increased expression of XS targets affect the bacterial fitness. As mentioned before, the presence of the prophage element CGP3 determines the essentiality of CgpS for *C. glutamicum* (Pfeifer et al., 2016). Castang and colleagues showed in a systematic transposon screen that *P. aeruginosa* cannot tolerate the double deletion of the genes encoding the XS paralogs MvaT and MvaU in the absence of suppressor mutations affecting the lifecycle of the Pf4 phage, indicating phage activity as detrimental fitness consequence (Castang and Dove, 2012). Interestingly, the essentiality of H-NS-like XS depends on the genetic background of the respective strain. While H-NS is essential in *Yersinia* species (Ellison and Miller, 2006; Heroven et al., 2004) and loss of H-NS results in severe growth defects in *Salmonella*, probably caused by increased expression of H-NS regulated pathogenic islands (Ali et al., 2014; Lucchini et al., 2006; Navarre et al., 2006), laboratory *E. coli* strains can tolerate H-NS deficiency to a certain extent (Barth et al., 1995; Navarre, 2016).

2.3.3. How do xenogeneic silencers recognize their targets?

As mentioned above, XS proteins show a clear preference for AT-rich DNA. These proteins interact with the DNA via their C-terminal DNA binding domain. Mutant variants of XS proteins which are able to oligomerize but lose their ability to bind the DNA, e.g. due to point mutations or the absence of the C-terminal domain, interfere with silencing, demonstrating that DNA binding is essential for silencing (Chen et al., 2008; Gehrke et al., 2019; Gordon et al., 2008; Pfeifer et al., 2016; Williamson and Free, 2005). High-resolution structures obtained with nuclear magnetic resonance (NMR) spectroscopy of the C-terminal DNA binding domains of H-NS (Gordon et al., 2011), Lsr2 (Gordon et al., 2010), MvaT

(Ding et al., 2015), and Rok (Duan et al., 2018) in complex with DNA revealed that they all insert with specific residues into the narrow, deep and electronegative minor groove of AT-rich DNA. The exocyclic 6-amino group present in GC-rich DNA sequences can sterically block the minor groove of the DNA. Thus, the absence of this group in AT-rich DNA can further facilitated the interaction of XS proteins with the DNA (Ali et al., 2012; Duan et al., 2018; Navarre, 2016; Pfeifer et al., 2019). However, the underlying molecular binding mechanisms are different (Figure 4). H-NS and Lsr2 binding is mediated via a convergently evolved Q/RGR motif: the prokaryotic AT-hook (Ali et al., 2012). Based on secondary structure predictions, Pfeifer and colleagues suggested that CgpS, the Lsr2-like XS of *C. glutamicum*, binds the DNA via a similar RGI motif (Pfeifer et al., 2016). In contrast, MvaT inserts into the DNA minor groove via a conserved lysine (R) and a downstream located GN motif (R-GN), the so-called AT-pincer, while additional conserved lysine residues interact with the DNA backbone (Ding et al., 2015; Duan et al., 2018). Rok has neither an AT-hook nor an AT-pincer motif. Instead, Rok uses a motif of three non-consecutive amino acids (N-T-R) to form hydrogen bonds with the DNA minor groove, while four lysine residues interact with the negatively charged phosphate backbone of the DNA (Duan et al., 2018) (Figure 4).

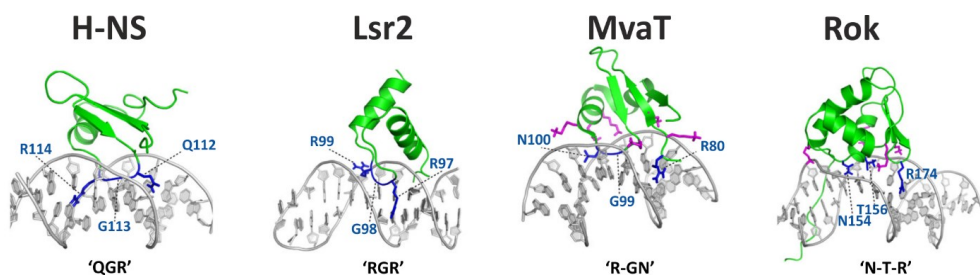


Figure 4: The DNA recognition mechanisms of the four XS protein classes based on structure models. H-NS and Lsr2: Amino acid residues forming the AT-hook motif of H-NS ('QGR') and Lsr2 ('RGR') are highlighted in blue. MvaT: Residues forming the AT-pincer of MvaT ('R-GN') are coloured in blue, while the lysine residues interacting with the sugar-phosphate backbone are shown in magenta. Rok: The three non-consecutive amino acids ('N-T-R') allowing Rok to form hydrogen bonds with the DNA minor groove are highlighted in blue. The four lysine residues interacting with the negative charged phosphate groups of the DNA are coloured magenta. Figure and information adapted from Duan et al. (2018).

Although all XS proteins are reported to bind specifically to AT-rich DNA and are able to silence in heterologous hosts (Gordon et al., 2008; Pfeifer et al., 2016; Tendeng et al., 2003), diversity in their recognition mechanisms leads to differences in target preferences. In vitro protein binding microarray (PBM) experiments with MvaT, H-NS, Lsr2 and Rok were previously performed (Ding et al., 2015; Duan et al., 2018; Gordon et al., 2011). For the analysis of their DNA binding affinities, microarray slides with short double stranded DNA oligonucleotides were used. These short DNA sequences differed in AT content, in the presence of TpA steps or A-tracts as well as in length of contiguous AT-stretches. Typically, these arrays were designed in such a way that all possible eight nucleotide long sequences

(8-mer) were present multiple times. To determine binding affinities and preferences of the XS proteins to the different DNA oligonucleotides, XS were N-terminally fused to a glutathione S-transferase (GST) tag allowing their detection on the microarray slide via GST-specific antibodies (Ding et al., 2015; Duan et al., 2018; Gordon et al., 2011). The screening for highly scored 8-mer sequences in DNA regions which were bound by the respective XS protein in ChIP-seq or ChIP-on-chip experiments revealed an overrepresentation of these 8-mers, suggesting that they are relevant for silencing *in vivo* (Duan et al., 2018; Gordon et al., 2011). These studies demonstrated that MvaT, H-NS and Rok prefer DNA stretches containing flexible TpA steps, which induce local widening of the DNA minor groove, while Lsr2 from *M. tuberculosis* prefers A-tracts instead of TpA steps (Ding et al., 2015; Duan et al., 2018; Gordon et al., 2011). Furthermore, these experiments showed that Lsr2, MvaT and H-NS uniformly favour contiguous AT-sequences without strict sequence specificity (Duan et al., 2018). In contrast, the unique binding mechanism of MvaT provides greater tolerance for G/C interruptions in comparison to H-NS and Lsr2, possibly due to the AT-pincer motif which leaves a cavity in the protein-DNA interface for exocyclic amino groups (Ding et al., 2015). Interestingly, Rok disfavours rigid A-tracts and even prefers single G/C interruptions within its target sequence. Additionally, a distinct number of sequence-specific Rok binding motifs containing flexible TpA steps were identified (AACTA, TACTA) (Duan et al., 2018). These motifs are highly underrepresented in the core genomes of *rok* encoding *bacilli* strains and even allow specific binding of Rok to targets that do not differ in GC content from the core genome, e.g. the promoter regions of *comK* and of its own gene (Duan et al., 2018; Smits and Grossman, 2010).

In our recent study, which is part of this thesis, we investigated the rules underlying target recognition of the Lsr2-like XS CgpS from *C. glutamicum*. Therefore, we performed bioinformatic analyses of recently obtained CgpS ChAP-seq data, *in vitro* binding studies as well as *in vivo* reporter assays (Wiechert et al., 2020a). Consistently with the previously mentioned *in vitro* binding studies for Lsr2, the genome-wide analysis of CgpS targets revealed a clear preference of CgpS towards consecutive AT-rich stretches. The fraction of CgpS targets increased with the length of the AT-rich stretch, while GC-interruptions tended to decrease binding, revealing similarities to the abovementioned results for Lsr2 from *M. tuberculosis* (Ding et al., 2015; Duan et al., 2018). However, in contrast to reports for Lsr2 which failed to identify a sequence-specific binding motif (Chen et al., 2008; Duan et al., 2018; Gehrke et al., 2019), our analysis revealed the overrepresentation of a 10 bp long AT-rich binding motif containing A/T steps (alteration of A to T and vice versa) (Figure 5A) within CgpS targets. The rather specialized function of CgpS as a silencer of prophage elements in *C. glutamicum* could be a possible explanation for this discrepancy. The identified motif was present in 51 of 54 bound promoters and often located close to the position of maximal CgpS binding (exemplarily shown in Figure 5B). Based

on the analysis of the GC-profiles of CgpS target promoters and their CgpS binding peaks, we grouped in our study the promoters into two different classes. Class 1 promoters, e.g. P_{lys} and P_{cg1999} , were typically characterized by a distinct drop in GC content and a bell-shaped CgpS binding peak and were mainly focused in our analyses (Wiechert et al., 2020a) (P_{cg1999} exemplarily shown in Figure 5B). In contrast, CgpS coverage peaks of class 2 promoters were wider and characterized by broader or multiple drops in GC content. We were able to confirm the importance of the identified motif for CgpS-mediated silencing by performing *in vivo* reporter studies with synthetic promoter variants based on the class 1 CgpS target promoter P_{lys} . To monitor promoter activities *in vivo*, we fused the P_{lys} variants to the reporter gene *venus*. A construct containing the motif was efficiently bound by CgpS, while CgpS was not able to silence the control promoter lacking this motif (Wiechert et al., 2020a). However, in contrast to the binding motif of Rok which is underrepresented in the host genome (Duan et al., 2018), our genome wide analysis of CgpS motif occurrence revealed that about 85% of these sequences are not bound by CgpS *in vivo* under standard conditions, indicating that the motif alone does not efficiently mediate CgpS binding (Wiechert et al., 2020a).

A sophisticated model for the mechanism of silencing was proposed for *E. coli* H-NS. *In vitro* studies with H-NS demonstrated high affinity of H-NS towards DNA enabling scanning of the molecule until the XS protein reaches sequence-specific motifs (tCG(t/a)T(a/t)AATT) serving as nucleation sites. Subsequent cooperative binding of additional XS proteins to adjacent, lower affinity sites leads to their oligomerization and to the formation of the tight nucleoprotein complex allowing silencing (Gulvady et al., 2018; Lang et al., 2007; Navarre, 2016; Sette et al., 2009). Based on our findings, we therefore suggested that the identified motif serves as nucleation site - but the formation of a stable CgpS nucleoprotein complex is additionally determined by the adjacent DNA regions. Consistently, an overlay of the GC contents of CgpS target promoters revealed a high degree of similarity in GC-profiles with a distinct drop in the area of maximal CgpS binding (Wiechert et al., 2020a). Furthermore, the identified binding motif was often located within these regions of low GC content featuring highest CgpS coverage, as exemplarily shown in Figure 5B.

To test our hypothesis that the GC content of the DNA sequences flanking the putative nucleation site contributes to CgpS silencing, we constructed and analysed two synthetic promoter variants which were based on the native CgpS target promoter P_{cg1999} (Wiechert et al., 2020a) (Figure 5B). While the sequence of the core promoter region which also contained the putative CgpS nucleation site was kept constant, the sequence of the adjacent flanks was changed. One variant ($P_{cg1999_A-T/G-C}$) was designed to mimic the native GC-profile by exchanging G to C and A to T and vice versa. This construct was efficiently silenced by CgpS. This demonstrated that, in contrast to the binding motif, sequence specificity does not play a role in the adjacent flanks. The second design (P_{cg1999_rand}) was based on a

randomized GC-profile in the adjacent flanks. This promoter was not silenced by CgpS, although the putative nucleation site was present, confirming that the GC-profile is important for silencing (Wiechert et al., 2020a).

These identified DNA features are consistent with previous observations of Pfeifer and colleagues (Pfeifer et al., 2016). By expressing *cgpS* of *C. glutamicum* in *E. coli* Δ *hns* mutants, the authors were able to complement a distinct mutant phenotype, indicating that CgpS can bind to H-NS targets. The applied assay was based on bromothymol blue salicin indicator plates allowing the colour-based visualization of the activity of enzymes encoded in the *bgl* operon (Pfeifer et al., 2016). This operon is suggested to be horizontally acquired and is natively silenced by H-NS (Sankar et al., 2009). Interestingly, the *bgl* promoter region also shows a distinct drop in GC content and at least two slightly truncated versions (8/10 and 9/10 bp) of CgpS motifs identified in native CgpS target sequences (Figure 5C). In conclusion, these findings support our hypothesis that the CgpS binding motif and a drop in GC content collectively mediate CgpS silencing.

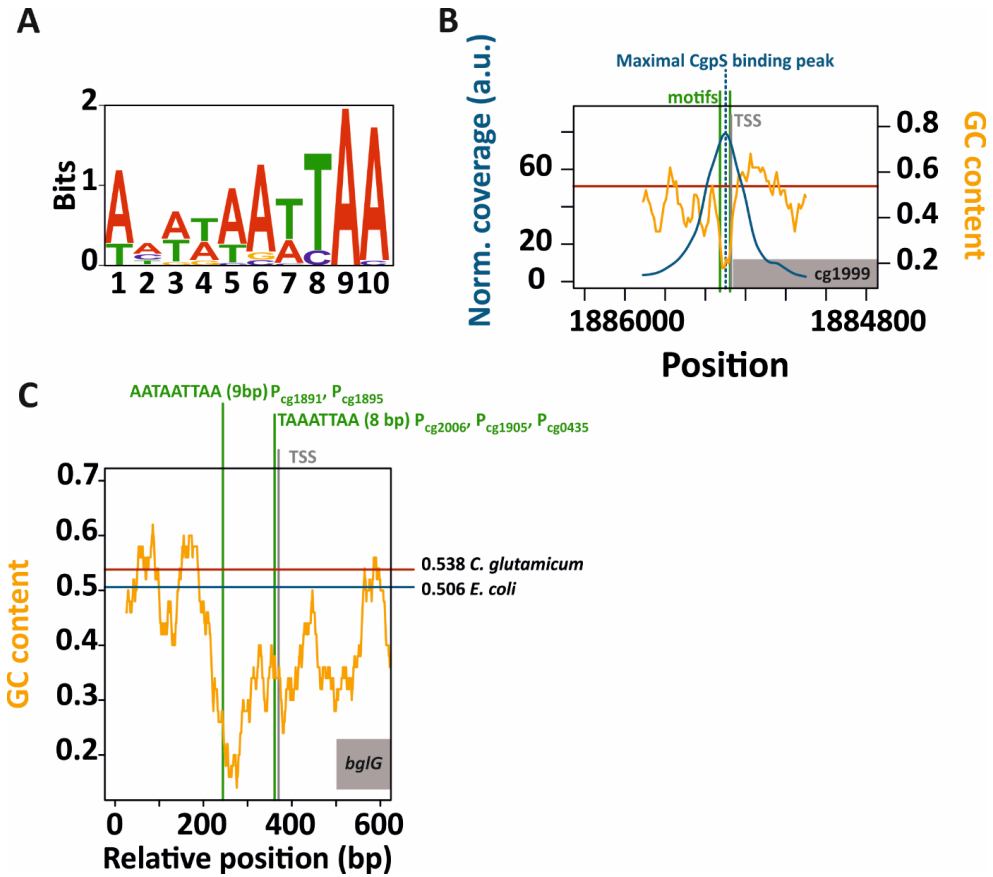


Figure 5: The sequence specific binding motif and the drop in GC-profile cooperatively support CgpS silencing. **A)** Identified binding motif which was present in 51 of 54 CgpS target promoters. Figure taken from Wiechert et al. (2020a). **B)** GC-profiles and CgpS binding peaks of the representative class 1 promoter P_{cg1999} . A rolling mean was applied to calculate the GC-profiles and CgpS coverage peaks based on previous ChAP-seq experiments (Pfeifer et al., 2016) as recently reported (Wiechert et al., 2020a). The position of TSS (Wiechert et al., 2020a) is given as vertical grey line and position of maximal CgpS binding (Pfeifer et al., 2016) as vertical blue dashed line. Positions of the putative binding motif (non-overlapping) from previous FIMO analyses (Wiechert et al., 2020a) are given as vertical green lines. The average GC content of the genome from *C. glutamicum* of 53.8% (Kalinowski et al., 2003) is shown as horizontal red dashed line. The corresponding genes are displayed as grey boxes. **C)** The *E. coli bglG* promoter features a drop in GC content and contains truncated variants of naturally occurring CgpS binding motifs (Wiechert et al., 2020a) located in areas of GC minimum and close to the TSS. The GC-profile of P_{bglG} was calculated and plotted as described in B). The average GC contents of genomes of *C. glutamicum* (red (Kalinowski et al., 2003)) and *E. coli* (blue (Mann and Chen, 2010)) are displayed as coloured horizontal lines. The positions of reference motifs are given, all of them are located within CgpS binding regions close to the maximal CgpS binding peak.

A model for CgpS binding and silencing based on our observations and inspired by the ideas for H-NS could be that CgpS initially binds to the putative sequence-specific nucleation sites. In the following, further CgpS molecules cooperatively bind to the adjacent flanks depending on the particular GC content in these regions, but apparently without strict sequence specificity. This might also explain the inverse correlation between CgpS binding and the GC-profile of its target regions (Figure 6). Another scenario could be that CgpS initially binds non-specifically to the DNA allowing the scanning of the DNA

for the sequence specific motif. In this case, the drop in GC content, meaning the stepwise increase in AT content, could direct CgpS towards these high affinity binding site allowing efficient binding and subsequent oligomerization.

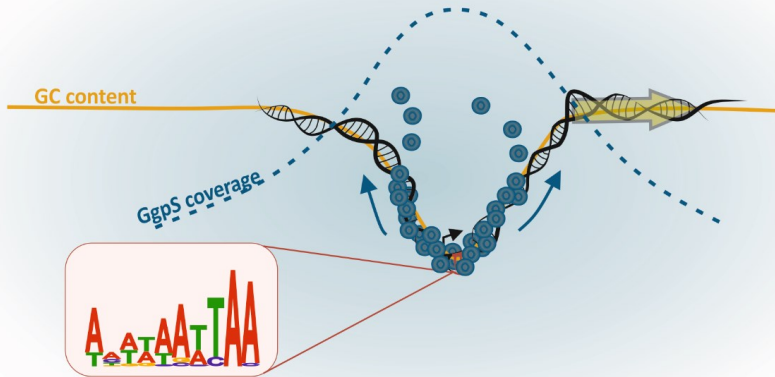


Figure 6: The proposed model for the CgpS target recognition mechanism. CgpS initially binds to its sequence-specific nucleation site containing multiple A/T steps, which is located in a region of low GC content (Wiechert et al., 2020a). In the following, further CgpS molecules cooperatively bind to the adjacent flanks depending on the particular GC content of these regions, but apparently without strict sequence specificity (Wiechert et al., 2020a).

As mentioned above, the synthetic promoter variant containing the correct motif but a randomized GC-profile (P_{cg1999_rand}) was not silenced by CgpS in vivo. However, this observation does not exclude partly CgpS binding, e.g. at the putative nucleation site. In vitro binding assays such as SPR analysis or electrophoretic mobility shift assays might demonstrate whether this variant is still bound by CgpS. Furthermore, protein binding microarray approaches as performed by Ding and colleagues (Ding et al., 2015) as well as by Duan and co-workers (Duan et al., 2018) could be used to study the in vitro affinity of CgpS for the identified motif in comparison to randomized sequences with same nucleotide composition. However, these in vitro approaches investigate binding of a XS protein in an isolated context, which does not necessarily lead to silencing. Our reporter-based analyses were applied to demonstrate CgpS-mediated silencing in vivo. It allows to investigate the result of the interplay of all parameters contributing to transcriptional repression of target genes in living cells and may therefore represent a promising approach for future investigations.

For instance, although we have confirmed the importance of the GC-profile based on one synthetic promoter variant and identified a certain degree of similarity in GC-profiles of different CgpS target

promoters (Wiechert et al., 2020a), the exact rules of how the shape of the GC-profile influences silencing remained unclear. What is the exact impact of the stepwise decreasing GC content and is CgpS able to silence a long promoter region containing only or at least mostly adenines and thymines? Based on these questions, an interesting experiment for future studies could be the expansion of our approach by constructing a library of synthetic promoters containing a functional core promoter region and the CgpS binding motif but differing in density and length of AT-rich stretches, which directly influence the GC-profile. By using flow cytometry, high-throughput screenings of this library might provide further insights into the importance of the surrounding GC-profile for *in vivo* CgpS silencing.

2.3.4. Formation of the nucleoprotein complex

Different studies demonstrated that DNA binding of XS proteins alone does not mediate efficient silencing and that oligomerization and the formation of the higher-order nucleoprotein complex are essential for repressing gene expression (Chen et al., 2008; Spurio et al., 1997; Winardhi et al., 2012). NMR analysis demonstrated that H-NS has a mainly α -helical N-terminal oligomerization domain and that H-NS molecules form dimers in solution. These dimers are capable of self-assembly resulting in the formation of large oligomers (Esposito et al., 2002; Smyth et al., 2000). Comparably, Lsr2, MvaT and Rok can form oligomers allowing the formation of higher-order nucleoprotein complexes (Chen et al., 2008; Duan et al., 2018; Winardhi et al., 2012).

The molecular mechanism of H-NS multimerization was studied in detail and is shown in Figure 7A. H-NS forms oligomers via two separated dimerization interfaces which are located within the N-terminal coiled-coil domain (Badaut et al., 2002; Ueguchi et al., 1996) (Figure 7A). The N-terminal dimerization interface consisting of three helices (H1, H2 and H3) is responsible for the formation of H-NS dimers in solution (Ali et al., 2012; Badaut et al., 2002; Ueguchi et al., 1996). High local H-NS concentrations, e.g. upon binding to the DNA, enables the interaction between different dimers via the central dimerization domain (helix-turn-helix motif between C-terminal end of helices H3 and H4), leading to elongation of the H-NS filament by head-to head/tail-to-tail multimerization (Ali et al., 2012; Arold et al., 2010) (Figure 7A). Oligomerization of XS proteins can result in silencing of the bound DNA regions by different mechanisms (Figure 7B): by binding in the core promoter region and consequently inhibiting binding of the RNA polymerase (I: occlusion), by preventing the RNA polymerase to escape the promoter (II: obstruction), by trapping the RNA polymerase in a formed DNA loop (III) or, putatively, by stimulating Rho-dependent termination (IV) (Landick et al., 2015; Lim et al., 2012).

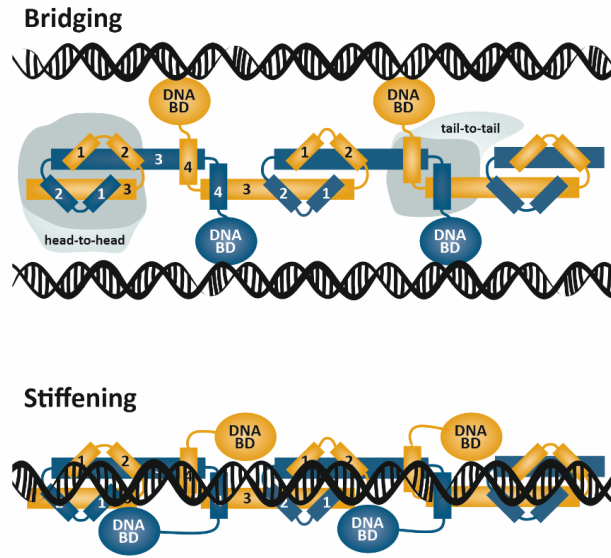
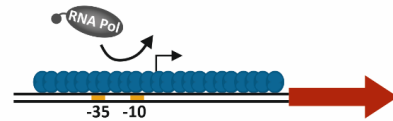
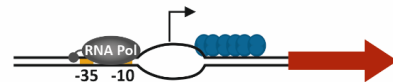
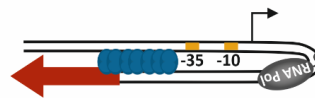
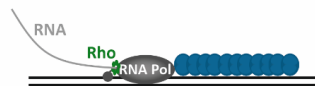
A**B****I) Occlusion****II) Obstruction elongation****III) Trapping****IV) Stimulation of Rho-dependent termination**

Figure 7: Mechanisms underlying xenogeneic silencing. **A)** Head-to head/tail-to-tail multimerization of H-NS is based on its two oligomerization interfaces: N-terminal dimerization interface formed by helices H1, H2 and H3 (head-to-head interaction) and the central dimerization domain (tail-to-tail interaction) formed as helix-turn-helix motif between the end of H3 and H4. H-NS molecules are alternatively coloured in blue and yellow and helices on the left filament site are labelled with helices numbers. Two binding modes for XS proteins were proposed: bridging and stiffening. DNA bridging is caused by DNA binding domains located on opposing faces of the helical XS protein filament which can interact with different sites of a DNA molecule. Rotations in the filament can result in DNA binding domains being located on the same face of the filament, allowing interaction with a single DNA molecule in the stiffening mode. Adapted from Ali et al. (2012), Singh et al. (2016). **B)** Mechanisms leading to xenogeneic silencing **I)** The XS nucleoprotein filament is formed along the RNA polymerase binding site leading to occlusion of the RNA polymerase. **II)** XS proteins bind and oligomerize downstream of the RNA polymerase binding site leading to obstruction of the elongation process. **III)** The bridging mode of XS proteins can induce a loop, which subsequently trap the RNA polymerase leading to silencing. **IV)** The XS nucleoprotein filament blocks the elongating RNA polymerase and putatively stimulates its susceptibility to Rho-dependent termination. Adapted from Landick et al. (2015),

Lim et al. (2012), Qin et al. (2019a). Models are mainly based on H-NS studies. [Abbreviations: RNA Pol, RNA polymerase; DNA BD, DNA binding domain].

Intensively applied *in vitro* assays such as electron microscopy, atomic force microscopy as well as magnetic tweezer and other single-molecule approaches revealed that the binding of XS proteins can lead to two distinct conformations: the bridging mode and the stiffening mode (Ali et al., 2012). The bridging state describes XS-mediated crosslinking of two distantly located sites of the DNA molecule resulting in loop formation and DNA bending (Dame et al., 2005) (Figure 7A). Silencing of the gene loci *bgl* and *proU* in *E. coli* is putatively caused by H-NS binding to regions located upstream and downstream of the promoters. Interactions between distantly bound H-NS molecules might lead to DNA bridging and is suggested to allow trapping of the RNA-polymerase (Nagarajavel et al., 2007). In addition to controlling gene expression, bridging is suggested to be involved in structural compaction of the chromosome (Dame et al., 2005). Stiffening, the second binding mode, was previously described by Amit and colleagues. Stiffening means the end-to-end extension of the H-NS filament leading to the formation of a rigid nucleoprotein complex which might lead to occlusion of the RNA-polymerase or to obstruction of elongation (Amit et al., 2003) (Figure 7).

These modes of action shown in different *in vitro* assays are still topic of controversial discussions. However, Liu and colleagues were able to demonstrate that the experimental conditions, in particular the concentrations of divalent cations such as Mg^{2+} and Ca^{2+} , were responsible for this observed discrepancy: high concentrations of Mg^{2+} lead to bridging, while low concentrations result in stiffening of H-NS (Liu et al., 2010). Later biochemical and biophysical assays performed by Will and colleagues demonstrated that the bridging and stiffening modes depend on the local concentration of H-NS and its corresponding binding sites as well as the binding affinity of H-NS for these sites which is directly influenced by Mg^{2+} . The authors concluded that both modes can play a physiological role in a bacterial cells and that silenced genes are predominantly bound via the stiffening mode (Will et al., 2018). Comparably, MvaT and Lsr2 binding and oligomerization was reported to lead to stiffening and bridging (Qu et al., 2013; Winardhi et al., 2012), while Rok seems to act via bridging (Qin et al., 2019b).

The binding mode of CgpS in terms of stiffening and bridging has not been characterized so far. By using previously reported approaches like scanning force microscopy (SFM) (Dame et al., 2005), electron microscopy (Amit et al., 2003) or single-molecule magnetic tweezer experiments (Gulvady et al., 2018; Liu and Gordon, 2012), the CgpS binding mode could be further analysed. However, as mentioned above, these *in vitro* approaches can be challenging since they do not necessarily reflect the physiological conditions within a cell. Alternatively, live-cell imaging with high resolution microscopy methods such as stochastic optical reconstruction microscopy (STORM) or photoactivated localization microscopy (PALM) might be suitable techniques to analyse the subcellular distribution of

CgpS within the cell. For instance, these methods provided insights into the clustered distribution of photoactivatable fluorescent protein fusions of H-NS (H-NS-mEos2) in *E. coli* (Wang et al., 2011). The combination of this approach with staining of the DNA in living cells might allow the visualization of the DNA conformation in regions bound by CgpS. A suitable DNA dye could be SYTOX, which was demonstrated to perform well in living Gram-negative and Gram-positive bacteria (Bakshi et al., 2014). Alternatively, chromosome conformation capture coupled with deep sequencing (HI-C) experiments allow the detection of physical proximity between DNA loci in the three-dimensional (3D) space, which are distantly localized in the DNA molecule (Verma et al., 2019). This approach could represent a suitable method to detect putative DNA bridges formed by CgpS in vivo. In HI-C studies, the chromosome of a cell is cross-linked with formaldehyde to stabilise contacts between distant loci. In a next step, restriction enzymes are used to digest the genome into smaller fragments. Subsequently, fragments which have been cross-linked, due to their physical proximity in the 3D chromosome organisation, are ligated. Typically, the implementation of a biotinylated adaptor into these ligation junctions allows their enrichment in pull down assays (Verma et al., 2019). To identify DNA bridges or loops which are directly associated with CgpS binding, a Strep-tagged CgpS variant which was used in previous ChAP-seq analyses from Pfeifer and colleagues (Pfeifer et al., 2016) might be a promising alternative for this enrichment step. Finally, these fragments are sequenced, which allows for the detection of ligated DNA loci and therefore the identification of originally bridged DNA (Verma et al., 2019).

In our current study, we performed plasmid-based analysis of CgpS target promoters which were fused to reporter genes to monitor their activity (Wiechert et al., 2020a). These promoters were efficiently silenced, indicating that the native genomic context of this regulatory elements is not important for silencing which might favour the stiffening mode theory. We further determined the positions of transcriptional start sites (TSSs) of CgpS target promoters relative to the position of maximal CgpS binding. Remarkably, in most target promoters, the TSSs were located close to the position of maximal CgpS coverage and GC minimum (exemplarily shown in Figure 5B), indicating that binding close to the TSS is important for efficient CgpS mediated silencing (Wiechert et al., 2020a). In line with our findings, H-NS was also reported to bind within the core promoter region of some of its targets, thereby preventing the binding of the RNA polymerase (Dame et al., 2002). This might support the hypothesis that CgpS silences its targets by occlusion of the RNA polymerase which would match the stiffening binding mode.

In our study, we mainly focused on class 1 CgpS target promoters (one distinct drop in GC content and a bell-shaped CgpS binding peak) (Wiechert et al., 2020a). In contrast, class 2 promoters (e.g. P_{cg1940} and P_{cg1936}) show broader or multiple drops in GC content and often putatively overlapping CgpS

binding peaks (Figure 8A). Remarkably, the identified CgpS binding motif was highly present in these promoters and often matched the different areas of low GC content. Hypothetically, the adjacent drops in GC-profile and the broad CgpS binding peaks could hint for bridging of these regions by CgpS. However, succeeding analysis with truncated variants of two class 2 promoters resulting in constructs featuring only one drop in GC content demonstrated that the upstream region is not essential for efficient CgpS silencing (Figure 8B). This indicates that bridging of adjacent regions with low GC content is not a prerequisite for this mechanism.

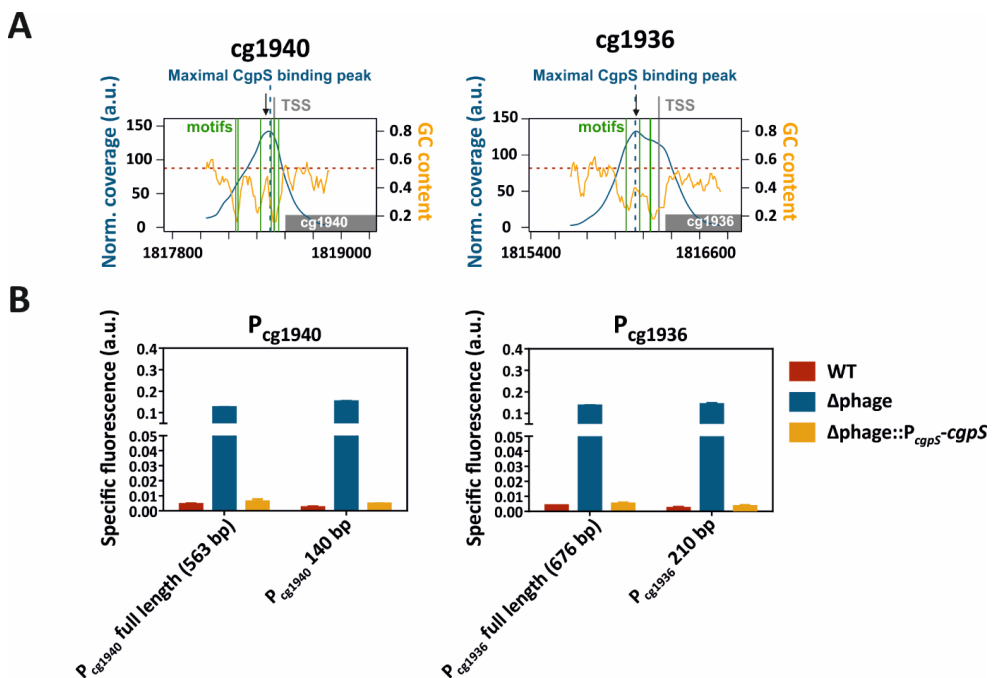


Figure 8: The upstream drops are not essential for silencing of class 2 CgpS target promoters featuring multiple drops in GC-profile. A) GC-profiles and CgpS binding peaks of class 2 promoter P_{cg1940} and P_{cg1936}. A rolling mean was applied to calculate the GC-profiles and CgpS coverage peaks based on previous ChAP-seq experiments (Pfeifer et al., 2016) as recently reported (Wiechert et al., 2020a). Highest ranked TSS (Wiechert et al., 2020a) are given as vertical grey lines and position of maximal CgpS binding (Pfeifer et al., 2016) as vertical blue dashed lines. Positions of the putative binding motif (non-overlapping) from previous FIMO analyses (Wiechert et al., 2020a) are given as vertical green lines. The average GC content of the genome from *C. glutamicum* of 53.8% (Kalinowski et al., 2003) is shown as horizontal red dashed line. The corresponding genes are shown as grey boxes. Black vertical arrows indicate the removed upstream regions in the truncated promoter variants. **B)** Reporter outputs (specific Venus fluorescence) after five hours of cultivation driven by full length or truncated promoter variants. 5'-ends of full-length constructs coincided with the upstream end of the CgpS binding peak. Promoters were plasmid-based (pJC1) analysed in *C. glutamicum* wild-type cells, in the prophage free strain Δphage (MB001 (Baumgart et al., 2013)) as well as in the prophage-free strain with reintegrated *cgpS* under its native promoter (Δphage::P_{cgps-cgps} (Wiechert et al., 2020a)). Cultivations were performed in CGXII minimal medium containing 100 mM glucose in a microtiter cultivation system as recently described (Wiechert et al., 2020a). The results demonstrated that the upstream drops in GC content of both class 2 promoters are not essential for silencing. All constructs showed significant reporter outputs in the prophage-free strain Δphage confirming that all constructs are transcriptionally competent. Silencing was fully restored after reintroduction of CgpS in the prophage-free strain (Δphage::P_{cgps-cgps}).

In a previous study, Kane and Dorman analysed H-NS mediated silencing of the *proU* promoter from *E. coli*. This promoter has two H-NS binding regions, the upstream (URE) and the downstream regulatory elements (DRE), flanking the core promoter which are suggested to allow the formation of H-NS bridges (*trans*-binding mode) leading to silencing (Kane and Dorman, 2011). Interestingly, removing the URE did not lead to promoter activation in wild-type cells, while the promoter showed high activity in a *hns* mutant strain, indicating that H-NS was still able to silence this promoter. The authors suggested a model in which the removal of the URE element leads to a reorganisation of the H-NS complex and to filament formation (*cis*-binding mode) within the DRE region. Subsequently, this *cis*-binding mode provides efficient silencing and inhibits transcription (Kane and Dorman, 2011). The comprehensive model of Kane and Dorman (Kane and Dorman, 2011) might also explain our results. At class 2 promoters, CgpS potentially binds to both drops and forms bridges between them leading to silencing. Removal of the upper region, which could represent the URE model, prevents bridging but triggers filament formation at the remaining high affinity binding site resulting in silencing.

2.3.5. The interplay of xenogeneic silencers and accessory factors

As mentioned above, oligomerization of XS proteins and their interaction with the DNA are critical steps for efficient silencing. In some cases, hetero-oligomer formation was reported. For instance, the paralogs MvaT and MvaU cooperatively regulate their target genes and have the ability to form hetero-oligomer complexes (Castang et al., 2008). The report of Castang and colleagues revealed that MvaT can influence binding of MvaU in specific DNA regions. However, MvaU was able to interact with other genomic regions independently of MvaT suggesting that heteromeric filaments are not essential for the silencing function of MvaU (Castang et al., 2008). Similar observations for H-NS and its paralog StpA propose that both proteins can form hetero-oligomer complexes, although a single *stpA* deletion had no detectable effect on gene expression (Müller et al., 2006).

In contrast, accessory factors of the Hha/YmoA family can strongly affect xenogeneic silencing efficiencies of H-NS-like proteins, e.g. by stabilizing the nucleoprotein complex over long areas of AT-rich DNA (Navarre, 2016). These proteins are characterized by molecular masses of about 8 kDa (half of the mass of H-NS) and structurally mimic the N-terminal oligomerization domain of H-NS (Banos et al., 2009). Hha-like proteins have a positively charged surface, which can contribute to silencing. Interactions of these accessory proteins with H-NS allow for cooperative regulation of gene expression. This interplay depends on the Hha binding motif within the N-terminal oligomerization domain of H-NS (Banos et al., 2009). Interestingly, all species of the *Enterobacteriaceae* family (e.g. *E. coli*, *Yersinia* sp. and *Salmonella* sp.) encode at least one member of the Hha/YmoA family of accessory factors, while

no homologs interact with other XS proteins or with H-NS from species outside of this family (Banos et al., 2009; Vivero et al., 2008).

Hha and its paralog YdgT preferentially influence H-NS mediated silencing of horizontally acquired DNA regions like virulence factors (e.g. SPIs from *Salmonella*), while ancestral H-NS target genes are often not influenced by these accessory proteins (Vivero et al., 2008). This indicates that these accessory proteins have a specialized regulatory function in silencing horizontally acquired DNA. Banos and colleagues suggested that these accessory proteins might enable H-NS to discriminate between self and non-self, thereby enabling stronger silencing of horizontally acquired DNA (Banos et al., 2009).

By analysing CgpS mediated silencing in a strain lacking all other phage genes (Δ phage::*P_{cgpS}-cgpS*), we confirmed in our study that CgpS is the only prophage-encoded protein responsible for silencing of the phage promoter *P_{lys}* (Wiechert et al., 2020a). We performed further comparable experiments with full length and truncated variants of the class 2 promoters *P_{cg1940}* and *P_{cg1936}* in the same strain (Figure 8). Silencing of all promoters was fully restored after reintegrating the *cgpS* gene, indicating that no further phage-encoded protein was involved in silencing of these promoters. Since we did not analyse all CgpS targets in this strain, we cannot exclude that some of them are silenced by a heteromeric nucleoprotein complex. Potentially, host-encoded accessory proteins could also be involved.

Previously, Pfeifer and colleagues performed transcriptome arrays with *C. glutamicum* cells expressing a truncated version of CgpS, which interferes with the CgpS nucleoprotein complex leading to prophage induction. While phage genes were strongly affected, only 2 of 32 targets outside of CGP3 region showed differential gene expression (Pfeifer et al., 2016). This observation might provide a first hint for differences in silencing and/or in the formation of the nucleoprotein complexes. Potentially, accessory proteins could stabilize the CgpS nucleoprotein complex outside of the prophage region. In this regard, further investigations would be necessary. However, it has to be kept in mind, that CgpS is a prophage-encoded XS protein that might play a rather specialized role in the control of the phage life cycle. Therefore, CgpS could possibly act independently of accessory factors. In contrast, H-NS from *E. coli* already evolved from a XS protein towards a global regulator of gene expression (Chib and Mahadevan, 2012), which is also fostered by its interaction with further accessory proteins.

2.4. Evolutionary network expansion by counter-silencing

Xenogeneic silencing globally sets the default state of AT-rich, foreign DNA to the off state - independent of potential effects on cellular fitness (Navarre, 2016; Navarre et al., 2007; Will et al., 2015). At first sight, silencing of beneficial xenogeneic genes seems to be counterintuitive but can be explained by different factors. One point is that a cell cannot differentiate whether invading DNA is

useful or harmful before it is expressed (Navarre, 2016). In general, foreign DNA has a greater chance to reduce the bacterial fitness and this global silencing strategy avoids potential fitness costs. Furthermore, since the value of a gene is defined by its time- and condition-dependent effect, even theoretically useful genes can result in fitness costs if their expression is not appropriately controlled (Sorek et al., 2007; Will et al., 2015). For instance, Ali and colleagues demonstrated that the strong growth defects observed in *Salmonella hns* mutant strains is caused by overexpression of the T3SS encoded in SPI-1. In contrast, an appropriately controlled T3SS mainly contributes to fitness and pathogenicity of this species by allowing invasion of the host and by triggering the inflammatory response (Ali et al., 2014). This example demonstrates the importance of evolving mechanisms, so-called counter-silencing strategies, to integrate foreign genes into existing regulatory networks of the host - thereby allowing their controlled expression at appropriate time points and environmental and physiological conditions (Will et al., 2014; Will et al., 2015). Evolutionary network expansion by counter-silencing allows cells to achieve control over silenced genes and to get access to novel beneficial traits (Will et al., 2014). Different counter-silencing strategies exist in bacteria which enable the transient alleviation of silencing in response to environmental conditions or physiological states (Navarre et al., 2007; Pfeifer et al., 2019; Stoebel et al., 2008). Remarkably, as result of the ongoing evolutionary arms race between bacteria and foreign DNA elements, pathogenicity islands, phages, and other MGEs also provide a variety of mechanisms enabling counteracting of host-mediated silencing (Ali et al., 2011; Navarre, 2016; Pfeifer et al., 2019). Mechanisms leading to counter-silencing can be grouped into different categories (Figure 9): I) Other NAPs or silencer-like proteins can interfere with the oligomerization of a XS protein preventing the formation of a stable silencer-DNA complex (e.g. H-NST (Williamson and Free, 2005)). Furthermore, DNA mimic proteins like the T4 phage protein Arn are suggested to be bound by H-NS leading to titration of the XS protein and to reduced H-NS binding to its genomic DNA targets (Ho et al., 2014). II) Sequence-specific DNA binding proteins such as transcription factors (TFs) compete with the silencer for binding to DNA regions (e.g. RovA (Heroven et al., 2004)). III) Alternative σ -factors not being affected by the silencer (e.g. by RpoS-mediated counter-silencing of *E. coli hdeAB* promoter (Shin et al., 2005)) can enable transcription. IV) Furthermore, protein-DNA interactions (e.g. SlyA (Corbett et al., 2007)) or environmental conditions (e.g. temperature (Falconi et al., 1998)) which induce alterations in promoter architecture can interfere with the silencer-DNA complex (Figure 9). For instance, Falconi and colleagues showed that H-NS represses the expression of the *virF* gene encoding an important regulator of virulence genes in *S. flexneri* only below 32°C. While under this conditions H-NS molecules bound at two distant sites in the *virF* promoter and cooperatively interact, higher temperatures induce conformational changes in the *virF* promoter structure, counteracting H-NS mediated silencing (Falconi et al., 1998). Remarkably, while the TF SlyA of *E. coli* appears to compete with H-NS for binding at certain DNA sequences (e.g.

at the *hlyE* gene (Lithgow et al., 2007)), SlyA is thought to antagonize H-NS silencing at other loci by remodelling the nucleoprotein complex without competing for binding (e.g. at the capsule gene cluster) (Corbett et al., 2007).

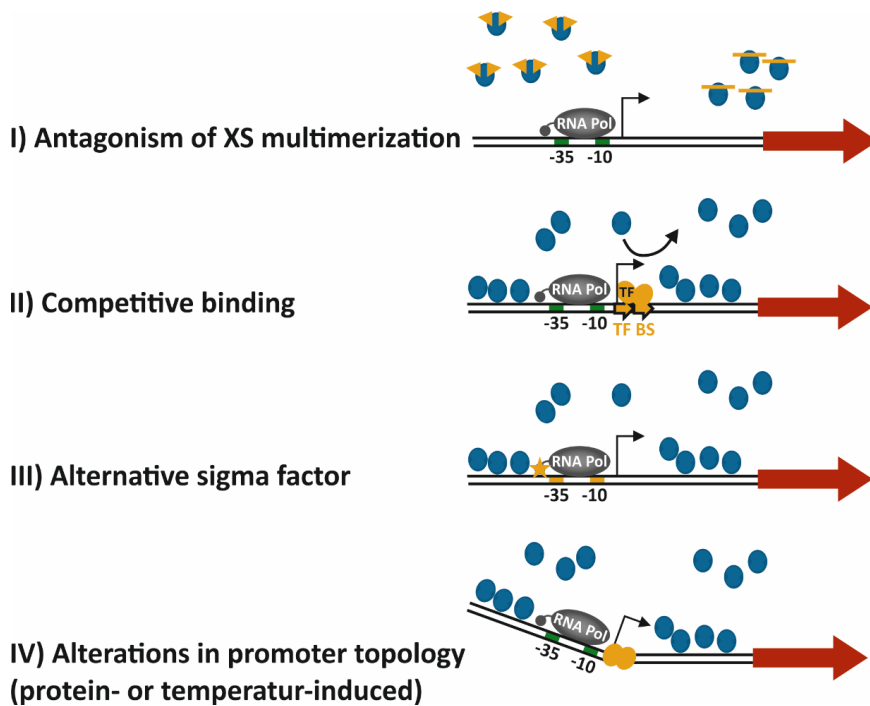


Figure 9: Different mechanisms of XS counter-silencing leading to the activation of transcription. I) Antagonists of XS multimerization can interfere with the oligomerization of the XS protein preventing the formation of a stable silencer-DNA complex (e.g. H-NST (Williamson and Free, 2005), 5.5 protein of phage T7 (Ali et al., 2011) orange triangles, left). Additionally, DNA mimic proteins (e.g. the T4 phage protein Arn; orange lines, right) are suggested to bind H-NS, leading to reduced H-NS binding at genomic targets (Ho et al., 2014). II) Sequence-specific DNA binding proteins such as TFs (e.g. RovA (Heroven et al., 2004); orange ovals) can compete with the silencer for DNA binding. III) Activation of transcription can be mediated by an alternative σ -factor (e.g. RpoS (Shin et al., 2005); orange star). IV) Alterations in the DNA topology caused by environmental changes (e.g. temperature-induced expression of *virF* (Falconi et al., 1998)) or DNA binding proteins (e.g. SlyA (Corbett et al., 2007); orange ovals) can disrupt the silencer-DNA complex. Adapted from Fang and Rimsky (2008). [Abbreviations: RNA Pol, RNA polymerase; TF, transcription factor; TF BS, TF binding site].

Furthermore, Brucoli and colleagues reported that small natural compounds like polyamides can bind into the minor groove of AT-rich DNA and interfere with DNA binding proteins, suggesting that these compounds might also antagonize binding of XS proteins (Brucoli et al., 2015). Remarkably, Rangarajan and Schnetz identified the transcribing RNA polymerase as further mechanism, which is able to relieve H-NS mediated repression. While poorly transcribed genes are repressed by H-NS, efficiently transcribed DNA regions escape silencing presumably by remodelling of the nucleoprotein complex (Rangarajan and Schnetz, 2018).

Examples of counter-silencing by interference between different NAPs as well as natively existing mechanisms of TF-mediated counter-silencing will be the topic of the following sections. In the here presented work, we performed a synthetic approach to decipher the rules underlying TF-mediated counter-silencing of Lsr2-like proteins using CgpS from *C. glutamicum* as a model (Wiechert et al., 2020a). I will discuss our results as well as findings from other synthetic counter-silencer approaches conducted previously for the XS protein H-NS in the following chapters.

2.4.1. Antagonism of XS multimerization

The oligomerization of XS proteins allowing the formation of higher-order nucleoprotein complexes is a prerequisite for their silencing function. Some NAPs cooperatively bind to their target regions leading to the formation of hetero-oligomer complexes with increased stability, as discussed for the H-NS/Hha complex (chapter 2.3.5. “The interplay of xenogeneic silencers and accessory factors.”). However, other proteins interfere with the multimerization mechanism or compete for XS binding sites leading to counter-silencing. Apparently, these strategies were evolved by horizontally acquired genomic islands to avoid silencing by the host XS protein. Examples are the H-NST and Ler proteins found in different *E. coli* strains (Williamson and Free, 2005; Winardhi et al., 2014).

H-NST is a horizontally acquired truncated version of H-NS lacking the C-terminal DNA binding domain and was identified in large genomic islands (ancestral MGE) of enteropathogenic (EPEC) and uropathogenic (UPEC) *E. coli* strains. By interfering with the N-terminal oligomerization domain of H-NS, H-NST has a dominant-negative effect on the formation of the nucleoprotein complex leading to reactivation of silenced genes (Williamson and Free, 2005). Surprisingly, H-NS encoded by the conjugative IncHI plasmid R27, where it is involved in the regulation of plasmid conjugation in response to temperature, exhibits a reduced sensitivity for H-NST interference allowing appropriate regulation of R27 conjugation (Baños et al., 2011).

The idea of counter-silencing by truncated variants of XS proteins was recently applied by different groups. Banos and colleagues heterologously expressed H-NST from EPEC in *Yersinia* allowing counter-silencing of the essential XS protein H-NS and the analysis of the regulatory network of H-NS (Baños et al., 2008). In another study, Pfeifer and co-workers overexpressed the N-terminal domain of the XS protein CgpS. Via interference with the formation of the CgpS nucleoprotein complex, this truncated variant led to prophage induction in *C. glutamicum* (Pfeifer et al., 2016). Remarkable, this effect was reproducible in a comparable experiment with N-terminal domains of Lsr2 variants from mycobacterial species (Pfeifer et al., 2016). Additionally, Gehrke and colleagues used a DNA binding deficient Lsr2 variant from *S. venezuelae* to activate expression of specialized metabolic clusters by counter-silencing Lsr2 in different *Streptomyces* species (Gehrke et al., 2019). Remarkably, strategies aiming at interfering with the formation of higher-order XS oligomers are also used by phages. For instance, the

5.5 protein of coliphage T7 interacts with the central oligomerization domain of H-NS and thereby inhibits H-NS repression, although H-NS is still bound to the DNA (Ali et al., 2011).

The horizontally acquired H-NS paralog Ler (locus of enterocyte effacement (LEE)-encoded regulator) of enterohemorrhagic *E. coli* (EHEC) and EPEC strains represents a further example of a NAP acting as an H-NS counter-silencer. Ler also binds to AT-rich DNA but has a highly different N-terminal oligomerization domain compared to H-NS (Winardhi et al., 2014). By performing magnetic tweezer experiments, Winardhi and colleagues showed that Ler binds to the DNA non-specifically and non-cooperatively and that the subsequent effects on DNA topology differ depending on the local concentration of Ler. DNA folding and wrapping was observed at low concentrations, while high amounts of bound Ler increased the rigidity of the DNA and replaced H-NS from the DNA, leading to counter-silencing (Winardhi et al., 2014). Interestingly, Levine and colleagues demonstrated that H-NS supports Ler binding in the presence of H-NS, thereby facilitating Ler-mediated counter-silencing (Levine et al., 2014).

HU is a conserved NAP which is highly abundant in eubacteria and has important functions in the regulation of cell growth, virulence, SOS response, and many other processes (Stojkova et al., 2019). Magnetic tweezer experiments and atomic force microscopy experiments hinted that HU and H-NS compete for the same DNA regions, putatively enabling HU to locally antagonize H-NS-mediated DNA condensations and silencing (van Noort et al., 2004). Fis, another highly abundant NAP is able to modulate the DNA topology (Ouafa et al., 2012) and was also proposed to counteract H-NS mediated gene silencing (Stoebel et al., 2008).

Bioinformatic analyses revealed that bacterial species frequently encode several XS proteins from the same class, while silencers from different classes do not co-exist in the same species (Perez-Rueda and Ibarra, 2015). In addition to the abovementioned results, Pfeifer and colleagues showed that the expression of *hns* from *E. coli* in wild-type *C. glutamicum* cells results in loss of CgpS mediated silencing and prophage induction (Pfeifer et al., 2016). These findings suggest that not related XS proteins compete for the binding at AT-rich genomic stretches, which hinders the formation of a silencing-competent nucleoprotein complex (Pfeifer et al., 2016).

2.4.2. Transcription factor-mediated counter-silencing in nature

The abovementioned NAP-based interfering mechanisms target the oligomerization process of XS proteins or compete with them for DNA binding. Thus, they often lead to global inhibition of the formation of higher-order nucleoprotein complexes. In contrast, TF-mediated counter-silencing can be considered as targeted process allowing the reactivation of single genes. TFs represent key players in the cellular regulatory network by activating or repressing gene expression in response to extra- and intracellular stimuli and their binding affinity for the DNA is determined by sequence-specific binding

motifs (von Hippel and Berg, 1986). As mentioned before, the control of beneficial, foreign gene expression is an important step for improving bacterial fitness (Will et al., 2015). Evolutionary network expansion by using existing regulatory circuits to control foreign gene expression, e.g. by developing TF-mediated counter-silencing mechanisms, is a common strategy found in bacteria (Navarre, 2016; Will et al., 2014; Will et al., 2015).

One of the best characterized system is the PhoPQ two-component system from *Salmonella*. This system is responsive to different environmental stimuli (low pH, antimicrobial peptides, low magnesium concentration) and represents a key player for virulence in *Salmonella* (Bader et al., 2003; Groisman et al., 1997; Miller et al., 1989). Upon stimulus perception, PhoQ phosphorylates PhoP leading to sequence-specific binding of the response regulator to the DNA. Will and colleagues systematically analysed the regulatory network of PhoP comprising ancestral core genes as well as horizontally acquired genes (Will et al., 2014). By analysing activities of promoters of both categories in different *Salmonella* mutant strains as well as in in vitro transcription assays, the authors demonstrated that ancestral promoters are controlled by classical PhoP-mediated activation. In contrast, transcription of horizontally acquired promoters depends on binding of PhoP and the TF SlyA, which cooperatively act as counter-silencing factors. Binding of SlyA and PhoP had no effect in the absence of H-NS, indicating that PhoP-mediated counter-silencing and classical activation are distinct mechanisms (Will et al., 2014).

Analyses of the promoter architectures of both categories revealed remarkable differences (Will et al., 2014; Zwir et al., 2012). Activated ancestral core genes typically exhibit a conserved promoter architecture with a PhoP binding site close to the -35 box and bound PhoP can recruit the RNA polymerase (Will et al., 2014; Zwir et al., 2012). In contrast, horizontally acquired targets typically show flexible promoter architectures regarding the number of operator sequences, the sequence of the binding site itself as well as the orientation of the binding motif and its distance to the TSS (Will et al., 2014; Zwir et al., 2012). This variability in promoter architectures indicates that, in contrast to classical activation, counter-silencing factors might not activate the promoter by recruiting the RNA polymerase. Instead, they enable transcription by interfering with the silencer-DNA complex (Will et al., 2014; Will et al., 2015). This flexibility in promoter architectures is assumed to facilitate evolutionary network expansion by counter-silencing in comparison to the de novo evolution of classical activator circuits (Will et al., 2014; Will et al., 2015).

In addition to the PhoPQ regulon, several diverse counter-silencing examples exist in nature highlighting the complexity of counter-silencing evolution. For instance, the important virulence regulator VirB from *S. flexneri* is suggested to counteract H-NS silencing by DNA binding, oligomerizing, and by DNA bending (Gao et al., 2013; Turner and Dorman, 2007). It is assumed that VirB is neither

involved in recruiting the RNA polymerase nor in promoting open complex formation, but that it antagonises H-NS mediated silencing (Turner and Dorman, 2007). In the absence of H-NS, VirB target promoters like P_{icsB} are constitutively active (Porter and Dorman, 1994), showing that counter-silencing and classical activation are distinct mechanisms. LeuO, a LysR-type TF found in *Salmonella enterica* and *E. coli*, leads to counter-silencing of H-NS by acting as boundary element: it binds between the nucleation site of H-NS and the promoter region, oligomerizes and bends the DNA around itself, thereby blocking the H-NS polymerization process and enabling transcription (Chen et al., 2003; Shimada et al., 2011). Further examples of TFs, which have been co-opted to act as H-NS counter-silencer, are the MarR-type regulators SlyA and RovA of *S. enterica* and *Yersinia tuberculosis* (Heroven et al., 2004; Navarre et al., 2005; Perez et al., 2008). MarR-type regulators, which play an important role in multiple antibiotic resistance, belong to a widespread, ancient family of TFs and typically function as environmentally responsive repressors (Aleksun et al., 2001; Perera and Grove, 2010). However, the SlyA/RovA lineage in *Enterobacteriaceae* has evolved an additional function as pleiotropic counter-silencer leading to a strong extension of their regulons including many virulence genes (Will et al., 2019). In a current review, Will and Fang suggested that the evolution of MarR-type regulators towards counter-silencing factors could be facilitated by similarities between their target sequences and those of XS proteins (Will and Fang, 2020). Furthermore, binding of these TFs induces distorting effects on the DNA leading to bending of the H-NS-DNA complex (Will and Fang, 2020). Several other examples of TF-mediated H-NS counter-silencing have been described comprising a large set of AraC-like proteins including GadX and GadW, HilC and HilD as well as ToxT from *V. cholerae* (Olekhnovich and Kadner, 2007; Tramonti et al., 2006; Yu and DiRita, 2002). In contrast to abovementioned examples, ToxT is assumed to have a dual function during counter-silencing: interfering with H-NS silencing and transcription activation by interacting with the RNA polymerase (Hulbert and Taylor, 2002; Yu and DiRita, 2002). A comparable dual activity was observed for the TF SsrB of *Salmonella* (Walthers et al., 2007) suggesting that classical activation and counter-silencing are distinct regulatory mechanisms but can be combined in a single TF.

In conclusion, counter-silencing of H-NS plays an important role in several cellular processes including activation of virulence genes and antibiotic resistance. Furthermore, a connection between counter-silencing and quorum sensing was recently reported. Binding of LuxR, the master quorum sensing TF of *Vibrio harveyi*, displaces H-NS from gene loci involved in quorum sensing (Chaparian et al., 2020). These reports combined with several other examples highlight that xenogeneic silencing and TF-mediated counter-silencing provide access to horizontally acquired DNA and thereby foster evolutionary network expansion and bacterial evolution. This might explain why using existing regulatory circuits, e.g. by co-opting a TF from its original function to act as counter-silencer, is a common strategy of bacteria to control expression of horizontally acquired genes. Although counter-

silencing reports mainly focused on the reactivation of targets of H-NS-like XS proteins in different species, they provide insights into the complexity of regulatory network expansion and into the variability of counter-silencing mechanisms.

However, while TF-mediated counter-silencing of H-NS-like proteins has been studied in detail, little is known about mechanisms allowing the reactivation of genes silenced by XS proteins of the other classes. For instance, to the best of our knowledge, only one native example for an Lsr2 counter-silencer has been reported (Kurthkoti et al., 2015). IdeR, a central regulator of iron homeostasis in *M. tuberculosis*, counteracts Lsr2-mediated repression of the *bfrB* locus encoding a bacterioferritin (Kurthkoti et al., 2015). As mentioned before, Lsr2 is a master regulator of virulence genes and might be involved in multidrug tolerance in *M. tuberculosis* and was therefore highlighted as promising drug candidate (Colangeli et al., 2007; Gordon et al., 2010; Gordon et al., 2011). Additionally, in *Streptomyces*, Lsr2 acts as a silencer of specialized metabolic clusters (Gehrke et al., 2019). Both examples underline the importance that motivated our recent study to understand the mechanisms of xenogenic silencing and counter-silencing of Lsr2-like proteins.

Binding of the TF is a critical step for efficient counter-silencing and this process directly depends on the presence of the corresponding TF binding site within the silenced promoter region. This was demonstrated in different mutant analyses lacking the respective TF and in studies with mutated binding sites preventing binding of the regulator (Caramel and Schnetz, 1998; Kane and Dorman, 2011; Wiechert et al., 2020a; Will et al., 2014). Regarding the evolution of counter-silencing, one could think of three different scenarios (partially reviewed by (Navarre, 2016; Will et al., 2015)): I) Accidentally, a foreign DNA element already contains a binding motif which is recognized by a host encoded TF. If the interplay of gene and stimulus-dependent TF binding increases the fitness of the host, this DNA element will be vertically transferred to the following bacterial generations. Otherwise, the resulting fitness costs will prevent its manifestation within the bacterial population. II) A second scenario would be that a foreign DNA element encodes an activating TF and contains the corresponding target promoter. Upon entering the bacterial cell, this target promoter might be silenced by host-encoded XS proteins leading to the addition of a repressive regulatory layer. Now, the TF might act in a dual fashion: original activity as activator and newly gained counter-silencing function. III) The third scenario is a try and error approach. The horizontally acquired DNA is initially repressed by a XS protein and resides as cryptic element within the bacterial genome. During evolution, random mutations within silenced promoter sequences can lead to the formation of TF binding sites. Binding of the corresponding TF interferes with the silencer DNA complex leading to counter-silencing and to the transcription of the following gene. Since binding of TFs can depend on a variety of stimuli (Seshasayee et al., 2011), those signals can be integrated into counter-silencing by co-opting corresponding host TFs. If the gene

product and its expression state fulfil the requirements of the bacterial cell, this mutation will provide a fitness advantage and will manifest within the bacterial population (Will et al., 2014; Will et al., 2015). In contrast, if the gene product reduces cellular fitness, this cell will die and mutations will be lost (Navarre, 2016; Will et al., 2015).

2.4.3. Synthetic disruptive counter-silencing

The flexibility in promoter architecture, which was reported by Will and colleagues for the PhoPQ regulon of *Salmonella* (Will et al., 2014), was reflected by our current study as well as in two other synthetic approaches aiming at counter-silencing H-NS target promoters (Caramel and Schnetz, 1998; Kane and Dorman, 2011; Wiechert et al., 2020a). In our study, we performed a comprehensive synthetic counter-silencer approach using the Lsr2-like XS protein CgpS of *C. glutamicum* as a model to decipher the rules underlying counter-silencing of Lsr2-like proteins (Wiechert et al., 2020a). In this plasmid-based approach, we used different phage promoters targeted by CgpS as platforms for our synthetic counter-silencer design. We artificially inserted the binding site of the TF GntR, the regulator of gluconate catabolism of *C. glutamicum*, within 12 different silenced phage promoters and analysed counter-silencing efficiencies by performing in vivo reporter assays. We could show that binding of GntR to various silenced promoters resulted in their activation, demonstrating that GntR can act as a counter-silencer in a set of different promoters (Wiechert et al., 2020a). We varied the position of the GntR binding site within two exemplary class 1 CgpS target promoters (P_{lys} and P_{cg1999} , one single drop in GC-profile and a bell-shaped CgpS binding peak) to analyse the potential and constraints of CgpS counter-silencing. Counter-silencing was achieved at different positions within P_{lys} in a range of 15 bp upstream and 10 bp downstream of the position of maximal CgpS coverage and highest efficiency was observed at this maximum position. The results for P_{cg1999} were in a comparable range, highlighting a certain degree of flexibility in promoter architectures. Positions outside of this region were not activated by GntR binding and often tended to be repressed. Remarkably, several constructs showed only low reporter outputs under non-inducing conditions demonstrating that they are still tightly controlled by CgpS (Wiechert et al., 2020a). In conclusion, almost all binding positions resulted in differential gene expression, highlighting the potential of xenogeneic silencing and counter-silencing to facilitate regulatory network expansion by providing tuneable promoter systems with low background activity. In line with the promoter studies of Will and colleagues (Will et al., 2014), our design allowed a certain degree of flexibility in terms of binding site orientation and position. Interestingly, the distances between the closest PhoP binding site and the TSS vary by only 34 bp (Will et al., 2014; Zwir et al., 2012), indicating that the range of binding site positions allowing counter-silencing was comparable to our study.

Similar results were previously described by Caramel and Schnetz (Caramel and Schnetz, 1998). The authors inserted the binding sites of the Isopropyl- β -D-1-thiogalactopyranoside- (IPTG-) dependent TF LacI and of the temperature-sensitive λ -repressor into the well-characterized *bgl* promoter of *E. coli*, which is silenced by H-NS. Observed counter-silencing effects by TF binding at different positions, which were in a similar window as discussed before (Wiechert et al., 2020a; Will et al., 2014), confirmed the abovementioned constraints of counter-silencing (Caramel and Schnetz, 1998). Furthermore, comparable to our results, this study highlighted that counter-silencing allows the conversion of a repressor into an activating regulator (Caramel and Schnetz, 1998). Examples for such an evolutionary conversion can also be found in nature as shown for the MarR-type repressors RovA and SlyA (Will et al., 2019). Another synthetic approach was based on the VirB protein from *S. flexneri* (Kane and Dorman, 2011) which natively acts as a counter-silencer by wrapping the DNA around itself (Turner and Dorman, 2007). By implementing the corresponding binding site at different positions and orientations into the heterologous *E. coli proU* promoter, which is silenced by H-NS but natively not bound by VirB, counter-silencing was achieved (Kane and Dorman, 2011).

In conclusion, the extensive analysis of the PhoP regulon in *Salmonella* and the two synthetic studies which focused on H-NS counter-silencing revealed important insights into the potential and constraints of H-NS counter-silencing. In our recent study, we performed the first systematic analysis of counter-silencing of a Lsr2-like XS protein (Wiechert et al., 2020a). Remarkably, the obtained results from all approaches exhibit a high degree of agreement, e.g. the flexibility in promoter architectures. However, in some points, the transferability shows limitations. For instance, we tested a set of different binding sites of heterologous and homologous effector-responsive TFs for the design of counter-silencer constructs based on the CgpS target promoter P_{lys} (Figure 10). Comparable to the *bgl* promoter-based study of Caramel and Schnetz (Caramel and Schnetz, 1998), we also tested constructs based on the LacI repressor. While the inserted LacI binding site only slightly influenced H-NS silencing and LacI binding efficiently reactivated the *bgl* promoter activity (Caramel and Schnetz, 1998), the introduction of this sequence into the P_{lys} promoter led to drastically increased background activities. Furthermore, LacI binding did not significantly further alleviate CgpS silencing (Figure 10). This scenario was repetitively shown for different TFs including IpsA from *C. glutamicum* and TrpR from *E. coli* (Figure 10). We assumed that the binding site composition, especially its GC content and length, strongly influence CgpS silencing independent of TF binding. This hypothesis was confirmed by the high reporter outputs driven from the P_{lys} promoter with the inserted LacI binding site in the absence of the corresponding TF (Figure 10). Obviously, the *bgl* promoter shows a higher degree of tolerance for the inserted LacI binding site (Caramel and Schnetz, 1998). In conclusion, the results of H-NS and our study revealed that differences between H-NS- and CgpS-mediated silencing exist.

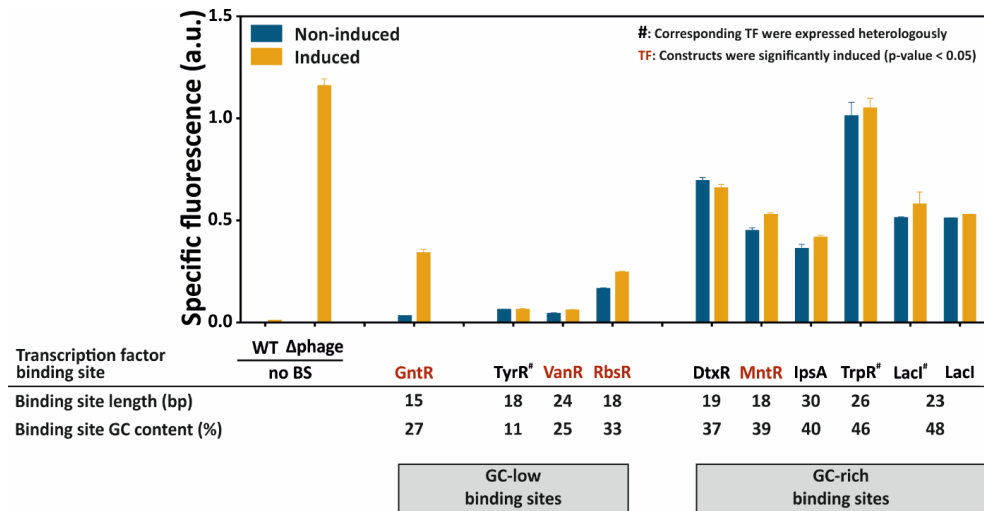


Figure 10: Impact of effector-responsive TF binding and of inserted operator sequences on promoter activities of synthetic variants of the CgpS target promoter P_{lys} . Different TF binding sites (BS) were inserted within the phage promoter P_{lys} directly upstream of the position associated with maximal CgpS binding obtained in previous ChAP-seq experiments by Pfeifer and colleagues (Pfeifer et al., 2016). Reporter outputs (Venus expression) driven by plasmid-based (pJC1) promoter constructs were analysed regarding silencing and counter-silencing in *C. glutamicum* wild-type cells after five hours of cultivation. The native phage promoter P_{lys} served as reference and was analysed in *C. glutamicum* wild-type cells as well as in the absence of CgpS in the prophage free strain Δphage (MB001 (Baumgart et al., 2013)). Cells were cultivated in a microtiter cultivation system under conditions that induced (yellow bars; +) or did not induce (blue bars; -) binding of the respective TF. In cases of constructs with binding sites of heterologous TFs, the corresponding TF encoding gene was inserted within the respective construct (#). For the LacI-based promoter variant, constructs with and without the *lacI* gene were tested. Cultivations were performed in CGXII minimal medium (Keilhauer et al., 1993). Used carbon sources and additives are listed in the following. For the ion-dependent TFs MntR and DtxR, the usually used concentrations of $MnSO_4$ (59 μM) and $FeSO_4$ (36 μM) were adjusted according to the TF, and amounts are given below. Effectors controlling TF binding are underlined, respectively. **No BS:** 100 mM gluconate; **GntR:** - 100 mM gluconate, + 111 mM glucose; **TyrR:** - 111 mM glucose + 3 mM Ala-Ala, + 111 mM glucose + 3 mM Ala-Ala + 0.3 mM Ala-Tyr; **VanR:** - 111 mM glucose + 2 mM vanillate, + 111 mM glucose; **RbsR:** - 111 mM glucose + 66.6 mM ribose, + 111 mM glucose + 66.6 mM gluconate; **DtxR:** - 111 mM glucose + 5 μM $FeSO_4$, + 111 mM glucose + 36 μM $FeSO_4$; **MntR:** - 55 mM glucose + 0.059 μM $MnSO_4$, + 55 mM glucose + 0.59 μM $MnSO_4$; **IpsA:** - 111 mM glucose + 50 mM myo-inositol, + 111 mM glucose; **TrpR:** - 111 mM glucose, + 111 mM glucose + 0.3 mM Ala-Trp; **LacI:** - 111 mM glucose + 100 μM IPTG, + 111 mM glucose. All cells were pre-cultivated in CGXII minimal medium under non-inducing conditions in microtiter plates. The lengths of the inserted binding sites as well their average GC contents are specified, and constructs were sorted based on the latter feature. Constructs which were significantly induced by binding of the TF (t-test, p-value < 0.05) are highlighted by red TF names. The figure demonstrates that CgpS mediated silencing is strongly affected by long and/or GC-rich inserted TF binding sites leading to high background expression levels. [Abbreviations: BS, binding site; a.u., arbitrary unit; WT, wild-type; TF, transcription factor].

In our study, we performed detailed plasmid-based counter-silencing analyses with class 1 promoters which were characterized by a single distinct drop in GC-profile and a bell-shaped CgpS binding peak resulting in a large set of counter-silencer constructs. However, most of the class 2 promoters showing broader or multiple drops in GC content and the usually broad or even overlapping CgpS coverage peaks failed to be counter-silenced when GntR binds at the annotated position of maximal CgpS binding (Wiechert et al., 2020a). We speculated that the annotation of the maximal CgpS coverage position of P_{CG1936} (Pfeifer et al., 2016), which was located between two areas of low GC content and distant to the TSS, might be incorrect (Figure 8A). In a following attempt, we inserted the GntR binding site at two different positions close to the TSS in a region of low GC content, but counter-silencing was not detectable

(Wiechert, Frunzke, unpublished). As discussed in chapter 2.3.4. ("Formation of the nucleoprotein complex."), we hypothesised that these broader peaks might stabilize silencing, e.g. by bridging the DNA between two CgpS high affinity sites, and thereby preventing GntR mediated remodelling of the nucleoprotein complex.

Although most class 1 promoters were efficiently counter-silenced in our plasmid-based approach, we must consider that we inspected the CgpS target promoters isolated from their native genomic context. For instance, CgpS coverage peaks located in high density and close distance in the CGP3 prophage region (Pfeifer et al., 2016) very likely influence each other, e.g. by forming bridges or determining the DNA topology together. Furthermore, high local concentrations of CgpS in the prophage region, a high density of CgpS binding sites as well as the surrounding GC-profile might influence CgpS target recognition and nucleoprotein complex stability, which could lead to higher silencing efficiencies. For instance, Will and colleagues demonstrated in biochemical and biophysical assays that the binding mode of H-NS strongly depend on the local concentration of H-NS and its corresponding binding sites as well as the binding affinity of H-NS (Will et al., 2018).

An important question therefore remains open: How well can our isolated plasmid system reflect the natively occurring mechanisms of silencing and counter-silencing? One promising approach for future studies could be the integration of the counter-silencer promoter into its native locus. Since native phage proteins have the risk to be toxic, e.g. the gene *lys* encodes a putative lysine, the native gene should either be translational inactivated (promoter activity measurements based on transcript level) or replaced by a reporter gene. This question will be further discussed in the outlook in chapter 2.6.

2.4.4. Counter-silencing and classical activation are distinct mechanisms

Previously, two mechanisms allowing counter-silencing were discussed: supportive and disruptive counter-silencing (Will et al., 2014). Some examples of supportive counter-silencing exist where the TF interferes with the silencer DNA complex but also directly interacts with the RNA polymerase to promote transcription, e.g. by recruiting the RNA polymerase (Hulbert and Taylor, 2002; Walther et al., 2007; Will et al., 2014; Yu and DiRita, 2002). However, other studies characterized a disruptive counter-silencing mechanism which is assumed to be independent of direct interaction between the TF and the RNA polymerase (Caramel and Schnetz, 1998; Kane and Dorman, 2011; Wiechert et al., 2020a), indicating that counter-silencing and activation are distinct regulatory mechanisms showing the possibility to be combined in a single TF. These discrepancies of the abovementioned studies likely reflect the complexity of regulatory networks and the individual evolutionary relationships between promoter and counter-silencing factor.

In accordance with the previous findings, we suggested that GntR-mediated counter-silencing is rather a disruptive mechanism than a supportive (Wiechert et al., 2020a). This hypothesis is based on three different observations:

- I) We observed highest counter-silencing efficiencies when GntR bound directly at the position of maximal CgpS coverage. These positions were often located in close distance to the TSSs (Wiechert et al., 2020a). Based on the scientific status quo, these positions would rather lead to the repression of gene expression by blocking transcription (Rojo, 1999; Rydenfelt et al., 2014). Indeed, GntR binding to different constructs led to counter-silencing in the wild type, but decreased promoter activities were observed in the absence of CgpS in the prophage-free strain Δ phage, showing that the regulatory output is strongly affected by the interplay of different DNA binding proteins (Wiechert et al., 2020a).
- II) In the native GntR target promoter P_{gntK} , the GntR binding site overlaps with the TSS (Frunzke et al., 2008) and GntR binding led to decreased promoter activities (Wiechert et al., 2020a), demonstrating that GntR binding close to the TSS acts as repressor. In accordance with the abovementioned examples RovA and SlyA (Will et al., 2019), our results nicely demonstrated that counter-silencing allows the conversion of a repressor to an activating counter-silencer (Wiechert et al., 2020a).
- III) None of our constructs, which was tested in the absence of CgpS in the prophage-free strain, showed significantly increased activity under conditions which induced GntR binding, demonstrating that its activating effect depends on the interplay with CgpS and not on a classical activation mechanism. Furthermore, all tested native phage promoters were strongly upregulated in this strain, indicating that they are constitutively active in the absence of CgpS (Wiechert et al., 2020a).

In accordance, previous studies from Will and colleagues suggested that open complex formation at AT-rich promoters is often not the rate-limiting step for transcription in the absence of XS proteins (Will et al., 2014). By performing combined differential DNA footprint analysis (DDFA) like KMnO_4 footprint assays with the H-NS target promoter P_{pagC} , the authors obtained comprehensive insights into the molecular mechanisms of silencing and counter-silencing. By systematically analysing the importance of different proteins for this process, the authors demonstrated that RNA polymerase binding is sufficient for the formation of a stable open complex in the absence of H-NS. In contrast, H-NS strongly inhibits this structural DNA change, defining its silencing effect. The open complex formation was restored in the presence of the counter-silencing factors PhoP and SlyA, explaining the molecular mechanism of counter-silencing: PhoP induces bending of the silencer-DNA filament leading to recovery of the open complex conformation (Will et al., 2014).

2.5. The potential of counter-silencer promoters for biotechnological and synthetic biological applications

Inducible expression systems represent key modules in biotechnological and synthetic biological applications, since they allow the precise and rational modulation of gene expression (Patek et al., 2013). They are frequently used to accurately coordinate metabolic fluxes in a variety of production processes and typically form the first layer of synthetic regulatory circuits (Liu et al., 2016; Xu et al., 2019). Synthetic Biology aims to provide suitable libraries of modular genetic parts, including promoters and TFs which can be assembled in genetic circuits (García-Granados et al., 2019). Various intensive promoter engineering studies and designed promoter libraries from this research field highlight the demand of appropriate expression systems which meet the requirements of specific application, e.g. metabolic engineering strategies (Alper et al., 2005; Blazeck and Alper, 2013; Han et al., 2019). A stringent but flexible control of gene expression is of particular importance for applications which have to deal with toxic products and pathway intermediates or with disturbances of the central carbon flux (Baritugo et al., 2018). Furthermore, gene function studies often do not depend on high expression rates, but on strictly controlled gene expression to allow the conditional knockdown of essential genes (Fitz et al., 2018; Shang et al., 2002).

In various bacteria, for instance in *C. glutamicum*, the well-established heterologous expression systems P_{tac} (Patek et al., 2013), P_{tet} (Lausberg et al., 2012) and P_{araBAD} (Ben-Samoun et al., 1999) are widely applied. However, they do not meet the requirements for all approaches due to inducer toxicity, high inducer costs, heterogeneous inducer uptake, high background expression levels and inappropriate induction levels (Baritugo et al., 2018; Patek et al., 2013; Yim et al., 2013; Zhang et al., 2012b).

Interestingly, the investigations of our synthetic promoter variants, which are controlled by xenogeneic silencing and counter-silencing, revealed that several of them have very low background expression levels and can be strongly induced by binding of the gluconate-dependent TF GntR (Wiechert et al., 2020a). Based on this observation, a further project of this PhD thesis was the detailed characterization of promising promoters to determine their potential as gene expression system in *C. glutamicum*. The results are summarized in our current manuscript: "Inducible expression systems based on xenogeneic silencing and counter-silencing and the design of a metabolic toggle switch" (Wiechert et al., 2020b). In contrast to inducers like IPTG, gluconate represents a non-toxic and cheap effector molecule (Wiechert et al., 2020b). The comparison of exemplarily chosen counter-silencer promoters with the heterologous expression systems $\text{LacI-}P_{tac}$ and $\text{TetR-}P_{tet}$ confirmed that our constructs have significantly lower background expression levels (Wiechert et al., 2020b). In the presence of the effector molecule gluconate which inhibits binding of GntR to the DNA, the counter-silencer promoters were in the OFF state. However, promoter activities increased after a certain period of time, probably

due to depletion of gluconate. Cultivation approaches with increasing amounts of gluconate revealed a gradual shift of the time point of promoter induction, highlighting the potential of counter-silencer constructs as expression systems with temporal tunability. By choosing defined amounts of gluconate, the increase in promoter activity could be correlated with the culture entry into stationary phase (Wiechert et al., 2020b), suggesting that counter-silencer promoters could be applied for approaches where a gene is supposed to be expressed after an initial growth phase.

The repertoire of homologous inducible promoters is comparatively small in *C. glutamicum* and includes the P_{malE} promoter induced by maltose (Okibe et al., 2010), the gluconate responsive promoters P_{git1} (Okibe et al., 2010), P_{gntK} (Hentschel et al., 2013; Letek et al., 2006), and P_{gntP} (Letek et al., 2006), the propionate inducible P_{prpD2} promoter (Plassmeier et al., 2013) as well as amino acid responsive biosensors like Lrp- P_{brnFE} (Mustafi et al., 2012) and LysG- P_{lysE} (Binder et al., 2012). Therefore, our counter-silencer promoters might represent suitable candidates to expand this limited set of inducible promoter systems.

We combined our synthetic constructs in a GntR-dependent promoter library comprising 44 regulatory elements which were repressed (16) or activated (28) by GntR binding (Wiechert et al., 2020b). Our promoter sets were characterized by small increment steps and covered a wide range of expression levels (repressed: 70-fold, activated: 100-fold) (Wiechert et al., 2020b), emphasizing that they show important features of high-quality promoter libraries (Fitz et al., 2018). Since only a few promoter libraries had been designed for *C. glutamicum* containing either synthetic, IPTG inducible regulatory elements (Rytter et al., 2014) or constitutive promoters (Rytter et al., 2014; Shang et al., 2018; Yim et al., 2013), we suggest that our synthetic, gluconate inducible library containing activated and repressed regulatory elements could be useful for different approaches in *C. glutamicum*.

Finally, it should be noted that it might also be worthwhile to investigate counter-silencing of naturally silenced genes in future research. For instance, Gehrke and colleagues showed that Lsr2 targets a variety of specialized metabolic clusters in *S. venezuelae* (Gehrke et al., 2019). If our findings about TF-mediated counter-silencing of the Lsr2-like XS protein CgpS are transferable to Lsr2 in *Streptomyces*, the specific and controlled reactivation of these clusters could be an interesting engineering target for the discovery of novel bioactive compounds. Furthermore, the implementation of TF binding sites within specific silenced promoters could be applied for studying the functions of one specific gene product within the cellular network. An interesting field of research could be the reactivation of phage genes, e.g. from *C. glutamicum*, which allows for their functional characterization within the native host.

2.5.1. Synthetic genetic toggle switches

Inspired by the genetic switch that governs the lysis-lysogeny decision of bacteriophage λ , Gardner and colleagues imitated this circuit design to construct a bistable synthetic toggle switch (Gardner et al., 2000). The regulatory network of bacteriophage λ combines the two repressors Cro and CI and their target promoters P_R and P_{RM} , each being repressed by the gene product of the other (Khalil and Collins, 2010). Comparably, Gardner and colleagues combined the IPTG-inducible expression system LacI- P_{trc} with the anhydrotetracycline- (ATc-) dependent TetR- P_{tetO-1} system, or with the temperature-sensitive λ repressor (CI_{ts}) and its corresponding promoter P_{slcon} and arranged them such that both promoters were repressed by the gene product of the other (Gardner et al., 2000). The additional expression control of a reporter gene (e.g. *gfpmut3*) by one of the promoters allowed them to monitor the state of the toggle (fluorescence on versus fluorescence off) (Gardner et al., 2000).

The analysis of reporter outputs driven by the native GntR target promoter P_{gntK} and the synthetic counter-silencer construct $P_{lys_CS_0}$ revealed a very similar, but inverted response to gluconate availability (Wiechert et al., 2020a). Based on this observation, we combined both promoters in a synthetic toggle switch and fused them to different reporter genes (*venus* and *e2-crimson*) to monitor the state of our toggle (Wiechert et al., 2020a) (Figure 11). Analysis of reporter outputs during long-term cultivations in the microfluidic cultivation system allowed us to characterize our toggle design in detail and demonstrated its robust and reversible functionality (Wiechert et al., 2020a; Wiechert et al., 2020b).

In contrast to the circuit architecture from Gardner and colleagues, which was based on the ratio of two repressors and two stimuli (Gardner et al., 2000), our design is controlled by only one specific TF, namely GntR, and one effector molecule, namely gluconate, allowing us to use native GntR levels (Wiechert et al., 2020a; Wiechert et al., 2020b). This simple design might be advantageous since it circumvents problems like inappropriate TF synthesis and degradation rates as well as insufficient repression efficiencies, which were discussed as major challenges for toggle functionality and stability in the previous publication (Gardner et al., 2000). Additionally, our modular circuit architecture is thought to allow the independent tuning of both toggle sides without affecting the response of the other side, demonstrating its potential for applications in Synthetic Biology. For instance, our GntR-dependent promoter library comprising 28 activated and 16 repressed synthetic regulatory elements provides a large set of potential building blocks allowing application-specific adjustments of the toggle (Wiechert et al., 2020b).

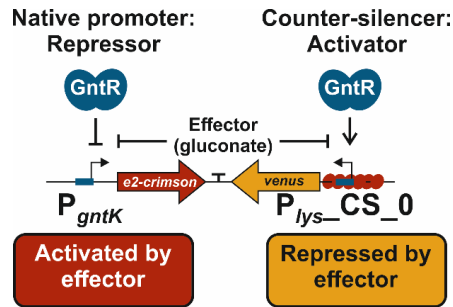


Figure 11: Design scheme of the GntR-dependent fluorescence toggle switch. The GntR-dependent toggle switch is based on the native GntR target promoter P_{gntK} and the synthetic GntR-dependent $P_{lys_CS_0}$ counter-silencer construct ($P_{lys_CS_0}$), both reacting inversely to gluconate availability. Both promoters were fused to different reporter genes ($P_{lys_CS_0}$ -*venus* and P_{gntK} -*e2-crimson*) to monitor promoter activities. Figure taken from Wiechert et al. (2020a).

2.5.2. Metabolic toggle switches to optimize flux balances by dynamic pathway regulation

Rationally engineered microbial cells enable improved production of value-added compounds from renewable feedstocks and represent an ecological, economical and sustainable alternative to production processes depending on limited fossil resources and high energy supply (Lee et al., 2012; Prather and Martin, 2008). The majority of bio-based products including natural and non-natural compounds are derived from intermediates of the central carbon metabolism, e.g. from tricarboxylic acid (TCA) cycle or glycolysis. Consequently, production processes frequently compete with pathways essential for microbial physiology and cell growth (Doong et al., 2018; Gupta et al., 2017; Soma et al., 2014). Permanent knockouts or knockdowns of competing reactions from the central carbon metabolism combined with the overexpression of production pathways (heterologous or endogenous) are established engineering strategies to redirect carbon flux towards the desired product (Becker et al., 2011; Blombach et al., 2008; Blombach et al., 2007; Huser et al., 2005; Krause et al., 2010; Litsanov et al., 2012). However, the permanent knockout of competing pathways and highly expressed heterologous biosynthesis routes can result in strong metabolic imbalances leading to severe growth defects and reduced overall productivity (Brockman and Prather, 2015a).

In contrast, the implementation of metabolic toggle switches or other synthetic genetic circuits allows the dynamic redirection of metabolic fluxes towards cell growth or product biosynthesis in several engineered *E. coli* strains (Brockman and Prather, 2015b; Doong et al., 2018; Farmer and Liao, 2000; Gupta et al., 2017; Lo et al., 2016; Soma et al., 2014; Soma et al., 2017; Tsuruno et al., 2015; Zhang et al., 2012a). By controlling timing of strain performance, cell growth as well as the overall productivity were significantly improved. For instance, Soma and colleagues constructed a metabolic toggle switch for isopropanol production in *E. coli*, which was inspired by the aforementioned circuit design from Gardner and co-workers (Soma et al., 2014). The conditional knockout of the citrate synthase GltA and

the simultaneous upregulation of a synthetic isopropanol production pathway enabled the switch between growth and production and circumvented the challenge that both pathways would otherwise compete for acetyl-CoA (Soma et al., 2014). Tsuruno and colleagues used a comparable circuit architecture to overcome competition between growth and product formation by dynamically redirecting the central carbon flux from glycerol towards 3-hydroxypropionic acid (Tsuruno et al., 2015). In *C. glutamicum*, the pyruvate dehydrogenase complex (PDHC) is an important target for metabolic engineering strategies aiming at increasing production of pyruvate-derived compounds like L-valine, isobutanol, ketoisovalerate and L-lysine (Blombach et al., 2007; Buchholz et al., 2013; Eikmanns and Blombach, 2014). Several of these successful approaches were based on the inactivation of PDHC, e.g. by deleting the *aceE* gene encoding the E1 subunit of this complex, to improve precursor supply. PDHC-deficiency results in strong growth defects on sugars like glucose, which can be complemented by the addition of acetyl-CoA replenishing carbon sources, such as acetate (Schreiner et al., 2005). The inactivation of the PDHC in $\Delta aceE$ deletion strains leads to decoupling of biomass and L-valine formation and cells mainly consume acetate during the initial growth phase (Blombach et al., 2007; Schreiner et al., 2005). However, acetate represents a costly and inefficient carbon source, which allows only low biomass yields (Blombach et al., 2007; Schreiner et al., 2005).

To overcome the challenges associated with the permanent knockout of the PDHC and to circumvent the competition of biomass and product formation, we implemented our GntR-dependent circuit design as metabolic toggle switch in a *C. glutamicum* L-valine production strain (Wiechert et al., 2020b) (Figure 12A, B). By replacing the native promoter of *aceE* with the gluconate-inducible P_{gntK} promoter, we could control cell growth in dependency of gluconate availability (Figure 12B, C). Remarkably, our dynamically controlled P_{gntK} -*aceE* strain was able to co-utilize glucose in the presence of gluconate leading to significantly improved biomass yields in comparison to those obtained for the previously established static $\Delta aceE$ deletion strain in the presence of acetate and glucose (Figure 12C). In contrast, both strains were not able to grow in media containing solely glucose (Figure 12C) and produced comparable amounts of L-valine (Figure 12D), indicating that controlling PDHC allowed the redirection of carbon flux towards product formation (Wiechert et al., 2020b).

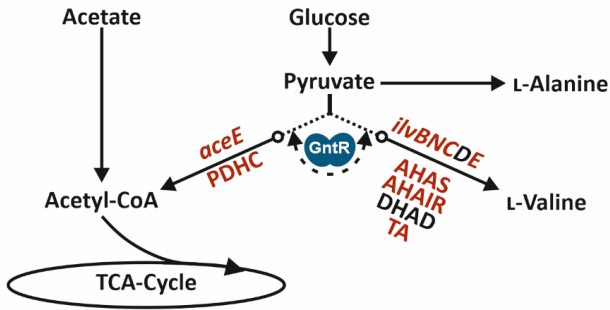
Engineering approaches of PDHC activity based on *aceE* promoter replacements to improve L-valine production were previously applied by two groups. Ma and colleagues applied a growth-phase dependent promoter of an industrial used L-leucine strain (P_{CP_2836} ; *C. glutamicum* CP) for phase-dependent control of the PDHC activity to overcome the competition between cell growth and L-valine production. While the PDHC was active and allowed the formation of biomass during exponential growth phase, the activity of the enzyme complex was strongly reduced during stationary phase resulting in accumulation of pyruvate (Ma et al., 2018). In the second study, the authors replaced the *aceE* promoter with mutated, constitutive promoter variants of *dapA* leading to decreased *aceE*

expression, acetate independent cell growth and increased L-valine productivity (Buchholz et al., 2013). However, in contrast to our effector-responsive system which is assumed to provide the opportunity to adjust PDHC activity during a production process, these strain designs are constrained with regard to fine-tuning.

The counter-part of our initial toggle design, the P_{lys} counter-silencer promoter, was used to control the plasmid-based overexpression of L-valine biosynthesis genes (Wiechert et al., 2020b) (Figure 12B). However, this design lowered the productivity compared to previously established production strains, which were based on overexpression plasmids containing the L-valine biosynthesis operons controlled by their native promoters (Figure 12D). We hypothesized that this could be caused by lower expression rates driven by the counter-silencer promoter and suggested the implementation of stronger promoters in future toggle designs (Wiechert et al., 2020b).

Another optimization strategy of our toggle design could focus on protein stability and degradation rates. Since we have controlled PDHC activity at the transcription level, we cannot exclude that when gluconate is depleted and *aceE* expression is downregulated, PDHC remains active to a certain extent. In this regard, degradation tags, e.g. SsrA tags which make proteins susceptible for tail-specific proteases (Herman et al., 1998), could accelerate AceE depletion and thereby improve the response of the system to changes in gluconate availability. The functionality of these signal peptides in *C. glutamicum* was already confirmed in previous studies (Hentschel et al., 2013; Huber et al., 2017).

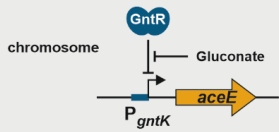
A



B

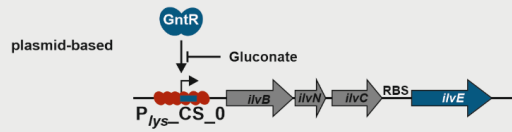
Host engineering:
precursor supply

P_{gntK} -*aceE*: PDHC is active in the presence of gluconate

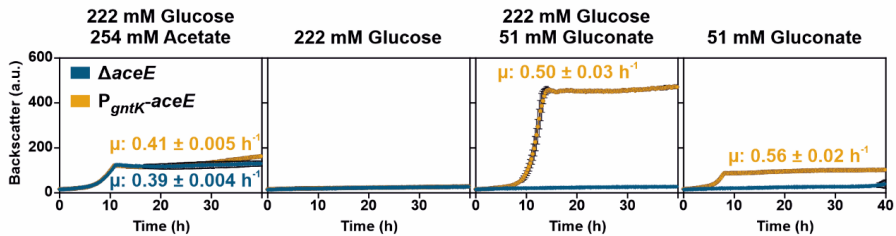


Pathway engineering:
precursor-product conversion

P_{lys-CS_0} -*ilvBNC-ilvE*: Overexpression of L-valine biosynthesis genes in the absence of gluconate



C



D

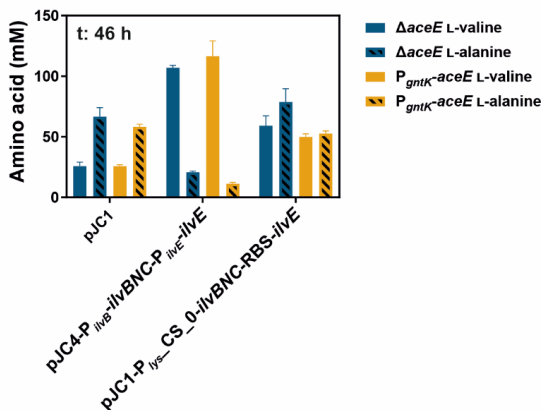


Figure 12: Application of the GntR-dependent toggle for the dynamic switch between growth and L-valine production. **A)** Schematic representation of relevant parts of the central carbon metabolism and the L-valine biosynthesis pathway of *C. glutamicum*. The GntR-dependent toggle controlled the redirection of the carbon flux allowing the conversion of pyruvate to either L-valine or acetyl-CoA entering the tricarboxylic acid cycle (TCA-Cycle). *aceE* encodes for the E1 subunit of the pyruvate dehydrogenase complex (PDHC), which converts pyruvate to acetyl-CoA. L-valine is formed from pyruvate in a four-step reaction pathway catalysed by acetohydroxyacid synthase (AHAS, *ilvBN*), acetohydroxyacid isomeroreductase (AHAIR, *ilvC*), dihydroxyacid dehydratase (DHAD, *ilvD*), and transaminase B (TA, *ilvE*). Dynamically controlled genes and their products are highlighted in red. **B)** Schematic overview of the genetic background of the dynamic L-valine production strain. The native promoter of the *aceE* gene was replaced by the GntR target promoter P_{gntk} allowing control of PDHC activity. The L-valine biosynthesis genes were combined in a synthetic operon by fusing *ilvE* via a linker containing an RBS sequence to the end of the operon *ilvBNC*. Its expression was controlled by the synthetic GntR counter-silencer promoter $P_{lys_CS_0}$. **C)** Growth of the strain with dynamically controlled *aceE* expression (P_{gntk} -*aceE*) in comparison to the previously established Δ *aceE* strain (Blombach et al., 2007; Schreiner et al., 2005). Both strains had been pre-cultivated in CGXII containing 222 mM glucose and 254 mM acetate before they were cultivated in a microtiter cultivation system in medium with either 222 mM glucose and 254 mM acetate, 222 mM glucose, 222 mM glucose and 51 mM gluconate or 51 mM gluconate. Calculated growth rates (μ) are given (colour-coded) when significant growth had been observed. **D)** L-valine titers of the *aceE* toggle strain (P_{gntk} -*aceE*) and the Δ *aceE* L-valine production strain harbouring either the empty control plasmid pJC1 (pJC1-*venus*-term (Baumgart et al., 2013)), plasmid-based L-valine biosynthesis genes controlled by the counter-silencer (pJC1- $P_{lys_CS_0}$ -*ilvBNC*-RBS-*ilvE*) or the natively regulated variant pJC4- P_{ilvB} -*ilvBNC*- P_{ilvE} -*ilvE* (pJC4-*ilvBNC*E; (Radmacher et al., 2002)). All strains were cultivated in CGXII supplemented with 222 mM glucose and 254 mM acetate. Bar plots represent the L-valine and L-alanine titers of biological triplicates and error bars the corresponding standard deviations after 46 h of cultivation. Figure and legend taken from Wiechert et al. (2020b).

2.5.3. Metabolic imbalances in static pathway engineering approaches

As previously mentioned, static downregulation of central metabolic reactions can lead to metabolic imbalances and to impaired cell growth, as well as to a reduced overall productivity of a fermentation process. One example of such a metabolic imbalance was recently described in our study “Impact of $\text{CO}_2/\text{HCO}_3^-$ availability in anaplerotic flux in pyruvate dehydrogenase complex deficient *Corynebacterium glutamicum* strains” (Krüger et al., 2019), which is part of this thesis. In this work, we systematically analysed the effect of imbalances of the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool on cell growth of *C. glutamicum* strains lacking the PDHC. In addition to the formation of acetyl-CoA, this enzyme complex catalyses an important decarboxylation reaction that leads to the release of CO_2 (de Kok et al., 1998). The results of our study strongly emphasize that the absence of the central decarboxylation reaction impairs the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool, resulting in elongated lag phases of PDHC-deficient strains in the presence of acetate and glucose (Krüger et al., 2019).

HCO_3^- is an important substrate of the anaplerotic reactions catalysed by the pyruvate carboxylase (PCx, encoded by *pyc*) and by the phosphoenolpyruvate carboxylase (PEPCx, encoded by *ppc*). Anaplerotic reactions are essential during growth on glycolytic substrates since they allow the replenishment of TCA cycle intermediates, which are consumed in anabolic reactions (Peters-Wendisch et al., 1998; Sauer and Eikmanns, 2005). The additional inactivation of the dominating anaplerotic enzyme PCx in the strain Δ *aceE* Δ *pyc* was shown to further improve L-valine production by increasing pyruvate supply (Blombach et al., 2008). However, we observed an elongated lag phase of more than 15 hours for the strain Δ *aceE* Δ *pyc* during cultivation in the presence of glucose and acetate, which could be complemented by different approaches aiming at improving the intracellular HCO_3^- availability. We assumed that higher HCO_3^- levels increased PEPCx activity allowing to complement the

inactivation of PCx. Interestingly, this growth defect depends on glucose since it was not observed during cultivation on acetate or after inactivation of the main glucose uptake system PtsG (Krüger et al., 2019).

In *C. glutamicum* wild-type cells, the glyoxylate cycle enables the replenishment of the TCA cycle intermediates during growth of on acetate as well as on the combination of glucose and acetate, but is turned off during growth on glucose (Wendisch et al., 2000; Wendisch et al., 1997). We characterized the phenomenon of glucose sensitivity of $\Delta aceE \Delta pyc$ cells in detail and suggested that the inactivation of PDHC and/or PCx led to metabolic imbalances of glycolytic intermediates resulting in inhibition of the glyoxylate cycle enzymes and to depletion of oxaloacetate, which caused the growth defect (Krüger et al., 2019).

In conclusion, our findings emphasised that static interruptions of central decarboxylation and carboxylation reactions aiming at improving productivity of microbial strains can result in severe metabolic imbalances with detrimental effects on cellular growth (Krüger et al., 2019), highlighting the demand for alternative engineering strategies, e.g. metabolic toggle switches.

2.6. Conclusion and outlook

Xenogeneic silencing and TF-mediated counter-silencing represent important drivers of bacterial evolution: they can provide access to new beneficial traits while buffering against putative detrimental fitness consequences of foreign DNA elements. Silencing and counter-silencing of H-NS-like XS proteins has been intensively addressed by previous studies, but up to now little was known about the medically and biotechnologically relevant Lsr2-like XS proteins of actinobacteria. This thesis contributes to the understanding of the underlying rules of xenogeneic silencing of the Lsr2-like XS protein CgpS from *C. glutamicum* and provides the first systematic analyses of TF-mediated counter-silencing of an Lsr2-like XS protein (Wiechert et al., 2020a). It shows that CgpS-mediated silencing is determined by a sequence-specific binding motif as well as a distinct drop in GC-profile and that binding of the TF GntR leads to counter-silencing at a variety of promoter architectures (Wiechert et al., 2020a). Furthermore, this work reveals for the first time the potential of synthetic counter-silencer promoters as tightly controlled expression systems and as modular part of genetic toggle switches for synthetic biological applications (Wiechert et al., 2020b). Finally, this thesis provides evidence that static interruptions of central catabolic pathway reactions aiming at improving product formation in biotechnological approaches can result in metabolic imbalances (Krüger et al., 2019) and that metabolic toggle switches allowing dynamic pathway regulation represent a promising alternative strategy (Wiechert et al., 2020b).

Nevertheless, the present work raises interesting questions, especially regarding the *in vivo* interference of XS and TFs during counter-silencing, the evolutionary process of regulatory network expansion by counter-silencing, and regarding putative mutual interactions of highly concentrated CgpS molecules in the prophage region.

An remaining question is the molecular effect on the nucleoprotein complex formed by the XS CgpS during binding of the TF GntR. Detailed biochemical footprint analysis performed by Will and colleagues revealed that counter-silencing does not depend on the complete relief of the XS protein H-NS and that structural changes of the nucleoprotein complex enable transcription (Will et al., 2014). Based on preliminary electrophoretic shift assays with GntR and CgpS, we got first hints that both proteins simultaneously bind to the DNA *in vitro* (Wiechert and Frunzke, unpublished). Additionally, none of our counter-silencing constructs reached the same level of promoter activity as it was observed in the absence of CgpS in the prophage-free strain, suggesting that GntR DNA binding does not fully displace CgpS from the DNA (Wiechert et al., 2020a). However, further studies are necessary to decipher the structural changes of the nucleoprotein complex during GntR-mediated counter-silencing. As an alternative to the previously performed *in vitro* footprint assays from Will and colleagues, *in vivo* ChIP- or ChAP-seq analyses with the synthetic constructs could provide comprehensive insights into the binding profile of both regulators during silencing (GntR DNA binding is inhibited) and counter-silencing (GntR is bound to the DNA). These approaches might show reduced and/or differentially shaped CgpS coverage peaks when CgpS and GntR compete for DNA binding. Additionally, no native CgpS counter-silencing factor has yet been identified. Genome-wide binding profiles of GntR, could allow for the identification of new targets and might reveal putative overlaps with known CgpS targets, thereby giving first hints for native GntR-mediated counter-silencing. In general, the large amounts of available ChIP- and ChAP-seq data sets of various TFs and XS proteins from different organisms could provide a promising platform for the systematic search for overlapping binding regions of TFs and XSs. Although this phenomenon is not necessarily linked to counter-silencing, as recently shown by Hünnefeld and colleagues for the MarR-type regulator MalR and CgpS from *C. glutamicum* (Hünnefeld et al., 2019), it could give a first hint for native TF-mediated counter-silencing effects.

Another interesting question focuses on the evolutionary network expansion by counter-silencing. Previously, it was convincingly discussed that, in comparison to the *de novo* evolution of classical activation circuits, counter-silencing facilitates regulatory network expansion by tolerating a higher degree of flexibility in promoter architectures (Will et al., 2014). This hypothesis was based on the detailed comparison of natively occurring counter-silencer promoters with conservatively activated

regulatory elements belonging to the same regulon (Will et al., 2014). Three synthetic approaches, including our own study, demonstrated that the insertion of TF binding sites allow the large development leap from a silenced promoter to a controllable counter-silencer promoter (Caramel and Schnetz, 1998; Kane and Dorman, 2011; Wiechert et al., 2020a). But what are the steps in between, and can we simulate this evolutionary process? Regarding this, one approach for future studies could be an adaptive laboratory evolution (ALE) experiment with silenced promoters, which have to evolve the mechanism of counter-silencing, in comparison to promoters, which need to develop a classical activation circuit. Promoter fusions with antibiotic resistance genes or other growth-related selection markers could provide a suitable platform for the identification of clones with desired mutations. CgpS target promoters from *C. glutamicum* could be used as starting point for the counter-silencer evolution. The choice of promoters of the second category, which need to evolve an activating circuit, represents probably a greater challenge. Those promoters should have the ability to functionally drive transcription, but this process should be dependent on a TF that activates gene expression, e.g. by recruiting the RNA polymerase. By disturbing native activating regulatory circuits, e.g. by deleting an activating TF in the genome, these requirements could be fulfilled for the corresponding target promoters. A comparative ALE experiment with both promoter categories could answer the question which mechanism – classical activation or counter-silencing - is more likely to be evolved. Furthermore, this experiment could provide comprehensive insights into the evolutionary process of co-opting a TF as a counter-silencing factor during regulatory network expansion.

A third interesting point is the effect of the high number of CgpS binding peaks located in high density and close distances within the AT-rich prophage element CGP3 (Pfeifer et al., 2016), which is probably associated with high local concentrations of bound but also unbound CgpS molecules. But do CgpS molecules influence each other, and is there even an attractive molecular interaction similar as observed during liquid-liquid phase separation (LLPS)? LLPS describes the dynamic condensation of proteins and nucleic acids into macromolecular condensates based on attractive molecular interactions which counterbalance entropy-driven effects like protein diffusion (Monterroso et al., 2019). Thereby, LLPS allows the compartmentalisation of cellular components independent of membranes and mainly contributes to their sub-cellular spatial organisation (Guilhas et al., 2019). In eukaryotic cells, LLPS is involved in many processes including chromatin rearrangements and three-dimensional genome organisation as well as transcriptional regulation (Wang and Liu, 2019). Guilhas and colleagues recently identified nanometre-sized droplets formed by LLPS which consist of components of the bacterial ParABS DNA segregation system. These structures were able to inhibit ParB (DNA binding protein) diffusion leading to trapping of ParB close to defined DNA regions (Guilhas et al., 2019). For future studies, it would be interesting to investigate whether XS proteins play a role

in the subcellular organisation of prophage elements in bacterial nucleoids via LLPS. Recently used methods in LLPS research such as super-resolution PALM microscopy and single-particle tracking PALM (sptPALM) (Guilhas et al., 2019) combined with protein reporter fusions represent promising approaches to investigate local accumulations of CgpS proteins. Furthermore, they might allow the analysis of the localization and diffusion dynamics of single CgpS molecules and the identification of putative trapping effects caused by high-affinity interactions between CgpS molecules.

As discussed in chapter 2.4.3. (“Synthetic disruptive counter-silencing.”), we inspected counter-silencing of CgpS target promoters plasmid-based and *in vivo*, but isolated from their native genomic context (Wiechert et al., 2020a). Therefore, another interesting point would be the effect of the TF GntR on these molecular interactions in the native genomic context. Here, super-resolution microscopy approaches could provide interesting insights into the CgpS-dependent organization of the prophage element when it is integrated into the bacterial genome as well as during the process of prophage induction.

2.7. References

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3. Publications and manuscripts

The “Contributor Roles Taxonomy” (CRediT) methodology describes a transparent standard for authorships of scientific publications and for defining author contributions roles (McNutt et al., 2018). The following table contains aspects of this taxonomy which were used to describe author contributions to publications and manuscripts presented in this thesis.

Contributor role	Role definition
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyse or synthesize study data.
Investigation/Experiments	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.
Methodology	Development or design of methodology; creation of models.
Project administration	Management and coordination responsibility for the research activity planning and execution.
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.
Visualization	Preparation, creation and/or presentation of the published work, specifically visualization/data presentation.
Writing – original draft	Preparation, creation and/or presentation of the published work, specifically writing the initial draft (including substantive translation).
Writing – review & editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.

- 3.1. Deciphering the rules underlying xenogeneic silencing and counter-silencing of Lsr2-like proteins using CgpS of *Corynebacterium glutamicum* as a model

Wiechert, J., Filipchyk, A., Hünnefeld, M., Gätgens, C., Brehm, J., Heermann, R. and Frunzke, J.

Published in *mBio*, 2020.

Contributor Role	Contributor
Conceptualization	JW 60 %, JF 40 %
Formal analysis	JW 85 %, AF 10 %, RH 5 %
Investigation/Experiments	JW 65 %, MH 7.5 %, CG 20 %, JB 2.5 %, RH 5 %
Methodology	JW 55 %, AF 10 %, MH 2.5 %, CG 10 %, JB 2.5 %, RH 5 %, JF 15 %
Project administration	JW 50 %, JF 50 %
Software	JW 50 %, AF 50 %
Supervision	JW 40 %, JF 60 %
Visualization	JW 95 %, AF 5 %
Writing – original draft	JW 70 %, JF 30 %
Writing – review & editing	JW 50 %, AF 5 %, MH 5 %, JF 40 %

Overall contribution JW: 70 %

This publication is the central work resulting from this doctoral thesis. All visualizations and data analyses were done by JW, except of the heat maps in Fig 1B and C which were done by AF and the analyses of the SPR measurements, done by RH. JW performed most of the experimental work and a large part of the bioinformatic analysis, comprising Fig. 1 A,D, and data evaluation of 1E (RNA samples were prepared by MH and TSS determination as well as first analyses steps of the raw sequencing data were done by the company Vertis Biotechnology AG (Freising, Germany)), Fig. 2A (Bar plot was based on data shown in the heat maps prepared by AH), sample preparation (protein and DNA fragments) for SPR analyses shown in Fig. 2C and 4D (SPR experiments were performed by RH and JB), Fig. 3, Fig. 4B and C, Fig. 5, Fig. 6, Fig S1, S2, S3, S4 and S6. CG performed experiments shown in Fig. 2B and D, Fig. 4A and Fig. S5. The original draft was mainly prepared by JW and JW took over a large part (50 %) of the revision and editing process.



RESEARCH ARTICLE
Molecular Biology and Physiology

Deciphering the Rules Underlying Xenogeneic Silencing and Counter-Silencing of Lsr2-like Proteins Using CgpS of *Corynebacterium glutamicum* as a Model

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ABSTRACT Lsr2-like nucleoid-associated proteins play an important role as xenogeneic silencers (XS) of horizontally acquired genomic regions in actinobacteria. In this study, we systematically analyzed the *in vivo* constraints underlying silencing and counter-silencing of the Lsr2-like protein CgpS in *Corynebacterium glutamicum*. Genome-wide analysis revealed binding of CgpS to regions featuring a distinct drop in GC profile close to the transcription start site (TSS) but also identified an overrepresented motif with multiple A/T steps at the nucleation site of the nucleoprotein complex. Binding of specific transcription factors (TFs) may oppose XS activity, leading to counter-silencing. Following a synthetic counter-silencing approach, target gene activation was realized by inserting operator sites of an effector-responsive TF within various CgpS target promoters, resulting in increased promoter activity upon TF binding. Analysis of reporter constructs revealed maximal counter-silencing when the TF operator site was inserted at the position of maximal CgpS coverage. This principle was implemented in a synthetic toggle switch, which features a robust and reversible response to effector availability, highlighting the potential for biotechnological applications. Together, our results provide comprehensive insights into how Lsr2 silencing and counter-silencing shape evolutionary network expansion in this medically and biotechnologically relevant bacterial phylum.

IMPORTANCE In actinobacteria, Lsr2-like nucleoid-associated proteins function as xenogeneic silencers (XS) of horizontally acquired genomic regions, including viral elements, virulence gene clusters in *Mycobacterium tuberculosis*, and genes involved in cryptic specialized metabolism in *Streptomyces* species. Consequently, a detailed mechanistic understanding of Lsr2 binding *in vivo* is relevant as a potential drug target and for the identification of novel bioactive compounds. Here, we followed an *in vivo* approach to investigate the rules underlying xenogeneic silencing and counter-silencing of the Lsr2-like XS CgpS from *Corynebacterium glutamicum*. Our results demonstrated that CgpS distinguishes between self and foreign by recognizing a distinct drop in GC profile in combination with a short, sequence-specific motif at the nucleation site. Following a synthetic counter-silencer approach, we studied the potential and constraints of transcription factors to counteract CgpS silencing, thereby facilitating the integration of new genetic traits into host regulatory networks.

KEYWORDS AT-rich DNA, Lsr2, actinobacteria, counter-silencing, horizontal gene transfer, regulatory networks, xenogeneic silencing

Horizontal gene transfer (HGT) is a major driver of bacterial evolution and plays an important role in creating genetic diversity (1). The rapid acquisition of beneficial new traits can create a competitive advantage for the recipient cells (1, 2). However, the chance that foreign DNA decreases the fitness of the cell is high, since it may lead to

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interference with regulatory networks, high transcriptional and translational costs, sequestration of cellular machineries, and cytotoxic gene products (3–8). Therefore, bacteria evolved a variety of immune systems allowing them to deal with foreign DNA (9). CRISPR-Cas and restriction modification systems are nuclease-based defense mechanisms enabling the recognition and targeted degradation of invading DNA (10–12). In contrast to these destructive immune systems, xenogeneic silencing enables the tolerance of foreign DNA and consequently fosters the acquisition of novel genetic material into the host chromosome (13). Xenogeneic silencing is based on specific nucleoid-associated proteins (NAPs), so-called xenogeneic silencers (XS) (3). Known XS proteins belong to one of four currently described classes: H-NS-like proteins of proteobacteria, like *Escherichia coli*, *Yersinia*, and *Salmonella* (14–16), MvaT/U-like proteins found in gammaproteobacteria of the *Pseudomonadales* order (17), Lsr2-like XS of *Actinomyces* (18, 19), and Rok, present in different bacilli, including *Bacillus subtilis* (20, 21). Although XS were convergently evolved and show only low sequence similarity within the different classes, the domain properties of their N-terminal oligomerization domains and their C-terminal DNA-binding domains are similar (19, 20, 22, 23). Their binding mechanisms are diverse, but they all preferentially bind to horizontally acquired DNA, which typically has a higher AT content than the genome of the recipient cell (4, 24). The broad distribution of XS among prokaryotes emphasizes the strong need to discriminate between self and non-self across phylogenetic clades (13). Even so, the GC content of microbial genomes dramatically varies, from 75% (*Actinobacteria*) to less than 20% (bacterial endosymbionts) (25, 26), and horizontally acquired regions typically feature a lower GC content than their resident genome, emphasizing base composition as a major discrimination factor shaping microbial genome evolution (3).

Several studies based on variants defective in oligomer formation revealed that binding of XS proteins to the DNA alone is insufficient for silencing (27–29). The formation of higher-order nucleoprotein complexes instead mediates silencing of the target genes by occlusion or trapping of the RNA polymerase, by interference with the transcription elongation complex, or by enhancing termination (30, 31). To get access to potentially encoded beneficial traits, cells must integrate foreign genes into preexisting regulatory circuits, allowing their controlled expression at appropriate time points and physiological or environmental conditions (32, 33). In contrast to classical activation, counter-silencing is based on the interference of a DNA-binding protein, e.g., a transcription factor (TF), with the silencer-DNA complex leading to transcription initiation without depending on the direct interaction with the RNA polymerase (32, 33). Counter-silencing of H-NS was addressed by several studies either by following a synthetic approach at well-studied promoters (34, 35) or by the analysis of the promoter architectures in the PhoPQ regulatory network (33). The recent study by Will et al. emphasizes that the principle of H-NS xenogeneic silencing and counter-silencing provides a certain degree of flexibility, fostering evolutionary network expansion (33).

Compared to H-NS in proteobacteria, much less is known about Lsr2-like XS proteins conserved throughout the actinobacteria. In *Mycobacterium tuberculosis*, Lsr2 acts as a master regulator of multiple virulence-associated genes (19, 22) and was suggested to be involved in the manifestation of multidrug tolerance (36). The essentiality of Lsr2 for this human pathogen makes this XS protein a highly promising drug candidate (37). In *Corynebacterium glutamicum*, the Lsr2-like XS protein CgpS also was shown to play an essential role as a silencer of cryptic prophage elements whose entrance into the lytic cycle would otherwise cause cell death (4, 38). In contrast to mycobacteria and corynebacterial species, *Streptomyces* species typically encode two Lsr2-like proteins. Here, the prototypical *lsr2* gene, showing the highest sequence identity to mycobacterial Lsr2, was recently described to silence the expression of specialized metabolic clusters (39). Considering the important role of Lsr2 proteins in the medically and biotechnologically important phylum of *Actinobacteria*, a detailed mechanistic understanding of Lsr2 binding *in vivo* is relevant as a potential drug target and for the identification novel bioactive compounds.

In this study, we set out to systematically assess the rules underlying silencing and

counter-silencing of Lsr2-like XS by using the Lsr2-like protein CgpS of *Corynebacterium glutamicum* as a model (4). To the best of our knowledge, this is the first detailed analysis of the counter-silencing mechanism of an Lsr2-like XS protein. Bioinformatic analysis of CgpS ChAP-seq (chromatin affinity purification and sequencing) data revealed a clear preference of CgpS toward AT-rich stretches containing A/T steps (alternation of A to T and vice versa). *In vivo* reporter studies with synthetic promoter variants verified the importance of a distinct drop in GC profile and revealed the overrepresentation of a short, sequence-specific motif at CgpS target regions. Insertion of TF operator sites at different positions within various CgpS target promoters was shown to counteract CgpS silencing, showing the most prominent effect at the position of maximal CgpS binding. With this approach, we provide important insights into the *in vivo* constraints of Lsr2 counter-silencing and contribute to an understanding of how bacteria can evolve control over the expression of horizontally acquired genes.

RESULTS

***In vivo* analysis of CgpS binding preferences.** Recent genome-wide profiling studies revealed that the Lsr2-like xenogeneic silencer CgpS preferentially binds to AT-rich DNA sequences in the genome of *C. glutamicum* ATCC 13032 (4). To determine the parameters affecting CgpS binding and silencing *in vivo*, we systematically analyzed the peak sequences obtained from CgpS ChAP-seq analysis (4) and subsequently verified our conclusion by testing the silencing of synthetic promoter variants. Remarkably, an overlay of the GC profiles of all 35 CgpS target promoters located within the prophage element CGP3 revealed a high degree of similarity with a distinct drop in GC content matching the position of maximal CgpS coverage (Fig. 1A). Genome-wide analysis of AT-rich genomic regions revealed that the fraction of CgpS-bound sequences increased with the length of the particular AT stretch. While increasing numbers of G/C interruptions (occurrence of G or C within an AT stretch) negatively influenced the proportion of CgpS-bound targets (Fig. 1B), a larger number of A/T steps (alternation of A to T and vice versa) increased the fraction of CgpS-bound sequences by trend (Fig. 1C). This trend became especially evident in the case of AT-rich stretches of medium length (14 to 30 bp).

Overall, this analysis suggested that long and consecutive AT stretches represent the main determinant of CgpS target binding. Individual inspection of CgpS-targeted phage promoters revealed a significant correlation between the CgpS peak maximum and the GC minimum in this area (Fig. 1D). Depending on the widths of the CgpS coverage peaks, promoters were grouped into two classes. Class 1 consists of promoters with peak widths between 500 and 850 bp, which typically show one distinct drop in GC profile, while CgpS coverage peaks of class 2 promoters are wider than 850 bp and the corresponding GC profiles often feature broader or multiple drops.

Due to efficient CgpS-mediated silencing of gene expression, most transcriptional start sites (TSS) of CgpS target promoters had not been identified in previous studies (40). It represents, however, an advantage of the chosen model system that expression of the majority of CgpS targets can be induced by triggering prophage induction using the DNA-damaging antibiotic mitomycin C. To provide comprehensive insights into the promoter architecture of CgpS targets, TSS were determined under conditions triggering phage gene expression (600 nM mitomycin C). For 46 out of all 54 CgpS target promoters, at least one TSS was identified (for 31 out of 35 prophage promoters) (see Table S1 in the supplemental material). Strikingly, the analysis of the relative distances between the positions of TSS and maximal CgpS binding revealed that in the majority of CgpS target promoters, TSS are located close to the position of maximal CgpS coverage and GC minimum (Fig. 1D and E; see Table S1 for the complete data set).

Design, build, and test: relevance of a DNA motif for CgpS binding and silencing. Bioinformatic analysis of CgpS target sequences confirmed the preference of CgpS for AT-rich DNA sequences. However, neither the distinct drop in GC content nor the occurrence of long and consecutive AT stretches were unique to CgpS targets (Fig. 1), indicating that additional parameters support CgpS to specifically recognize its

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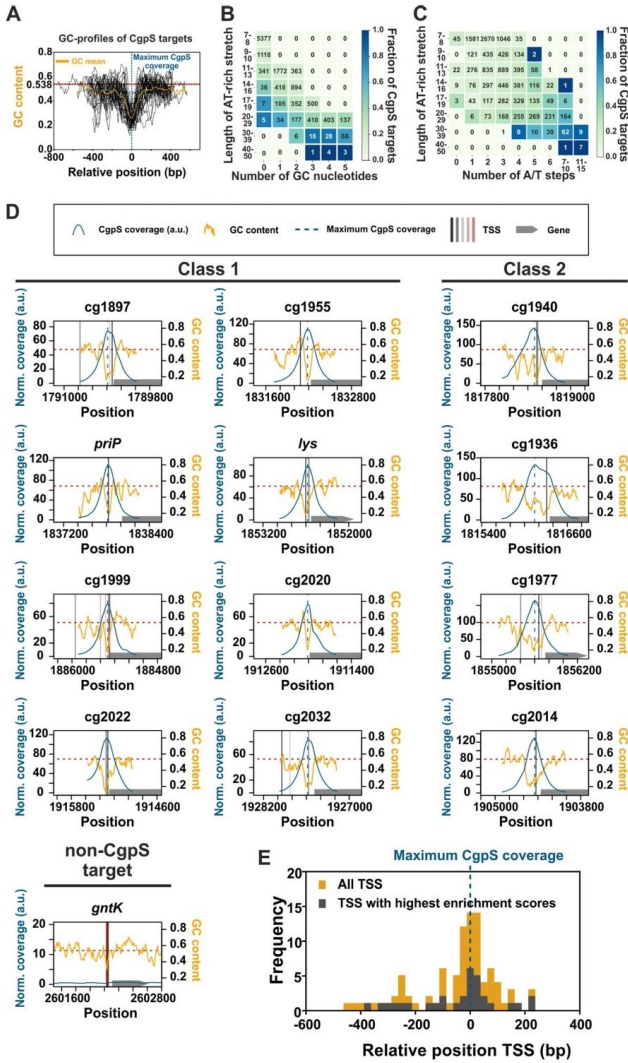


FIG 1 CgpS preferentially binds to long and consecutive AT stretches. (A) Overlay and calculated mean (orange curve) of GC profiles of CgpS target promoters located within the CGP3 prophage ($n = 35$) (4). Profiles were calculated by a (Continued on next page)

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targets. Interestingly, a MEME-CHIP analysis (41) on CgpS-bound promoter sequences revealed a 10-nucleotide-long AT-rich binding motif (E value, 5.2×10^{-9}) containing A/T steps (Fig. 2A), which was found in 51 of 54 bound promoter regions. Remarkably, the presence of this motif within AT-rich stretches of different lengths significantly increased the fraction of CgpS-bound sequences by a factor of up to 2.8-fold (Fig. 2A). However, the genome-wide search for motif occurrence using the online tool FIMO (Find Individual Motif Occurrences) (42) revealed that about 85% of the motifs (669/785) within the *C. glutamicum* genome were not bound by CgpS, indicating that the motif alone is not sufficient to permit CgpS binding.

In the following experiments, we used an *in vivo* approach to test whether the combination of the motif and the drop in GC profile are sufficient for CgpS-mediated silencing of gene expression. For this purpose, different synthetic promoter variants were designed based on the 50- to 70-bp core promoter regions of the phage genes P_{cg1999} and P_{lys} . Both promoters were highly active in the absence of CgpS, indicating that the chosen core regions efficiently drive transcription. In the case of P_{cg1999} , the DNA sequence containing the core promoter elements (-10 and -35 box and TSS) and the predicted binding motif (shown in Fig. 2A) was kept constant (Fig. 2B). The adjacent sequence was either designed to mimic the native GC profile of P_{cg1999} (exchange of A to T and G to C and vice versa, $P_{cg1999_A-T/G-C}$) or contained a randomized sequence varying in GC profile and sequence (P_{cg1999_rand}). The resulting promoter designs were fused to a gene encoding the yellow fluorescent protein Venus. In line with our hypothesis, the construct $P_{cg1999_A-T/G-C}$, featuring the native GC profile, was efficiently silenced by CgpS in the wild-type strain and displayed even lower reporter output than the native phage promoter P_{cg1999} (Fig. 2B). Surface plasmon resonance (SPR) analysis revealed CgpS binding kinetics and affinities for this synthetic promoter (equilibrium dissociation constant [K_D], 42 nM) similar to those of the corresponding native CgpS target promoter P_{cg1999} ($K_D = 58$ nM). CgpS also interacted with the control promoter fragment P_{cg336} , but with much lower affinity ($K_D = 381$ nM) and very fast dissociation rates (Fig. 2C). In the prophage-free strain Δ phage, which lacks the phage-encoded *cgpS* gene, the reporter output was significantly higher for all tested promoter fusions, confirming that all designs functionally drive transcription. Silencing of the promoter variant with randomized adjacent flanks was strongly impaired, demonstrating that the motif-containing 50-bp core promoter region alone did not mediate silencing. This highlights the importance of the overall drop in GC content observed at CgpS target promoters (Fig. 2B).

The relevance of the identified motif was verified using synthetic promoter designs of the phage promoter P_{lys} . Here, constructs carrying only parts of the predicted motif (70-bp core) did not permit silencing, while constructs covering the motif entirely enabled silencing (Fig. 2D). In all P_{lys} -based synthetic constructs, the native GC profile

FIG 1 Legend (Continued)

rolling mean with a window size of 50 bp and a step size of 10 bp. The GC profiles of the promoters were normalized regarding the orientation and position of the maximal CgpS binding peak (blue line), which was defined for all sequences as position 0. The mean GC content of the *C. glutamicum* genome (69) is shown as a red line (53.8%). (B and C) Genome-wide analysis of CgpS binding to consecutive AT stretches of different lengths considering G/C interruptions (occurrence of G or C within an AT stretch) (B) or number of A/T steps (allowing up to five G/C interruptions) (C). A/T steps are defined as alterations of A to T and vice versa. The value in the array represents the number of stretches found in the *C. glutamicum* genome fitting the respective criteria, while the color indicates the fraction of CgpS targets per array. (D) Inverse correlation of GC profiles and CgpS coverage of promoters. CgpS coverage obtained from previous ChAP-seq experiments (4) was calculated with a rolling mean with a window size of 50 and a step size of 10. All identified TSS (see Materials and Methods and Text S1) are shown in Table S1 and represented as vertical black, gray, and red lines (mapped according to their enrichment scores: black > shades of gray > red). Positions of maximal CgpS coverage and average GC content are shown as described for panel A. The corresponding genes are shown as gray arrows. Promoters were grouped into two classes based on the width of the region bound by CgpS (class 1 promoters, 500 to 850 bp, typically featuring one distinct drop in GC profile; class 2 promoters, >850 bp, often broader and containing multiple drops in GC content). As a negative control, the non-CgpS target promoter of the gene *gntK* is shown. a.u., arbitrary units. (E) Frequency distribution of relative positions of all new identified TSS (yellow) of CgpS target promoters referred to the position of maximal CgpS binding. TSS showing the highest enrichment scores per gene are highlighted in gray.

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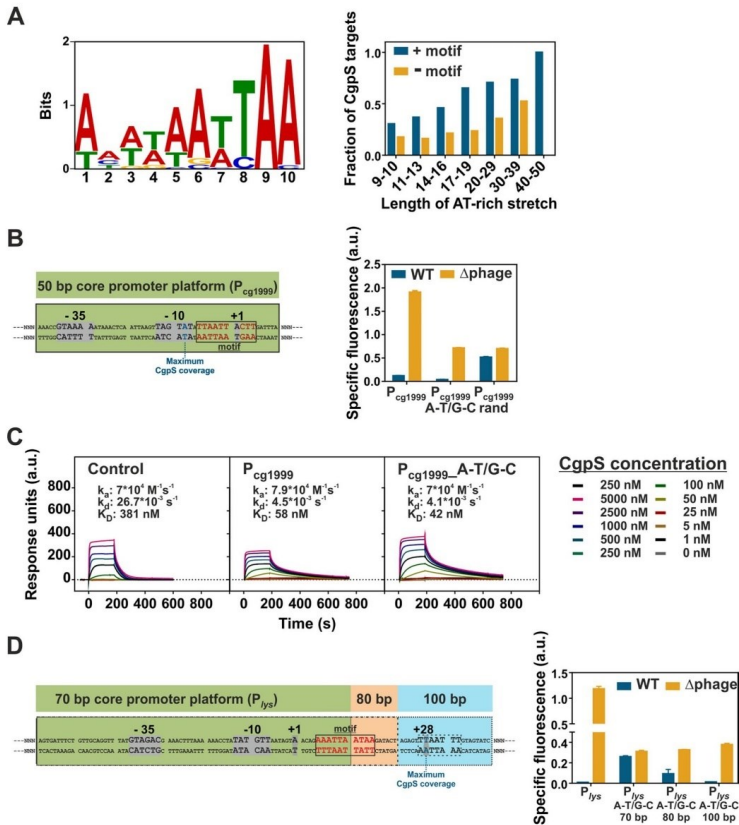


FIG 2 Synthetic *in vivo* approach to dissect the relevance of the GC profile and a sequence-specific binding motif for CgpS silencing. (A) Identified 10-bp CgpS binding motif using MEME-ChIP (41) analysis found within 51 of 54 CgpS target promoters (4) (E value, 5.2×10^{-9}). The bar plot represents the genome-wide fraction of CgpS targets in AT stretches of different length, allowing up to 5 G/C interruptions with or without the identified motif. (B) CgpS silencing of synthetic constructs ($P_{cg1999-A-T/G-C}$ and $P_{cg1999-rand}$), based on a 50-bp core promoter region (green box) of the phage gene *cg1999*. The fixed 50-bp DNA sequence covered the -10 and -35 box, positions of the TSS (40), and potential binding motif (gray box). The adjacent sequence (N upstream, 260 bp; N downstream, 48 bp) was either adjusted to maintain the native density of AT stretches ($P_{cg1999-A-T/G-C}$, exchange of A to T and G to C) or randomized ($P_{cg1999-rand}$). (C) Surface plasmon resonance analysis of CgpS binding to the synthetic promoter $P_{cg1999-A-T/G-C}$ (423 bp) compared to that of the negative-control $P_{cg1999-rand}$ (424 bp) and the corresponding native CgpS target P_{cg1999} (423 bp). k_a , association constant; k_d , dissociation constant. (D) CgpS silencing of synthetic constructs based on fixed 70- to 100-bp promoter regions of the phage gene *lys*. The 70-bp sequence (green box) covered the -10 and -35 box and TSS but only half of the putative motif. The 80-bp region (green and orange boxes) covered the motif completely, and the 100-bp region (all boxes) additionally covered the position of maximal CgpS coverage. The adjacent sequences (N upstream, 304 bp; N downstream, 70 to 100 bp) were adjusted to maintain the native density of AT stretches (A-T/G-C). (B and D) Reporter outputs (Venus) of the native and corresponding synthetic variants (plasmid backbone pJ1) in wild-type and Δ phage (Δ *cgpS*) strains after 5 h of cultivation in a microtiter cultivation system in CGXII medium containing 100 mM glucose. Shown are mean values and standard deviations from biological triplicates. All synthetic sequences are listed in Table S21.

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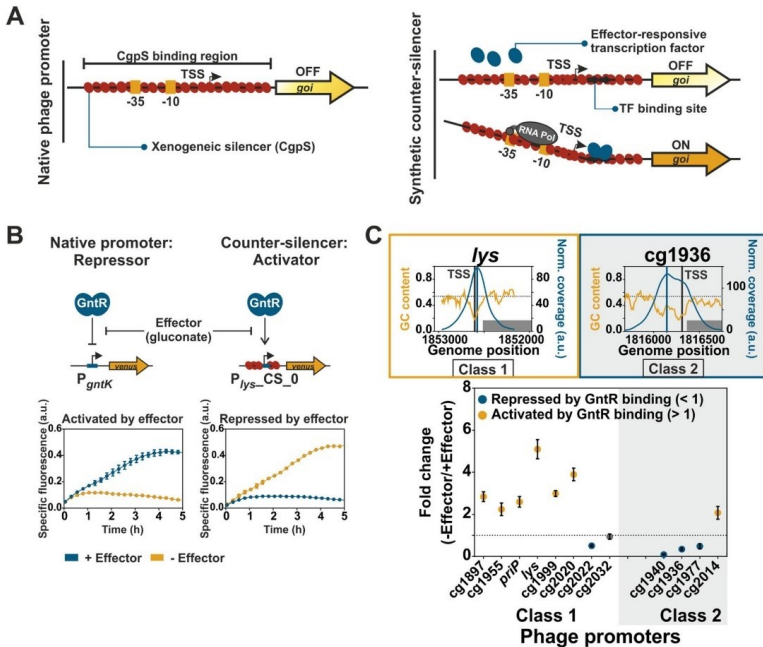


FIG 3 Synthetic approach to study disruptive counter-silencing. (A) Schematic overview of a native CgpS target promoter (phage) and the corresponding synthetic counter-silencer construct. (B) Signal inversion by synthetic counter-silencing. Comparison of the reporter outputs of P_{gntK} , the native target promoter of the regulator of gluconate catabolism GntR (43), and the synthetic GntR-dependent counter-silencer promoter P_{lys-CS_0} . (C) *C. glutamicum* wild-type strains harboring the plasmid-based constructs (pJC1) were cultivated in the absence of the effector (111 mM glucose) or in its presence (100 mM gluconate) in a microtiter cultivation system. Graphs represent the means and error bars the standard deviations from biological triplicates. Backscatter and fluorescence were measured at 15-min intervals. (C) Counter-silencing efficiency of different phage promoters with inserted GntR binding sites located directly upstream of the position of maximal CgpS binding. Promoters were grouped into two classes based on the width of the region bound by CgpS (class 1 promoters, 500 to 850 bp, often one distinct drop in GC profile; class 2 promoters, >850 bp, often broader or multiple drops in GC content). CgpS coverage and GC profiles of two representative promoters are shown. The highest-ranked TSS are marked as vertical gray lines and the position of maximal CgpS binding as vertical blue lines. GC profiles of all used phage promoters are shown in Fig. 1D. *C. glutamicum* wild-type cells harboring the plasmid-based (pJC1) counter-silencers were cultivated in the presence (100 mM gluconate) or absence (100 mM glucose) of the effector molecule gluconate in a microtiter cultivation system. Fold change ratios of Venus reporter outputs in the absence and in the presence of the effector were calculated based on the specific reporter outputs after 5 h of cultivation (Fig. S1A). Dots represent the means and error bars the standard deviations from at least biological triplicates. Yellow dots demonstrate counter-silencing (activated by GntR binding), while blue dots represent repression (repressed by GntR binding). Promoters, which did not show significant changes in reporter output, are shown as gray dots (t test; $P < 0.05$).

of the sequence flanking the core promoter region was mimicked but the DNA sequence was changed (A to T and G to C and vice versa). This *in vivo* analysis of synthetic phage promoter variants revealed that efficient CgpS silencing depended on both specific DNA sequences (binding motif) and the drop in GC profile.

Synthetic disruptive counter-silencing. Disruptive counter-silencing was previously described as a mechanism that may provide access to horizontally acquired genes silenced by nucleoid-associated proteins (32). To study the potential and constraints of evolutionary network expansion by counter-silencing of CgpS target promoters, a synthetic counter-silencer (CS) design was applied in this study (Fig. 3A). At native

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target promoters (e.g., P_{cg1999} or P_{lys}), oligomerization of the xenogeneic silencer CgpS leads to the formation of a nucleoprotein complex inhibiting transcription (4, 31). In the following experiments, we used a set of 12 different phage promoters as a basis for synthetic CS constructs and inserted the operator sequence of an effector-responsive transcription factor (TF) into the silenced promoter regions. We postulated that binding of the TF to its operator sequence would interfere with the silencer nucleoprotein complex and thereby mediate counter-silencing (Fig. 3A). To avoid interference of the inserted operator site with CgpS-mediated silencing, we chose the operator site of the functionally redundant TFs GntR1 (Cg2783) and GntR2 (Cg1935) (summarized as GntR in the following), which bind to a well-defined short (15 bp) and AT-rich (GC content, 27%) DNA motif (43). One of the native targets of GntR is the promoter of the *gntK* gene, which is repressed by binding of GntR. The P_{gntK} promoter and the synthetic promoter constructs were fused via a consistent linker containing a ribosomal binding site (RBS) to the reporter gene *venus* and were inserted into the plasmid pJc1. The effector molecule gluconate was shown to act as an inducer triggering the dissociation of GntR from its operator site (43), consequently leading to derepression of P_{gntK} (Fig. 3B).

Monitoring of fluorescent outputs driven by phage-based synthetic promoter constructs allows the *in vivo* analysis of silencing and counter-silencing efficiencies. GntR operator sites were indeed confirmed as suitable candidates for the construction of counter-silencers, since the insertion into different phage promoters led to only slightly increased background expression levels in the wild-type strain in the presence of the effector molecule (Fig. S1A). The insertion of a GntR binding site (BS) within the CgpS-silenced phage promoter P_{lys} (P_{lys-CS_0}) led to effector-dependent reporter outputs. GntR binding resulted in an increased reporter output of the counter-silencer construct P_{lys-CS_0} when glucose was added as a carbon source, while gluconate (effector addition) triggered the dissociation of GntR, leading to silencing of promoter activity by CgpS (Fig. 3B). This is especially remarkable considering that the binding site was inserted at the position of maximal CgpS coverage close to the annotated TSS (27 bp downstream [Table S1]). Based on textbook knowledge, this position would rather fit to a repressor function (44, 45). In the case of P_{gntK} , the GntR binding site overlaps the TSS, leading to repression of gene expression (43). In the context of xenogeneic silencing, however, GntR binding appeared to efficiently interfere with CgpS silencing. Thus, in contrast to the native GntR target P_{gntK} , the synthetic P_{lys} -counter-silencer promoter was activated in the absence of the effector molecule. Although both promoters (P_{gntK} and P_{lys-CS_0}) were completely different and had only the 15-bp-long GntR binding site in common, they showed very similar but inverted responses to effector availability (Fig. 3B). This demonstrates the potential of the counter-silencing principle to convert a repressor to an activating, tunable counter-silencer, thereby facilitating the expansion of regulatory networks.

Disruptive counter-silencing is most efficient at the CgpS nucleation site. To systematically assess the constraints of counter-silencing, 12 representative phage promoters of both classes (eight class 1 and four class 2) were selected as targets to test the efficiency of synthetic counter-silencing. The GntR binding site was inserted directly upstream of the previously identified position of maximal CgpS binding obtained from ChAP-seq analysis (4). To study counter-silencing efficiency, all constructs were analyzed in *C. glutamicum* wild-type cells in the presence and absence of the effector molecule gluconate. The ratio of maximal (– effector; GntR binding) and minimal (+ effector; GntR dissociation) reporter outputs was used to compare the counter-silencing efficiency of the different constructs (Fig. 3C and Fig. S1). Overall, counter-silencing appeared to be more efficient in class 1 promoters typically featuring a bell-shaped CgpS peak and a distinct drop in GC profile. Here, six out of eight constructs showed an effector-responsive counter-silencing behavior. In contrast, only one of four class 2 promoters was activated by GntR binding. The broader regions bound by CgpS are probably stabilizing the silencer-DNA complex, compensating for the local interference

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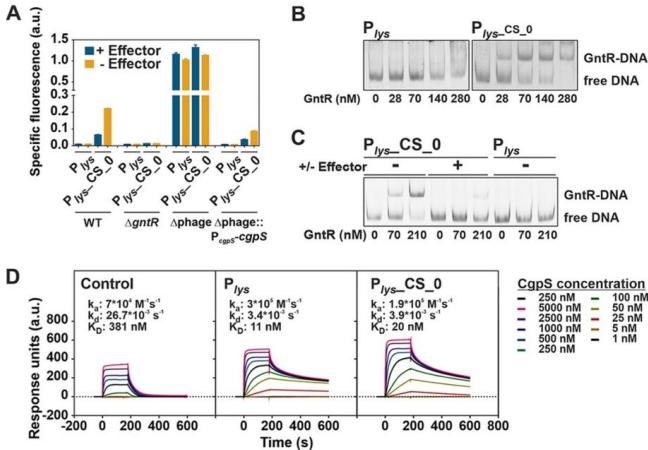


FIG 4 Silencing is mediated by Cgp5, while counter-silencing depends of GntR binding. (A) Reporter output (*venus* expression) of different *C. glutamicum* strains carrying the native P_{lys} promoter or the counter-silencing design P_{lys-CS_0} after 5 h of cultivation. Both constructs were analyzed in *C. glutamicum* wild-type cells, in a $gntR1-gntR2$ double deletion strain, in the phage-free strain Δ phage (lacking the phage-encoded *cgp5*), and in its variant with reintegrated *cgp5* under the control of its native promoter (Δ phage:: P_{cgp5} -*cgp5*). Cells were cultivated in a microtiter cultivation system in CGXII medium supplemented with either 100 mM gluconate (+ effector) or 100 mM fructose (– effector). (B) EMSA of GntR binding to DNA fragments covering the synthetic counter-silencer promoter P_{lys-CS_0} (533 bp, 14 nM) or the native phage promoter P_{lys} (518 bp, 14 nM). (C) Impact of the effector molecule gluconate on binding of GntR to the synthetic counter-silencer construct. EMSA was performed as described for panel B, but GntR and the DNA fragments were incubated either in the presence of the effector (100 mM gluconate) or in its absence (100 mM glucose). (D) Surface plasmon resonance analysis of Cgp5 binding kinetics to biotinylated DNA fragments covering the negative-control P_{cgs336} (424 bp), the native phage promoter P_{lys} (424 bp), or the corresponding synthetic counter-silencer construct (439 bp) that were captured onto a streptavidin-coated sensor chip. Different concentrations of Cgp5 were passed over the chip using a contact (association) time of 180 s, followed by a 420-s dissociation phase. The increase in response units correlates with increasing Cgp5 concentrations.

effects caused by GntR binding. The general functionality of promoter variants (Fig. S1A) was confirmed in the strain Δ phage, where all variants showed a significant fluorescent signal (Fig. S1B). Interestingly, GntR binding to $P_{cgs2014}$ led to counter-silencing in the wild type but to slight repression in the Δ phage strain, suggesting that only the destructive interference between Cgp5 and GntR facilitates efficient transcription of the downstream gene.

Silencing is mediated by Cgp5 binding, and counter-silencing depends on GntR binding.

Cgp5 as silencer and GntR as counter-silencer are the two key components of the synthetic counter-silencer approach presented in this study. To confirm their presumed functions, mutant analysis and *in vitro* binding assays with both proteins were performed. The reporter outputs of the native phage promoter P_{lys} , as well as of the corresponding counter-silencer construct (P_{lys-CS_0}) were analyzed in *C. glutamicum* wild-type cells and different mutant strains. In the wild type, the counter-silencing construct showed the expected increase of reporter output upon GntR binding (– effector). In line with the assumed counter-silencing function of GntR, both constructs featured a low reporter output in the $\Delta gntR$ strain lacking both functionally redundant GntR1 and GntR2 regulators (Fig. 4A). To confirm the relevance of the inserted GntR operator sequence, different mutated variants were tested as well. Here, neither the insertion of a randomized operator sequence, identical in length and nucleotide

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composition, nor a mutated operator site, where only one conserved base in the GntR motif was exchanged, led to counter-silencing of the P_{lys} promoter (Fig. S2). These results confirmed that counter-silencing directly depends on GntR binding. However, the insertion of a reverse-oriented GntR binding site within the silenced promoter allowed counter-silencing, showing that this mechanism does not depend on the directionality of the binding site (Fig. S3). P_{lys} and the corresponding counter-silencer construct showed strongly increased promoter activities in the Δ phage strain in the absence of CgpS, suggesting that CgpS is responsible for silencing. Effector-dependent activation was abolished in the absence of CgpS, indicating that GntR acts as a counter-silencer rather than as a classical activator. Reintegration of the *cgpS* gene into the Δ phage strain, resulting in Δ phage:: P_{cgpS} -*cgpS*, confirmed CgpS as the only factor responsible for silencing of the native phage promoter P_{lys} and, thus, emphasized that CgpS function does not depend on further phage-encoded accessory proteins (Fig. 4A).

As a further piece of evidence, electrophoretic mobility shift assays (EMSA) were performed to confirm the specific binding of GntR to the synthetic counter-silencing construct (P_{lys} -CS_0) *in vitro*. In contrast to the native phage promoter, the P_{lys} fragment containing the GntR operator site showed a significant shift at low GntR concentrations, confirming specific GntR binding to P_{lys} -CS_0 (Fig. 4B). Addition of the effector molecule gluconate led to dissociation of GntR (Fig. 4C), which is in agreement with previous reports (43). Surface plasmon resonance analysis of CgpS binding to DNA fragments covering either P_{lys} or the synthetic counter-silencer construct P_{lys} -CS_0 showed comparable high-affinity binding of CgpS to both promoters ($K_{D, P_{lys}}$ 11 nM; P_{lys} -CS_0, 20 nM) (Fig. 4D).

Impact of operator site position. When analyzing the promoter architecture of horizontally acquired gene clusters, previous studies revealed a certain variability (33). To systematically assess the potential and constraints of the counter-silencing mechanism for evolutionary network expansion, we analyzed the impact of operator site position on counter-silencing efficiency. Therefore, the GntR binding site was inserted at different positions using the prophage promoter P_{lys} as a test case (Fig. 5A). Position 0 is defined as the position located directly upstream of the nucleotide featuring maximal CgpS binding in ChAP-seq studies (4). The position of maximal CgpS binding was located 27 bp downstream of the TSS. *C. glutamicum* wild-type cells harboring the plasmid-based constructs [pJC1- P_{lys} ::GntR BS_pos(variable)-*venus*] were cultivated in the presence or absence of the effector molecule gluconate. Induced and noninduced reporter outputs were strongly influenced by the binding site position. This demonstrated that the inserted binding site itself, depending on its position, already interferes with the silencer-DNA complex (Fig. 5B). Comparison of the fold change ratio of reporter outputs in the absence and presence of the effector gluconate revealed that the construct with the GntR binding site located directly upstream of the maximal CgpS binding peak (position 0) showed the highest dynamic range (~5-fold). This dynamic range decreased when the operator was inserted between 15 bp upstream (-15) and 10 bp downstream (+10) of the maximal CgpS binding peak, but constructs still showed counter-silencing in the absence of the effector (Fig. 5C). However, analyzing the -15 promoter variant in the absence of CgpS (Δ phage) revealed repression caused by GntR binding, demonstrating again that the observed counter-silencing effect is a result of regulatory interference (Fig. S4). GntR binding sites located at greater distances led in most cases to relatively low reporter outputs. Here, the expression level tended to be higher when GntR binding was inhibited, suggesting GntR acts mainly as a repressor of gene expression at these positions (Fig. 5B and C). A similar trend was observed for the phage promoter $P_{cgl1999}$ (Fig. S5). Altogether, these results demonstrated that the impact of GntR binding on promoter activity strongly depends on the context of xenogeneic silencing. While interference with CgpS binding triggered promoter activation by counter-silencing, GntR binding in the absence of CgpS often lowered the reporter output. Analysis of reporter outputs driven by 5'-truncated promoter variants of P_{lys} and P_{lys} -CS_0 revealed that the region >89 bp upstream of

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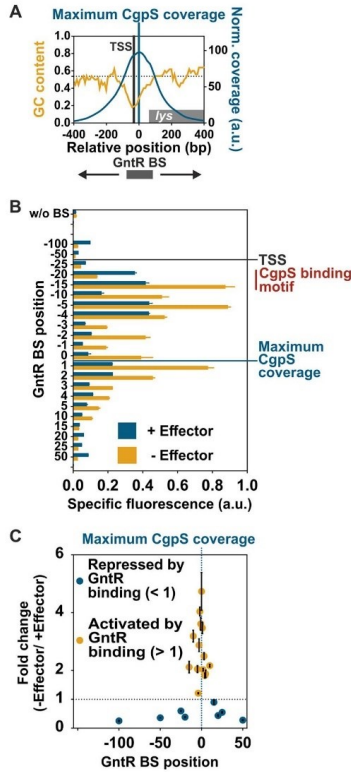


FIG 5 Impact of GntR operator position on inducibility of P_{lys} -based promoter constructs. (A) Inverse correlation of GC profile and CgpS binding coverage (4) of the phage promoter P_{lys} . The transcriptional start site (TSS) and the position of maximal CgpS binding are shown as vertical lines. Binding site positions (also used in panels B and C) refer to the sequence base associated with maximal CgpS binding. The position directly upstream of this nucleotide was defined as position 0. (B) Impact of inserted GntR binding site position on specific reporter outputs in the presence (gluconate) and absence (glucose) of the effector molecule after 5 h of cultivation. Positions of TSS and maximal CgpS coverage are marked by horizontal lines, and the range of the putative CgpS binding motif is shown. (C) Impact of GntR binding site position on counter-silencing efficiency of P_{lys} -based promoter constructs. Ratio of specific reporter outputs, shown in panel B, were used for the calculation of their inducibility (fold change). Cells harboring the plasmid-based synthetic promoter constructs were grown in CGXII medium supplemented with either 111 mM glucose or 100 mM gluconate. Bars (B) and dots (C) represent the means and error bars the standard deviations from at least biological triplicates.

the maximal CgpS binding peak is not required for silencing or counter-silencing (Fig. S6).

Implementation in a genetic toggle switch. The P_{lys} counter-silencing construct ($P_{lys_CS_0}$) and the native GntR target promoter P_{gntK} showed a very similar promoter output but an inverted response to effector availability. While P_{gntK} is repressed by

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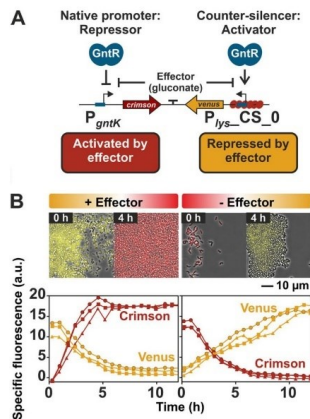


FIG 6 Implementation of P_{gntK} and $P_{lyS_CS_0}$ in a genetic toggle switch. (A) Scheme of the designed toggle switch based on the native GntR target promoter P_{gntK} and the synthetic GntR-dependent P_{lyS} counter-silencer construct. In order to monitor their activities, the promoters were fused to different reporter genes ($P_{lyS_CS_0}$ -*venus* and P_{gntK} -*e2-crimson*). The promoter reporter gene fusions were oriented in opposite directions and separated by a short terminator sequence. (B) Dynamic switch between both reporter outputs. *C. glutamicum* wild-type cells harboring the plasmid-based toggle were cultivated in a microfluidic cultivation system (46) with continuous supply of CGXII medium supplemented either 111 mM glucose or 100 mM gluconate and analyzed by time-lapse microscopy at 20-min intervals. Switch of carbon source supply was performed after the first 17 h. This time point was defined as T_{01} . The graphs show the specific fluorescence of three independent microcolonies (circles, squares, and triangles) over time, and images display one representative colony.

binding of GntR, the counter-silencer promoter is activated by GntR binding in the absence of the effector molecule gluconate (Fig. 3B). Both promoters were combined in a gluconate-dependent, GntR-controlled genetic toggle switch. To monitor the switching between different expression states, $P_{lyS_CS_0}$ was fused to the reporter gene *venus*, while the native GntR target promoter P_{gntK} was fused to the reporter gene *e2-crimson* (Fig. 6A). Since the toggle output is only regulated by GntR binding, native GntR levels could be used for toggle control, avoiding a negative impact of artificial TF levels on cellular growth. *C. glutamicum* wild-type cells harboring the plasmid-based toggle ($pJC1$ - $P_{lyS_CS_0}$ -*venus*- T - P_{gntK} -*e2-crimson*) were cultivated in a microfluidic chip device (46) in minimal medium containing either gluconate (+ effector) or glucose (− effector) as carbon source. The carbon sources were switched after the first 17 h. The following time-lapse microscopy analysis revealed that the output of this synthetic toggle is reversible, as shown by the rapid changes in reporter outputs (Fig. 6B). This overall design principle allows the control of the toggle by only one specific effector-responsive TF and features a robust and reversible response to effector availability, highlighting the potential of this toggle for biotechnological applications, for example, to switch between biomass production and product formation.

DISCUSSION

The nucleoid-associated protein Lsr2 is conserved throughout the phylum of *Actinobacteria*, where it plays an important role in the xenogeneic silencing of horizontally acquired genomic regions (4, 18, 19, 39). The Lsr2-like protein Cgp5 was recently described as a classical XS protein silencing the expression of cryptic prophage elements and further horizontally acquired elements in *C. glutamicum* (4).

CgpS binds DNA with a distinct drop in GC content close to the TSS. In this study, we systematically assessed the promoter architecture of CgpS targets as well as the constraints underlying silencing and counter-silencing of target gene expression. The genome-wide analysis of CgpS-bound regions obtained from ChAP-seq analysis revealed that CgpS targets share a distinct drop in GC content (Fig. 1). The binding to AT-rich DNA is a common feature of XS proteins (3) and was shown to represent an important fitness trait to avoid spurious transcription and the sequestering of the RNA polymerase (8). The importance of this drop in GC content was confirmed by measuring the CgpS-mediated silencing of $P_{cgp1999}$ -based synthetic variants, where changes in GC profile abolished silencing (Fig. 2B). The analysis of relative distance between the TSS and position of maximal CgpS binding emphasized that binding of CgpS close to the TSS is important for efficient silencing (Fig. 1E).

CgpS recognizes a sequence-specific binding motif containing A/T steps. *In vitro* protein binding microarray experiments revealed a clear preference of the xenogeneic silencers H-NS, MvaT, and Rok for DNA stretches containing flexible TpA (thymine-pyridine) steps, while no positive effect of TpA steps was observed for Lsr2 from *Mycobacterium tuberculosis* (20, 47). Our design-test-build approach, where different synthetic promoter variants were tested *in vivo* concerning CgpS-mediated silencing, however, revealed a certain degree of sequence specificity of CgpS toward a binding motif containing A/T steps. While the GC content was kept constant, alteration of the proposed motif in the P_{cgp} promoter significantly affected *in vivo* silencing (Fig. 2). A scenario that has been proposed for H-NS features a high affinity toward DNA (K_D of 60 nM), allowing the scanning of DNA until it reaches high-affinity sites, triggering the nucleation of the tight nucleoprotein complex required for silencing (3, 48, 49). Based on the systematic analysis of truncated promoter variants, we clearly defined the region required for efficient *in vivo* silencing by CgpS (see Fig. S6 in the supplemental material). It is important to note that we almost exclusively relied on *in vivo* approaches, including ChAP-seq analysis and reporter assays, to define the parameters affecting silencing and counter-silencing at the systems level. Although *in vitro* analysis of protein-DNA fragments (often linear DNA) is frequently applied and has provided valuable insights into the binding behavior of XS proteins (20, 47, 49), the conditions do not reflect physiologically relevant situations (DNA topology, protein-protein interaction, and interference); consequently, the results have to be interpreted with caution.

GntR-dependent counter-silencing is a disruptive mechanism. While xenogeneic silencing neutralizes the potentially negative effect of invading foreign DNA, counter-silencing allows the integration into the host regulatory network and thereby provides access to horizontally acquired genes. This principle has been almost exclusively studied for H-NS in proteobacteria, and several types of TFs were shown to counteract H-NS silencing *in vivo* (15, 50–53). In the case of Lsr2, the mycobacterial iron-dependent regulator IdeR represents, to the best of our knowledge, the only example of an investigated Lsr2 counter-silencer. IdeR enables the iron-dependent activation of ferritin by alleviating Lsr2 repression at the *bfrB* locus (54).

During bacterial evolution, mutations leading to the formation of TF operator sequences within silenced promoters allow the controlled expression of the previously silenced genes by TF-mediated counter-silencing. In this work, the artificial insertion of the 15-bp short operator sequences of the gluconate-dependent TF GntR within different CgpS target promoters allowed us to study the potential and constraints of counter-silencing of this Lsr2-like XS protein. Binding of GntR to several CgpS target promoters led to transcription initiation of the silenced phage promoters, demonstrating that small changes in the DNA sequence added a further regulatory layer for expression control.

All tested CgpS target promoters showed significant reporter output in the absence of CgpS, confirming that they promote transcription and that CgpS inhibits promoter activity, presumably by hindering open complex formation or by trapping the open complex once it has formed (55). Previous studies already suggested that, without

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xenogeneic silencing, open complex formation is typically not the rate-limiting step at AT-rich promoters of horizontally acquired genes, meaning they are constitutively active (33, 56, 57). In the case of the H-NS target promoter *pagC*, an *in vitro* approach demonstrated RNA polymerase binding and open complex formation in the absence of additional factors, confirming that this promoter alone is transcriptionally competent (33).

In general, two different mechanisms of counter-silencing are conceivable. In one scenario (disruptive mechanism), the interference of TF and XS protein leads to a local disruption of the XS nucleoprotein complex, thereby enabling binding of the RNA polymerase to the DNA. Another possibility is that counter-silencing allows for supportive contacts between the RNA polymerase and the TF itself or more distal DNA regions (supportive mechanism). In this study, binding of GntR to the promoter constructs in the absence of the XS Cgp5 resulted in reduced reporter outputs, although counter-silencing was observed in the wild type. This result strongly speaks for a disruptive rather than a supportive GntR-mediated counter-silencing mechanism.

GntR binding close to the Cgp5 nucleation site is a prerequisite for efficient counter-silencing. By the systematic analysis of promoter variants with varied positions of the GntR operator site, we clearly defined the window where binding of a specific TF led to counter-silencing. Counter-silencing of the Lsr2-like XS Cgp5 was most efficient at positions close to the position of maximal Cgp5 binding in a range of approximately 25 bp (Fig. 5 and Fig. S5), demonstrating that the position of GntR binding is critical for counter-silencing.

Previous studies by Will et al. revealed that counter-silencing and classical activation are different mechanisms of gene regulation (33). While TFs acting as activators typically bind to conserved promoter architectures and promote transcription either by changing the DNA conformation or by recruiting the RNA polymerase (58), the principle of counter-silencing allows a higher degree of flexibility in terms of promoter architecture (33). For the PhoPQ regulon of *Salmonella enterica* serovar Typhimurium, it was shown that PhoP activates core promoters featuring a precise operator position overlapping the -35 box. In contrast, horizontally acquired PhoP target genes show rather diverse promoter architectures, and here transcriptional activation is achieved by counter-silencing of H-NS. In these reported examples, the distances between the TSS and the closest PhoP binding site vary by only 34 bp (33, 59). This is in a range similar to that for our results and those obtained in previous studies for the H-NS target promoter P_{Cgp5} , where the insertion of TF operator sites counteracted H-NS silencing in a comparable window (34).

It is intriguing that in the context of xenogeneic silencing binding of a TF at positions close to the TSS leads to promoter activation, where it would cause a block of transcription at classical promoters (44, 60). Indeed, GntR binding in the absence of the XS Cgp5 resulted in reduced reporter outputs of several synthetic promoter constructs tested in this study (Fig. S1 and S4). However, counter-silencing was observed in the presence of Cgp5 in the wild type. These results demonstrate the potential of the counter-silencing principle to convert a repressor to an activating, tunable counter-silencer, thereby facilitating the integration of horizontally acquired DNA into host regulatory networks. Overall, these data illustrate how interference of TFs is shaping global regulatory networks and that the regulatory impact of TF binding is strongly affected by competition with other DNA-binding proteins.

MATERIALS AND METHODS

Bacterial strains and cultivation conditions. All bacterial strains and plasmids used in this project are listed in Table S2A to C in the supplemental material. The strain *C. glutamicum* ATCC 13032 (61) was used as the wild-type strain. Detailed information about general growth conditions, microtiter cultivations used to monitor cell growth and fluorescence (62), and cultivations in the microfluidic chip device (46, 63) is available in the supplemental material (Text S1).

Recombinant DNA work. All standard molecular methods, such as PCR, DNA restriction, and Gibson assembly, were performed according to previously described standard protocols (64, 65) or according to the manufacturer's instructions. All plasmids were constructed by Gibson assembly. Details on plasmid construction are provided in Table S2C. DNA sequencing and synthesis of oligonucleotides used for

amplification of DNA fragments (inserts for Gibson assembly [Table S2D], biotinylated DNA fragments for surface plasmon resonance [SPR] spectroscopy [Table S2E], and DNA fragments for electrophoretic mobility shift assays [EMSA] [Table S2F]) and sequencing (Table S2G), as well as synthesis of DNA sequences (Table S2H), were performed by Eurofins Genomics (Ebersberg, Germany). Chromosomal DNA of *C. glutamicum* ATCC 13032 was used as the PCR template and was prepared as described previously (66). Detailed information about construction of strain Δ phage: P_{gbs} -*cgpS* via two-step homologous recombination (67) and the design of disruptive counter-silencing constructs is available in the supplemental material (Text S1).

Determination of TSS. The determination of TSS and data analysis were performed with *C. glutamicum* wild-type cells by Vertis Biotechnology AG (Freising, Germany) using the Cappable-seq method developed by Ettwiller and Schildkraut (68). Prophage induction was triggered by adding mitomycin C. Detailed information about cultivation conditions, RNA preparation, and data analysis can be found in the supplemental material (Text S1). Relevant TSS were assigned to phage and nonphage *CgpS* target promoters when they were located in the promoter region 500 bp upstream of the start codon and directed in gene orientation (Table S1). Multiple TSS assigned to the same promoter were ranked depending on their enrichment scores.

Plots of *CgpS* coverage and GC profiles. Normalized *CgpS* coverage values obtained from previous ChAP-seq analysis of Pfeifer and colleagues (4) and GC content of the reference *C. glutamicum* genome BX927147 (69) were plotted to the corresponding genome positions. Both parameters were calculated by a rolling mean with a window size of 50 bp and a step size of 10 bp using R (<http://www.R-project.org>) (70). The position of maximal *CgpS* coverage was centered, and the promoter orientation was normalized (start codon of the corresponding gene is located on the right site). Identified TSS positions were added. Ends of graphs are defined by the range of the *CgpS* binding peaks identified in previous ChAP-seq analysis (4). Plotting was performed either by R (70) or by GraphPad Prism 7.00 (GraphPad Software, La Jolla, CA).

Analyses of AT-rich stretches in *CgpS* binding regions. The *C. glutamicum* genome (BX927147 [69]) was scanned for AT-rich stretches using a custom python script (submitted to GitHub at https://github.com/afilipch/afp/blob/master/genomic/get_at_stretches.py). For further details, see the supplemental material (Text S1).

Protein purification, SPR spectroscopy, and EMSA. Information about purification of Strep-tagged *CgpS* and His-tagged GntR and the performed *in vitro* binding assays (SPR spectroscopy and EMSA) can be found in the supplemental material (Text S1).

Data availability. The custom python script used for scanning for AT-rich stretches is available in the GitHub repository (https://github.com/afilipch/afp/blob/master/genomic/get_at_stretches.py). Data from previously reported ChAP-seq analysis (4) are available at the GEO database (<https://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE141132.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, PDF file, 0.2 MB.

FIG S1, TIF file, 0.2 MB.

FIG S2, TIF file, 0.2 MB.

FIG S3, TIF file, 0.1 MB.

FIG S4, TIF file, 0.1 MB.

FIG S5, TIF file, 0.3 MB.

FIG S6, TIF file, 0.2 MB.

TABLE S1, XLSX file, 0.03 MB.

TABLE S2, DOCX file, 0.1 MB.

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3.2. Inducible expression systems based on xenogeneic silencing and counter-silencing and the design of a metabolic toggle switch

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Contributor Role	Contributor
Conceptualization	JW 70 %, JF 30 %
Formal analysis	JW 100 %
Investigation/Experiments	JW 70 %, GC 20 %, AW 10 %
Methodology	JW 65 %, GC 15 %, AW 10 %, JF 10 %
Project administration	JW 70 %, JF 30 %
Software	-
Supervision	JW 60 %, JF 40 %
Visualization	JW 100 %
Writing – original draft	JW 90 %, JF 10 %
Writing – review & editing	JW 50 %, JF 50 %

Overall contribution JW: 85 %

All data analyses and the visualization of the data was performed by JW. JW performed most of the presented experimental work or was involved in the execution. GC did the qRT-PCR experiment (Fig. 3C), the microtiter growth experiment with L-valine production strains (Fig 6C) as well as the glucose measurements (Fig S3). The cultivation approaches for comparison of productivity of different L-valine production strains was conducted by GC and JW together. The HPLC measurements were done by JW and AW from the group of Dr. Tino Polen from the Forschungszentrum Jülich. The manuscript text was mainly written by JW.

Inducible expression systems based on xenogeneic silencing and counter-silencing and the design of a metabolic toggle switch

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Abstract

Inducible expression systems represent key modules in synthetic regulatory circuit designs and metabolic engineering approaches. However, many established systems are often limited in terms of applications due to high background expression levels and inducer toxicity. In bacteria, xenogeneic silencing (XS) proteins are involved in the tight control of horizontally acquired, AT-rich DNA. The action of XS proteins may be opposed by interference with a specific transcription factor, resulting in the phenomenon of counter-silencing, thereby activating gene expression. In this study, we harnessed this principle for the construction of a synthetic promoter library consisting of phage promoters targeted by an Lsr2-like XS protein of *Corynebacterium glutamicum*. Counter-silencing was achieved by inserting the operator sequence of the gluconate-responsive transcription factor GntR within silenced promoter regions. The GntR-dependent promoter library is comprised of 28 activated and 16 repressed regulatory elements featuring effector-dependent tunability. For selected candidates, background expression levels were confirmed to be significantly reduced in comparison to established heterologous expression systems. Finally, a GntR-dependent genetic toggle switch was implemented in a *C. glutamicum* L-valine production strain allowing the dynamic redirection of carbon flux between biomass and product formation.

Introduction

Inducible expression systems allow the rational and precise control of transcription and are the most frequently used mechanisms for controlling gene expression in synthetic biology and metabolic engineering approaches (Liu et al., 2016; Patek et al., 2013). They usually form the first layer in synthetic regulatory circuits allowing the response to extra- and intracellular stimuli and are applied for the accurate coordination of metabolic fluxes in various microbial production strains (Liu et al., 2016; Xia et al., 2019). In many bacterial model strains, for instance in the industrial platform strain *Corynebacterium glutamicum*, the widely used expression systems P_{tac} (Patek et al., 2013), P_{araBAD} (Ben-Samoun et al., 1999) and P_{tet} (Lausberg et al., 2012; Radmacher et al., 2005) are key parts used for circuit design. They are well established but limited in terms of applications due to considerably high background expression levels, heterogeneous inducer uptake, inducer toxicity or high inducer costs (Baritugo et al., 2018; Patek et al., 2013; Yim et al., 2013; Zhang et al., 2012b). Especially approaches where gene products are toxic to the recipient cell or disturb the central carbon flux are in demand for very stringent control of gene expression (Baritugo et al., 2018). Besides the set of heterologous expression systems, the repertoire of homologous inducible promoter systems is comparatively small for *C. glutamicum* (Binder et al., 2012; Hentschel et al., 2013; Letek et al., 2006; Mustafi et al., 2012; Okibe et al., 2010; Plassmeier et al., 2013) and only a few promoter libraries were constructed containing either constitutive promoters (Rytter et al., 2014; Shang et al., 2018; Yim et al., 2013) or synthetic, Isopropyl- β -D-1-thiogalactopyranoside- (IPTG-) inducible regulatory elements (Rytter et al., 2014).

In a recent project, we designed synthetic promoter constructs for *C. glutamicum*, which were regulated by the mechanisms of xenogeneic silencing and counter-silencing. Xenogeneic silencing is based on specialized nucleoid-associated proteins, so-called xenogeneic silencers (XS), which preferentially bind to horizontally acquired AT-rich DNA (Navarre, 2016; Pfeifer et al., 2019; Will et al., 2015). Known XS proteins are grouped into four classes including H-NS-like XS protein from proteobacteria (Navarre et al., 2006; Oshima et al., 2006), Lsr2-like proteins present in *Actinomycetes* (Gordon et al., 2008), MvaT/U-like XS of gammaproteobacteria of the order *Pseudomonadales* (Tendeng et al., 2003), and Rok found in different *Bacillus* strains (Smits and Grossman, 2010). The major function of the prophage encoded Lsr2-like XS protein CgpS from *C. glutamicum* is the silencing of cryptic prophage elements to maintain their lysogenic state (Pfeifer et al., 2016). CgpS binds to its target promoters and oligomerization of this XS protein leads to the formation of a nucleoprotein complex tightly inhibiting transcription (Pfeifer et al., 2016) (silencing, Figure 1). In our recent study, we inserted the operator site of the regulator of gluconate catabolism GntR at different positions within various CgpS target promoters serving as platforms for the construction of synthetic counter-silencer promoters (Wiechert et al., 2020). Binding of the transcription factor (TF) GntR to its operator

sequence in the absence of gluconate leads to repression of its native target promoter P_{gntK} (Frunzke et al., 2008). In contrast, binding of GntR to the synthetic counter-silencer promoter was shown to interfere with the nucleoprotein complex allowing the RNA polymerase to bind and to initiate transcription (Wiechert et al., 2020) (counter-silencing, Figure 1).

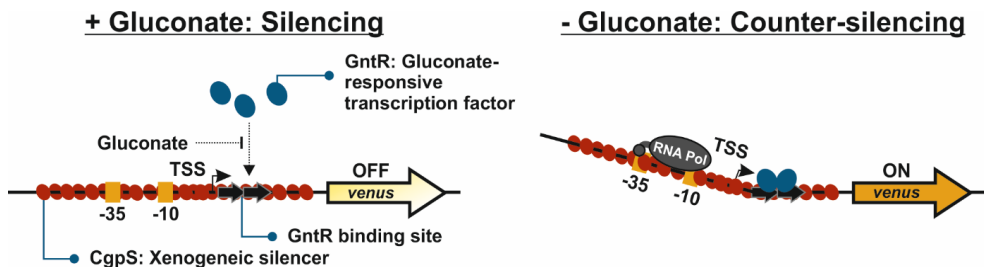


Figure 1: Schematic overview of the GntR-dependent mechanism of counter-silencing. Synthetic counter-silencer constructs are based on phage promoters targeted by the xenogeneic silencer CgpS (red ovals). The 15 bp short operator sequence (black arrows) of the regulator of gluconate catabolism GntR (blue ovals) (Frunzke et al., 2008) was inserted within the silenced promoter regions. In the absence of the effector molecule gluconate, binding of GntR to its operator sequence will interfere with the silencer-DNA complex leading to transcription initiation. Adapted from (Wiechert et al., 2020).

Appropriate inducible expression systems are key modules for the design of synthetic regulatory circuits such as toggle switches and are frequently applied in metabolic engineering strategies aiming at improving the performance of bacterial production strains (Xia et al., 2019). Several natural and non-natural biotechnological products such as amino acids, organic acids and alcohols are derived from metabolites from the central carbon metabolism such as the glycolysis and the tricarboxylic acid (TCA) cycle, thereby competing with pathways essential for bacterial growth and physiology (Doong et al., 2018; Gupta et al., 2017; Soma et al., 2014; Soma et al., 2017; Tsuruno et al., 2015). To achieve a redirection of carbon flux towards the molecule of interest, recent rationally engineered production strains were often based on permanent knockouts or knockdowns of genes concerning the central carbon metabolism (Becker et al., 2011; Blombach et al., 2008; Blombach et al., 2007; Hüser et al., 2005; Krause et al., 2010; Litsanov et al., 2012; Peters-Wendisch et al., 2005). This may, however, have detrimental effects on cellular fitness resulting in metabolic imbalances as well as in impaired growth rates and final cell densities, thereby reducing the overall productivity of a fermentation process (Brockman and Prather, 2015b). As an alternative strategy for these static engineering approaches, recent synthetic biology studies aimed at the dynamic redirection of carbon flux between cell growth and product biosynthesis by using metabolic toggle switches or other synthetic genetic circuits for the control of metabolic flux (Brockman and Prather, 2015a; Doong et al., 2018; Farmer and Liao, 2000; Gupta et al., 2017; Lo et al., 2016; Soma et al., 2014; Soma et al., 2017; Tsuruno et al., 2015; Zhang et al., 2012a). For instance, Soma and colleagues constructed a metabolic toggle switch for isopropanol production in *Escherichia coli*. This synthetic genetic circuit allows switching between growth and

production mode by controlling the conditional knockout of the citrate synthase GltA and the simultaneous upregulation of a synthetic isopropanol production pathway, both competing for acetyl-CoA (Soma et al., 2014; Soma et al., 2017). Using a comparable circuit architecture, Tsuruno and colleagues constructed a metabolic toggle switch for the redirection of the central carbon flux from glycerol towards 3-hydroxypropionic acid (Tsuruno et al., 2015).

In this work, we systematically tested and compared a broad set of synthetic promoters based on the principle of xenogeneic silencing and counter-silencing. The resulting promoter library comprised 44 synthetic promoters regulated by GntR in a gluconate-dependent manner. Comparison to established expression systems revealed very low background expression and good tunability of the resulting constructs. Finally, a GntR-based genetic toggle switch was implemented in *C. glutamicum* for the dynamic switch between cell growth and L-valine production. By controlling the activity of the pyruvate dehydrogenase complex (PDHC), the redirection of carbon flux towards L-valine biosynthesis was regulated.

Results and Discussion

A synthetic promoter library based on the principles of xenogeneic silencing and counter-silencing

Xenogeneic silencing and counter-silencing represent efficient mechanisms for the tight control of gene expression (Wiechert et al., 2020). Following a synthetic counter-silencing approach, we recently demonstrated that binding of the TF GntR within promoters silenced by the XS protein CgpS allows their effector-responsive reactivation (Wiechert et al., 2020). However, depending on the architecture of the phage promoter and the position of the inserted GntR binding site, counter-silencing promoters strongly vary in dynamic range as well as minimal and maximal promoter activities (Wiechert et al., 2020). Here, we systematically tested and compared a broad set of gluconate-dependent, inducible promoters, which are based on the counter-silencing principle. Figure 2 provides an overview of synthetic promoter variants activated by counter-silencing (Figure 2B) or repressed (Figure 2C) by GntR binding (p -values < 0.05). Promoter activity was measured by means of the fluorescence output (production of the Venus reporter protein) over time (Wiechert et al., 2020). Constructs activated by GntR-mediated counter-silencing showed a 100-fold range in maximal reporter output (-gluconate; 0.009 to 0.89 a.u. under the applied conditions) with small increment sizes and a large range of non-induced background levels (+gluconate; ranging from 0.005 to 0.43 a.u. under the applied conditions). Fold-change ratios of the individual promoters (-gluconate/+gluconate) ranged from 1.2-fold to 5-fold. However, not all synthetic promoter variants were activated by GntR binding. Depending on the core promoter part and the position of the binding, GntR binding led to the repression of gene expression in some cases. These constructs showed a 70-fold range in induced expression strength (+gluconate). Remarkably, several synthetic promoter variants appeared to have very low background expression levels. Lowest background expression levels among constructs repressed by GntR binding were observed for P_{cg1977} 0, while the minimum background activity of activated counter-silencer constructs was detected for P_{prip} +5. In *C. glutamicum*, standard systems used for inducible gene expression are heterologous expression systems like the IPTG inducible promoters P_{lac} , P_{tac} and P_{trc} (Patek et al., 2013), the arabinose-dependent promoter P_{araBAD} (Ben-Samoun et al., 1999), the anhydrotetracycline inducible promoter P_{tet} (Lausberg et al., 2012; Radmacher et al., 2005) and the heat induced $P_{R}P_L$ promoter of bacteriophage λ (Tsuchiya and Morinaga, 1988). Remarkably, the set of native inducible promoter systems is comparatively small. Described were promoters induced by gluconate namely P_{git1} (Okibe et al., 2010), P_{gntK} (Hentschel et al., 2013; Letek et al., 2006) and P_{gntP} (Letek et al., 2006), the P_{male} promoter induced by maltose (Okibe et al., 2010), the propionate inducible P_{pripD2} promoter (Plassmeier et al., 2013) as well as the amino acid responsive biosensors Lrp- P_{brnFE} (Mustafi et al., 2012) and LysG- P_{lysE} (Binder et al., 2012). So far, only a few promoter libraries have been constructed

containing either constitutive promoters (Rytter et al., 2014; Shang et al., 2018; Yim et al., 2013) or synthetic, IPTG-inducible regulatory elements (Rytter et al., 2014).

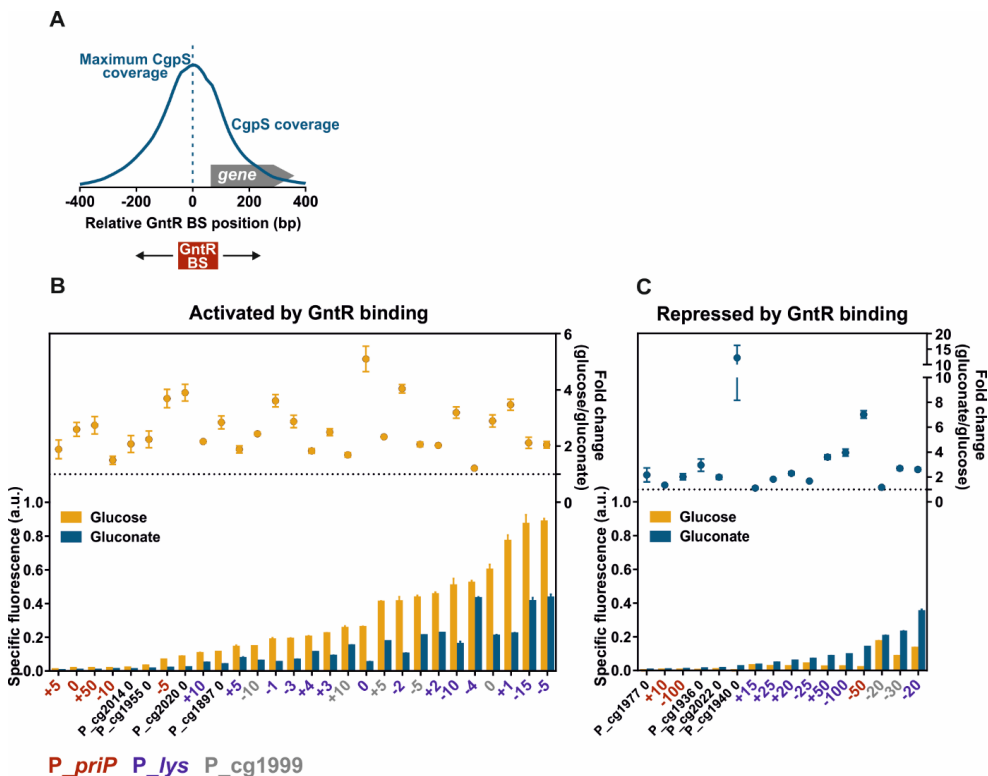


Figure 2: A synthetic GntR-dependent promoter library based on different Cggs target promoters with inserted GntR binding sites. The library consists of different recently described counter-silencer promoters (Wiechert et al., 2020) as well as additional promoter variants based on the phage promoter P_{priP} following the same design approach. **A)** Definition of the binding site position. Schematic overview of a representative phage promoter which is bound by Cggs. The positions of the GntR binding site (BS) were referred to the nucleotide position associated with maximal Cggs binding (Pfeifer et al., 2016). The position directly upstream of this nucleotide was defined as position 0, negative numbers describe upstream and positive numbers downstream positions. **B/C)** Shown are reporter outputs driven by the promoters in the presence (gluconate) and absence (glucose) of the effector molecule after five hours of cultivation and the corresponding fold-change ratios. Promoters are grouped into two sets depending on their response to gluconate availability: activation by GntR binding (counter-silencing) **(B)** and repression by GntR binding **(C)** (p -values < 0.05). Constructs based on the phage promoter P_{priP} , P_{lys} , or P_{cg1999} are color-coded and names are reduced to the position of the GntR binding site. Cells harbouring the plasmid-based synthetic promoter constructs were grown in CGXII medium supplemented with either 111/100 mM glucose or 100 mM gluconate. Bars represent the means and error bars the standard deviation of at least three biological replicates. Names indicate the platform promoter and the position of the GntR binding site positions. Specific fluorescence values were background corrected by subtracting values of strains harbouring the control plasmid $pC1$ -*venus-term* (no promoter in front of *venus*) (Baumgart et al., 2013).

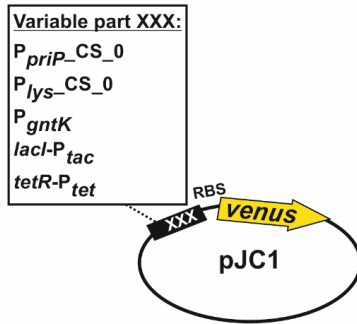
The here presented set of promoters being either activated or repressed by GntR binding provided a gluconate-dependent promoter library covering a broad range of promoter activities which may be highly useful for synthetic biology or metabolic engineering approaches demanding for tightly controlled and inducible regulation of gene expression. In favor for biotechnological applications, the

effector molecule gluconate is a non-toxic, degradable and cost-efficient effector molecule compared to the frequently used inducer IPTG.

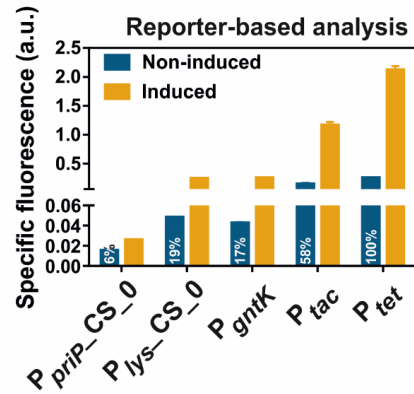
Counter-silencing-based promoters permit tight control of gene expression

To validate the performance of counter-silencing constructs as expression systems, the synthetic promoters $P_{priP_CS_0}$ and $P_{lys_CS_0}$ were exemplarily compared with the native GntR target promoter P_{gntK} and the well-established expression systems P_{tac} and P_{tet} on protein (Venus) and transcript level (*venus* mRNA). Therefore, all promoters were fused to the reporter gene *venus* via a consistent linker containing a ribosomal binding site to avoid differences in translation efficiency and were integrated into the plasmid pJC1. Since the genes encoding for the TFs LacI and TetR are not present in the *C. glutamicum* genome, the *lacI* and *tetR* genes were additionally inserted into the respective plasmids (Figure 3A). Analysis of the reporter levels under inducing and non-inducing conditions revealed a wide range of expression levels for the different constructs (Figure 3B). The lowest background level was observed for the P_{priP} -based counter-silencer (6% of P_{tet}) which also showed the lowest induced reporter output. The P_{lys} counter-silencer and the native GntR target promoter P_{gntK} showed similar, but inverted response to gluconate availability. Both showed at least 5-fold lower background levels when compared to P_{tet} emphasizing their potential for application demanding stringent control of gene expression (Figure 3B). Similar ranges were observed on transcript levels using quantitative real-time PCR (qRT-PCR). $P_{lys_CS_0}$ and P_{gntK} showed only 44 and 27% of the background level of P_{tet} , respectively, and background expression of $P_{priP_CS_0}$ was even reduced to 15% (Figure 3C), confirming that synthetic counter-silencer promoters represent suitable tools for tightly controlled gene expression.

A



B



C

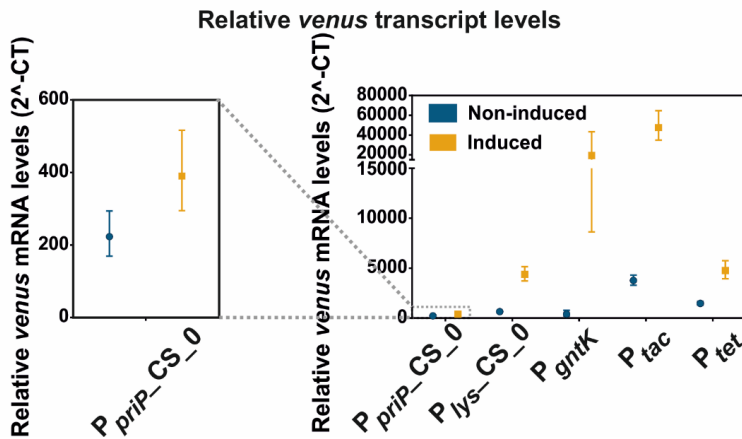


Figure 3: Comparison of counter-silencing constructs with established expression systems. A) For the comparison of the counter-silencer constructs P_{lys-CS_0} and P_{priP-CS_0} (Wiechert et al., 2020) as well as the native GntR target promoter P_{gntK} with the established expression systems P_{tac} and P_{tet}, all of the different promoters were cloned into the plasmid pJC1 and fused to the reporter gene *venus* via a consistent linker containing a ribosomal binding site (RBS; AGGAG (Pfeifer-Sancar et al., 2013)). C. *glutamicum* wild type cells harbouring the plasmid-based constructs were cultivated in a microtiter cultivation system under inducing and non-inducing conditions depending on the particular promoter construct: P_{priP-CS_0} and P_{lys-CS_0}: +: 100 mM glucose, -: 100 mM gluconate; P_{gntK}: +: 100 mM gluconate, -: 100 mM glucose; P_{tac}: +: 100 μM IPTG, -: 0 μM IPTG; P_{tet}: +: 235 nM Atc, -: 0 nM Atc B) Reporter outputs of the native GntR target promoter P_{gntK} as well as P_{priP}- and P_{lys}-based counter-silencer constructs (P_{priP-CS_0}; P_{lys-CS_0}) in comparison to the established expression systems P_{tac} and P_{tet} after five hours of cultivation. All strains were pre-cultivated in CGXII containing 100 mM gluconate. Bars show the mean and error bars the standard deviation of specific fluorescence of biological triplicates. Indicated numbers represent the percentage of the background expression level of P_{tet}. C) Promoter-derived relative *venus* transcript levels measured by quantitative real-time PCR after five hours of cultivation under non-inducing and inducing conditions. Symbols represent the means and error bars the range of relative *venus* mRNA levels measured in biological and technical duplicates. All strains were pre-cultivated under non-inducing conditions.

Tunability of native and synthetic GntR target promoters

Tunability of promoters, for example by varying the amount of effector molecule, is an important feature for their applicability as bacterial expression systems in synthetic and biotechnological applications.

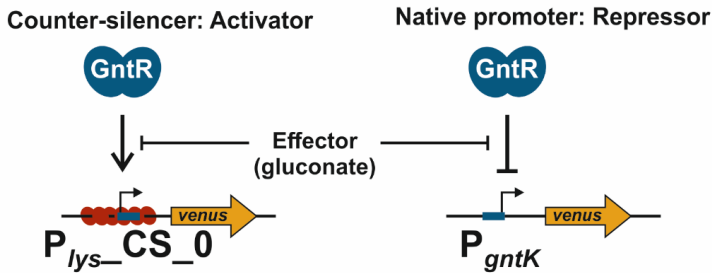
In this study, the impact of varying gluconate concentrations on temporal dynamics and dynamic ranges of promoter activities of the native GntR target promoter P_{gntK} and the counter-silencer promoter $P_{lys_CS_0}$ were characterized. Both promoters inversely react to GntR binding and, therefore, to gluconate availability (Wiechert et al., 2020) (Figure 4). GntR binding within the synthetic counter-silencer promoter $P_{lys_CS_0}$ leads to promoter activation due to efficient interference between GntR and the CgpS-DNA complex (Wiechert et al., 2020). In contrast, binding of GntR to the native target promoter P_{gntK} leads to promoter repression (Frunzke et al., 2008) (Figure 4A).

Highest $P_{lys_CS_0}$ -derived reporter outputs were reached in the absence of gluconate (0 mM), meaning that the default state in the absence of the effector molecule of this construct is ON. In contrast, maximal P_{gntK} promoter activity was observed in the presence of highest gluconate concentrations (100 mM), demonstrating the inverted response of both systems (Figure 4B). The default state in the absence of gluconate of P_{gntK} promoter activity is OFF and the signal remained stable in this state after cells had entered the stationary phase (Figure 4B). In contrast, in the presence of 100 mM gluconate, $P_{lys_CS_0}$ promoter activities stayed low during the first 11 hours of cultivations, but reporter outputs strongly increased shortly after cells had entered the stationary phase (Figure S1). At this time point, gluconate was probably fully consumed allowing GntR to bind to the DNA and to interfere with the silencer-DNA complex. Small amounts of 1 mM supplemented gluconate also led to reduced reporter outputs in the beginning of the cultivation, however, the fluorescence signal already increased after two hours of exponential growth phase. Increasing amounts of supplemented gluconate (10 and 50 mM) gradually shifted the time point of induction, demonstrating the potential of counter-silencer promoters as expression systems with temporal tunability (Figure 4B). In contrast, a positive correlation between gluconate concentrations and reporter outputs was observed for P_{gntK} (Figure 4B) demonstrating the opportunity of fine-tuning maximal promoter activity. Comparably to the tunability of maximal P_{gntK} activity by varying the gluconate concentration, the previously designed P_{tet} -based expression system pCLTON1 showed an ATc-dependent gradual increase of reporter outputs (Lausberg et al., 2012), while a T7 expression system differentially reacts to increasing amounts of the effector molecule IPTG (Kortmann et al., 2015).

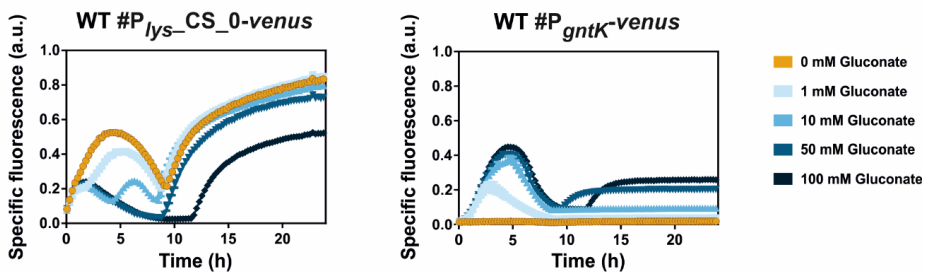
Upon transport into the cell, gluconate is phosphorylated by the gluconate-specific kinase GntK to 6-phosphogluconate which is further metabolized in the pentose phosphate pathway (Frunzke et al., 2008). To reduce degradation rates of the effector molecule gluconate, we deleted the *gntK* gene

encoding for the gluconate kinase GntK. Analysis of activities of the counter-silencer promoter $P_{lys_CS_0}$ in a *C. glutamicum* $\Delta gntK$ deletion strain revealed the gradual temporal shift of the time point of reporter output increase. However, the time periods of low promoter activity were significantly extended and already 10 mM gluconate led to an almost complete repression of $P_{lys_CS_0}$ promoter activity (Figure 4C, D). On the other hand, constitutively high effector concentrations ($\Delta gntK$) led to more than 2.5-fold higher P_{gntK} reporter outputs in comparison to the wild type (Figure 4C, D). The assumed increased and more stable intracellular gluconate levels were only reduced by dilution effects during cell growth comparable to the use of non-degradable structural sugar analogues like IPTG. However, the higher levels of gluconate can affect cell growth of $\Delta gntK$ (Figure S1), therefore, fine-tuning of effector concentrations must be considered for potential applications of this strain.

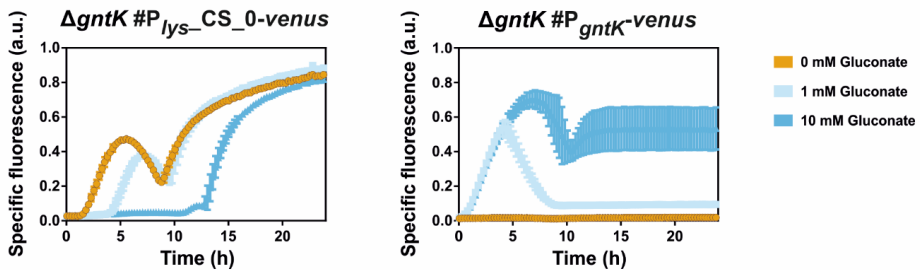
A



B



C



D

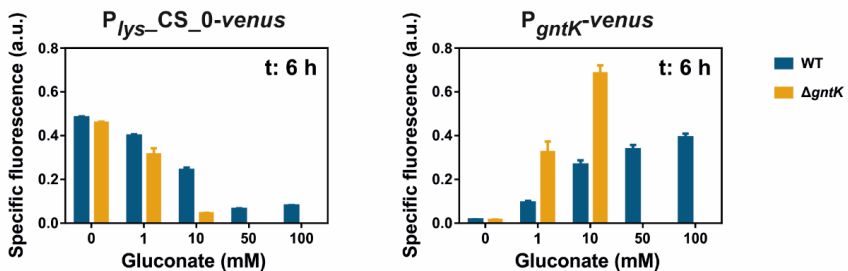


Figure 4: Tunability of the counter-silencer construct $P_{lys_CS_0}$ and the native GntR target promoter P_{gntK} . A) Shown are schematic overviews of both promoter constructs. B/C) Reporter outputs (specific Venus fluorescence) of *C. glutamicum* wild type (WT) (B) or *C. glutamicum* strain $\Delta gntK$ (C) harbouring the plasmid-based constructs pJC1- $P_{lys_CS_0}$ - $venus$ or pJC1- P_{gntK} - $venus$. Graphs show the mean and error bars the standard deviation of specific Venus fluorescence of biological triplicates over time. Increasing gluconate concentrations are indicated as shades of blue. D) Bar plots show the reporter outputs (specific Venus fluorescence, from B and C) after six hours of cultivation. Cells were cultivated in a microtiter cultivation

system in CGXII medium supplemented with glucose (100 mM in analysis of pJC1- P_{gntK} -*venus* and 111 mM for characterization of pJC1- $P_{lys_CS_0}$ -*venus*) and either no or varying amounts of gluconate as effector. Backscatter and fluorescence were measured at 15 min intervals. Corresponding backscatter values are given in Figure S1.

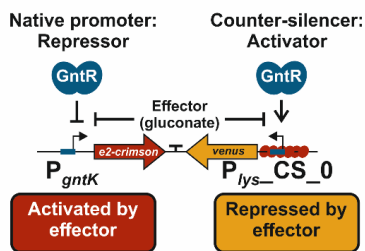
A GntR-based genetic toggle switch

Based on their similar, but inverted response to gluconate availability, the native GntR target promoter P_{gntK} and the synthetic P_{lys} counter-silencing construct ($P_{lys_CS_0}$) were recently combined in a gluconate-dependent, GntR-controlled bistable genetic toggle switch (Wiechert et al., 2020). Switching between different expression states was monitored by fusing the counter-silencer promoter $P_{lys_CS_0}$ to the reporter gene *venus* and the native GntR target promoter P_{gntK} to the reporter gene *e2-crimson* (Figure 5A). Previous analysis of the toggle state and its switching dynamics had confirmed its principal functionality. The addition of gluconate led to a switch in reporter outputs from Venus to E2-Crimson, while the removal of gluconate had the inverted effect (Wiechert et al., 2020). In this study, we characterized the reversibility and robustness of the GntR-dependent toggle system in long-term experiments. Therefore, we cultivated *C. glutamicum* wild type cells harbouring the plasmid-based toggle design in a microfluidic cultivation system starting with continuous supply of CGXII minimal medium containing 100 mM glucose as carbon source. The cells had been in a stable Venus-dominant state until the cultivation medium was switched to medium containing 100 mM gluconate after the first 3.5 hours of cultivation. Gluconate interfered with binding of GntR to its operator site leading to P_{gntK} activation and restoring of silencing of $P_{lys_CS_0}$, visualized by the following switch of the toggle from the Venus- to the E2-Crimson-state within the next eight hours of cultivation. In the following, a stable expression profile was observed demonstrating the robustness of the toggle design. During cultivations, single cells occurred which stayed in one of the both toggle states. However, these cells had also stopped to divide hinting that they were in a dormant state. Three consecutive medium switches revealed the dynamic reversibility of the toggle switch (Figure 5B).

Previously, Gardner and colleagues designed a bistable synthetic toggle switch inspired by the genetic switch of bacteriophage λ governing the lysis-lysogenic decision (Gardner et al., 2000). This network consists of two repressors, namely Cro and CI, and their corresponding promoters P_R and P_{RM} , each being repressed by the gene product of the other (Khalil and Collins, 2010). Comparably, in the modular synthetic toggle switch variants constructed by Gardner and colleagues, the authors imitated this circuit design by combining the IPTG-dependent $\text{LacI-}P_{trc}$ expression system with the temperature-sensitive λ repressor (CI_{ts}) and its corresponding promoter P_{LsIcon} or the anhydrotetracycline (ATc) inducible TetR- P_{tetO-1} system (Gardner et al., 2000). This design principle was successfully applied as metabolic toggle switch for improving product formation in engineered *E. coli* strains (Soma et al., 2014; Soma et al., 2017; Tsuruno et al., 2015). However, especially inappropriate TF synthesis and

degradation rates and insufficient repressor strengths were discussed as major challenges for toggle stability and functionality (Gardner et al., 2000). In contrast to this circuit architecture, the here presented toggle is controlled by only one effector molecule, namely gluconate, and one specific TF, namely GntR. This design is simple but also very robust and allows the use of native TF levels, thereby circumventing challenges like TF synthesis and degradation rates. Furthermore, due to the circuit architecture, both sides of the toggle are independent of each other allowing tuning of one side of the toggle without affecting the other.

A



B

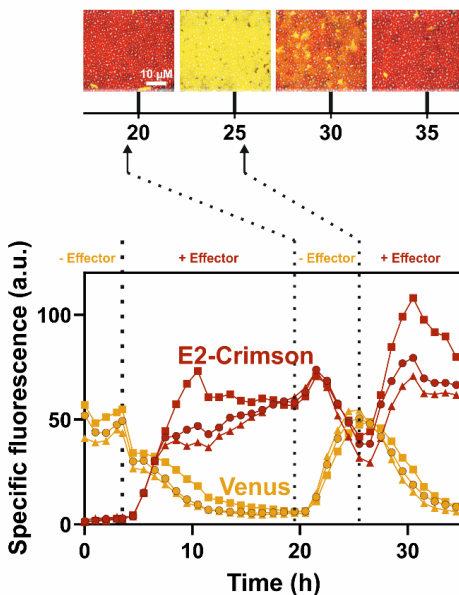


Figure 5: Reversibility of the GntR-dependent toggle switch. **A)** Schematic overview of the GntR-dependent toggle switch (adapted from (Wiechert et al., 2020)). **B)** Reversible switch between both reporter outputs. *C. glutamicum* wild type cells harbouring the plasmid-based toggle were cultivated in a microfluidic cultivation system (Grünberger et al., 2015) with continuous supply of CGXII medium supplemented with either 100 mM glucose or 100 mM gluconate and analyzed by time-lapse microscopy at 15 min intervals. Cells were pre-cultivated in shaking flasks in the absence of the effector molecule gluconate (100 mM glucose). After the first 3.5 h (left dotted vertical line) of cultivation in the microfluidic chip in the absence of gluconate, the medium supply was switched to CGXII supplemented with 100 mM gluconate. Further switches of effector supply were performed after 19.5 and 25.5 h (dotted vertical lines). The graphs show the background corrected specific

fluorescence (Venus and E2-Crimson) of three independent microcolonies (circles, squares, triangles) over time and images display one representative colony (triangle) after 20, 25, 30 and 35 h of cultivation.

Implementation of a metabolic toggle switch for dynamic control of L-valine production

In a next step, the GntR-dependent toggle switch was implemented for the dynamic control of growth and L-valine biosynthesis in *C. glutamicum*. L-valine, an essential amino acid for human and animals, is used for a variety of application ranging from supplementation in human and animal nutrition, as precursor for herbicides, antibiotics and anti-viral drugs and as compound in cosmetic industries (Oldiges et al., 2014). L-valine is formed from two molecule of the precursor pyruvate in a pathway comprising four reaction steps catalyzed by acetohydroxyacid synthase (AHAS, *ilvBN*), acetohydroxyacid isomeroreductase (AHAIR, *ilvC*), dihydroxyacid dehydratase (DHAD, *ilvD*) and transaminase B (TA, *ilvE*) (Figure 6A). The theoretical yield for L-valine production from glucose is one mol L-valine per mol glucose (Oldiges et al., 2014). Several, previously established L-valine production strains were constructed by engineering the activity of the PDHC, which catalyzes the competing conversion of pyruvate to acetyl-CoA (Figure 6A). Engineering strategies ranged from the deletion of the *aceE* gene encoding the E1 subunit of the enzyme complex, which leads to a permanent PDHC inactivity, to a reduction of *aceE* expression by promoter exchanges (Blombach et al., 2008; Blombach et al., 2007; Buchholz et al., 2013; Ma et al., 2018; Schreiner et al., 2005). Growth of cells with inactive PDHC is decoupled from L-valine production and depends on costly carbon sources like acetate, which enables only low biomass yields (Blombach et al., 2007; Schreiner et al., 2005). After acetate depletion, glucose is consumed and converted to L-valine (Blombach et al., 2007). The dynamic control of precursor supply and of flux through biosynthetic routes, for example by applying synthetic regulatory circuits like the presented GntR-dependent toggle, can represent an alternate strategy to the static permanent knockout approaches. For instance, this design may allow to overcome the growth dependency on acetate.

For the construction of the toggle-based L-valine production strain, the native *aceE* promoter was replaced by the gluconate-dependent promoter P_{gntK} . P_{gntK} is repressed in the absence of gluconate leading to PDHC downregulation (Figure 6B). Growth analysis of the P_{gntK} -*aceE* strain in comparison to the previously designed $\Delta aceE$ deletion strain revealed that both strains did not grow in medium containing glucose as sole carbon source for 40 hours of cultivation (Figure 6C). Both strains grew in medium containing glucose and acetate but showed relatively low final backscatter values (Figure 6C). In contrast, the P_{gntK} -*aceE* strain reached 3.4-fold higher backscatter values in medium containing 222 mM glucose and 51 mM gluconate in comparison to the acetate-glucose approach (molarity of carbon: 254 mM acetate > 51 mM gluconate, factor 1.7) (Figure 6C). The gluconate-induced *aceE* expression allowed the co-utilization of glucose, as shown by the significantly reduced backscatter values when

cells were cultivated in medium containing only 51 mM gluconate (Figure 6C). Remarkably, no differences in cell growth were observed between *C. glutamicum* P_{gntK} -*aceE* and wild type cells in minimal medium containing 100 mM gluconate (Figure S2). The costs for gluconate are in a comparable range as those for glucose, while acetate represents a more costly carbon source (<https://www.sigmaaldrich.com>, Table S1) highlighting the economic potential of the dynamic gluconate controlled PDHC activity.

Previously applied successful engineering strategies targeting PDHC activity were based on *aceE* promoter replacements leading to reduced or growth phase-dependent *aceE* expression and acetate-independent cell growth (Buchholz et al., 2013; Ma et al., 2018). These approaches were either based on weaker, but constitutive mutated promoter variants of *dapA* (Buchholz et al., 2013) or on a growth-regulated promoter of an industrial used L-leucine strain (P_{CP_2836} ; *C. glutamicum* CP) (Ma et al., 2018). However, in contrast to an effector-responsive expression system like the gluconate-dependent promoter P_{gntK} (Figure 4), both regulatory circuits are independent of inducers and cannot be influenced by extracellular supplementation of effector molecules. Thus, fine-tuning of strain performance is constrained, for instance regarding the time point of switching or maximal promoter activities.

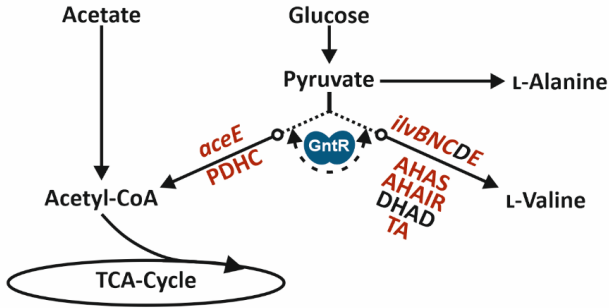
In the absence of gluconate, when the PDHC is inactive in the toggle strain, pyruvate is available for L-valine production. Therefore, the counter-silencer $P_{lys_CS_0}$, as counterpart of the toggle, was used to control the overexpression of the L-valine biosynthesis genes *ilvBNCE* (combined in a synthetic operon) (Figure 6B). The plasmid-based construct was analyzed in the dynamic P_{gntK} -*aceE* strain and in the static $\Delta aceE$ deletion strain in comparison to the same strains harbouring the empty plasmid pJC1 (pJC1-*venus*-term) or the previously designed plasmid pJCA- P_{ilvB} -*ilvBNC*- P_{ilvE} -*ilvE* (pJCA-*ilvBNCE*) (Radmacher et al., 2002). pJCA- P_{ilvB} -*ilvBNC*- P_{ilvE} -*ilvE* containing L-valine biosynthesis genes under control of their native promoters served as reference for strain performance. For comparison of L-valine productivity, all strains were cultivated in CGXII minimal medium containing 222 mM glucose and 254 mM acetate. This cultivation condition fully erased the benefit of dynamic *aceE* and *ilvBNCE* expression (*aceE*: OFF state; *ilvBNCE*: ON state); however, it allows comparison of strain performance independent of cell densities. Analysis of the culture supernatant revealed that after 46 hours of cultivation glucose was fully consumed by the production strains, while L-valine titers reached maximal values (Figure S3). Both platform strains P_{gntK} -*aceE* and $\Delta aceE$ produced comparable amounts of L-valine during cultivations in the absence of gluconate indicating that the PDHC was inactive in the toggle strain and that this redirected carbon flux towards L-valine (Figure 6D, Table 1).

The toggle strain $P_{gntK-aceE}$ #pJC1- $P_{lys_CS_0}$ - $ilvBNC$ -RBS- $ilvE$ produced 1.9-fold higher amounts of L-valine than cells harbouring the empty plasmid pJC1 (Figure 6D, Table 1), confirming the importance of increased levels of biosynthesis enzymes. However, in comparison to the previously characterized plasmid pJC4- P_{ilvB} - $ilvBNC$ - P_{ilvE} - $ilvE$ (pJC4- $ilvBNCE$) (Radmacher et al., 2002), L-valine titers were reduced by about 50% in strains $\Delta aceE$ and $P_{gntK-aceE}$. In contrast, pyruvate-derived by-product formation (L-alanine) was 4-5-fold increased (Figure 6D, Table 1). This shift in amino acid concentrations hinted towards limitations in the conversion of pyruvate to L-valine, apparently caused by lower promoter activity of the counter-silencer ($P_{lys_CS_0}$) compared to the native promoters P_{ilvB} and/or P_{ilvE} and the resulting lower enzyme levels. AHAS ($ilvBN$) is the key enzyme of the L-valine biosynthesis pathway and catalyzed the first reaction step. AHAS is feedback inhibited by branched chain amino acids like L-valine (Eggeling et al., 1987; Elišáková et al., 2005) and its biosynthesis intermediate α -ketoisovalerate (Krause et al., 2010). In previous AHAS engineering approaches, a genomically encoded, feedback resistant enzyme variant led to increased L-valine production (Elišáková et al., 2005), suggesting that this reaction step can be a bottleneck for efficient substrate product conversion. Consistently, several engineering approaches succeeded in increasing L-valine titers by increasing the gene dosage of L-valine biosynthesis genes by plasmid-based expression (Blombach et al., 2007; Elišáková et al., 2005; Radmacher et al., 2002; Sahm and Eggeling, 1999).

In conclusion, the production performance of the here presented toggle strain was not optimal in comparison to previously established L-valine production strains, probably due to lower $ilvBNCE$ transcription levels. However, the GntR-dependent promoter library contains much stronger counter-silencer promoters (Figure 2) which might represent promising candidates for further toggle strain optimizations.

In the absence of gluconate, both platform strains ($P_{gntK-aceE}$ and $\Delta aceE$) produced comparable amounts of L-valine indicating that the PDHC was inactive in the toggle strain redirecting carbon flux towards L-valine (Figure 6D, Table 1). However, higher cell densities of the *C. glutamicum* $P_{gntK-aceE}$ strain observed during co-utilization of 222 mM glucose and 51 mM gluconate (Figure 6C) showed on the one hand the potential to overcome growth dependency on costly and inefficient carbon sources like acetate and, on the other hand, the potential to improve substrate product conversion by accelerating biomass formation. Further in-depth analyses of this effect are indispensable for final evaluation of the dynamically controlled $P_{gntK-aceE}$ strain.

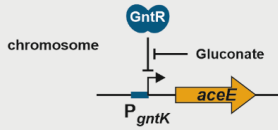
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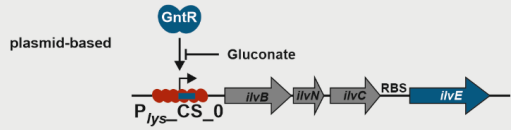
Host engineering:
precursor supply

P_{gntK} -*aceE*: PDHC is active in the presence of gluconate

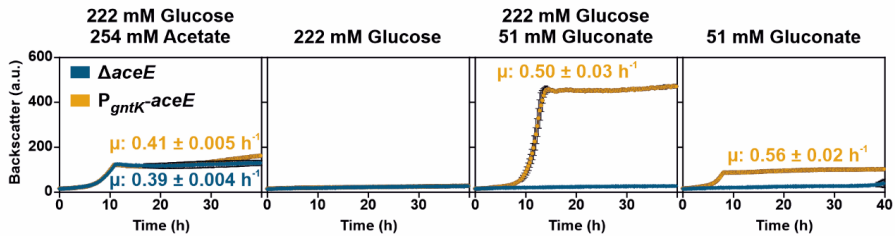


Pathway engineering:
precursor-product conversion

$P_{lys_CS_0}$ -*ilvBNC-ilvE*: Overexpression of L-valine biosynthesis genes in the absence of gluconate



C



D

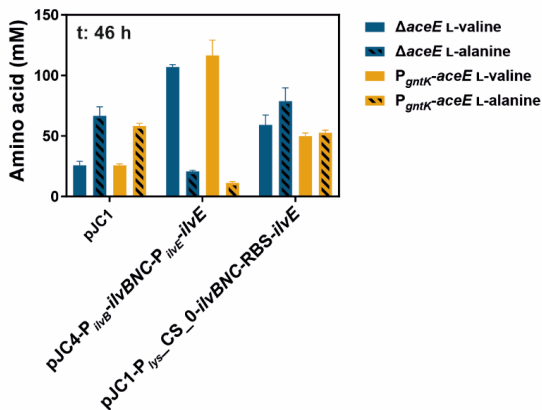


Figure 6: Application of the GntR-dependent toggle for the dynamic switch between growth and L-valine production. **A)** Schematic representation of relevant parts of the central carbon metabolism and the L-valine biosynthesis pathway of *C. glutamicum*. The GntR-dependent toggle controlled the redirection of the carbon flux allowing the conversion of pyruvate to either L-valine or acetyl-CoA entering the tricarboxylic acid cycle (TCA-Cycle). *aceE* encodes for the E1 subunit of the pyruvate dehydrogenase complex (PDHC), which converts pyruvate to acetyl-CoA. L-valine is formed from pyruvate in a four-step reaction pathway catalyzed by acetohydroxyacid synthase (AHAS, *ilvBN*), acetohydroxyacid isomeroreductase (AHAIR, *ilvC*), dihydroxyacid dehydratase (DHAD, *ilvD*), and transaminase B (TA, *ilvE*). Dynamically controlled genes and their products are highlighted in red. **B)** Schematic overview of the genetic background of the dynamic L-valine production strain. The native promoter of the *aceE* gene was replaced by the GntR target promoter P_{gntK} allowing control of PDHC activity. The L-valine biosynthesis genes were combined in a synthetic operon by fusing *ilvE* via a linker containing an RBS sequence to the end of the operon *ilvBNC*. Its expression was controlled by the synthetic GntR counter-silencer promoter $P_{lys_CS_0}$. **C)** Growth of the strain with dynamically controlled *aceE* expression (P_{gntK} -*aceE*) in comparison to the previously established Δ *aceE* strain (Blombach et al., 2007; Schreiner et al., 2005). Both strains had been pre-cultivated in CGXII containing 222 mM glucose and 254 mM acetate before they were cultivated in a microtiter cultivation system in medium with either 222 mM glucose and 254 mM acetate, 222 mM glucose, 222 mM gluconate and 51 mM gluconate or 51 mM gluconate. Calculated growth rates (μ) are given (colour-coded) when significant growth had been observed. **D)** L-valine titers of the *aceE* toggle strain (P_{gntK} -*aceE*) and the Δ *aceE* L-valine production strain harbouring either the empty control plasmid pJC1 (pJC1-*venus*-term (Baumgart et al., 2013)), plasmid-based L-valine biosynthesis genes controlled by the counter-silencer (pJC1- $P_{lys_CS_0}$ -*ilvBNC*-RBS-*ilvE*) or the natively regulated variant pJC4- P_{ilvB} -*ilvBNC*- P_{ilvE} -*ilvE* (pJC4-*ilvBNC*E; (Radmacher et al., 2002)). All strains were cultivated in CGXII supplemented with 222 mM glucose and 254 mM acetate. Bar plots represent the L-valine and L-alanine titers of biological triplicates and error bars the corresponding standard deviations after 46 h of cultivation. L-valine and L-alanine titers, glucose concentrations and cell densities (OD_{600}) are shown in Figure S3. Further details are given in Table 1.

Table 1: Product (L-valine) and by-product (L-alanine) formation of strains P_{gntK} -*aceE* and Δ *aceE*. Corresponding bar plots are shown in Figure 6D. Given are the amino acid concentrations, L-valine titers, cell dry weight- (CDW-) specific L-valine titers and L-valine yields after 46 h of cultivation. Means and standard deviations of biological triplicates are listed.

Plasmid	Strain	OD_{600}	L-valine (mM)	L-alanine (mM)	L-valine titer (g/l)	Specific titer (g L-valine/ g CDW)	Yield (g L-valine/ g glucose)
pJC1	Δ <i>aceE</i>	45.9 ± 1.0	25.9 ± 2.6	66.7 ± 6.0	3.0 ± 0.31	0.26 ± 0.02	0.087 ± 0.007
	P_{gntK} - <i>aceE</i>	48.7 ± 1.5	25.8 ± 0.9	58.3 ± 1.8	3.0 ± 0.11	0.25 ± 0.02	0.076 ± 0.003
pJC4- P_{ilvB} - <i>ilvBNC</i> - P_{ilvE} - <i>ilvE</i>	Δ <i>aceE</i>	41.1 ± 0.8	106.9 ± 1.7	20.8 ± 0.8	12.5 ± 0.2	1.22 ± 0.01	0.313 ± 0.005
	P_{gntK} - <i>aceE</i>	44.3 ± 1.1	116.5 ± 10.4	11.3 ± 1.0	13.7 ± 1.22	1.23 ± 0.1	0.341 ± 0.03
P_{ilvE}-<i>ilvE</i>							
pJC1- $P_{lys_CS_0}$ - <i>ilvBNC</i> -RBS- <i>ilvE</i>	Δ <i>aceE</i>	43.3 ± 0.4	59.2 ± 6.7	78.8 ± 9.0	6.9 ± 0.78	0.64 ± 0.07	0.175 ± 0.022
	P_{gntK} - <i>aceE</i>	46.6 ± 0.1	50.0 ± 2.0	52.6 ± 1.8	5.85 ± 0.24	0.5 ± 0.02	0.146 ± 0.006

Conclusion

Tightly controlled, inducible promoter systems are key modules in biotechnological and synthetic biological applications and the most frequently used principle for controlling gene expression (Liu et al., 2016; Patek et al., 2013). In this study, we demonstrated the potential of synthetic counter-silencer promoters as expression system allowing a more stringent control of gene expression in comparison to well-established systems such as P_{tac} and P_{tet} . These promoters were tightly repressed by CgpS-mediated xenogeneic silencing and significantly induced by GntR-dependent counter-silencing. Synthetic promoter variants were combined in a GntR-dependent bilateral promoter library comprising in total 44 regulatory elements which were repressed (16 promoters) or activated (28 promoters) in the presence of the non-toxic and cheap effector molecule gluconate. These sets of regulatory elements provided a wide range of promoter activities, which could fulfil the requirements of various applications.

The effect of varying effector concentrations on the dynamics and timing of promoter activities was analyzed in detail for exemplary chosen synthetic GntR target promoters revealing that counter-silencer constructs are expression systems with temporal tunability.

Furthermore, we performed a comprehensive characterization and application of a GntR-dependent toggle switch allowing opposed expression of two sets of target genes. This toggle is controlled by only one TF (GntR) and one effector molecule (gluconate) and combines two promoters – a native target promoter and a synthetic counter-silencer promoter – both inversely responsive to gluconate dependent GntR binding (Wiechert et al., 2020). Finally, the GntR-dependent toggle was successfully applied as metabolic toggle switch for the dynamic redirection of carbon flux between the growth-related formation of acetyl-CoA and L-valine biosynthesis, both competing for the precursor molecule pyruvate.

In conclusion, our results demonstrated the high potential of promoters being controlled by xenogeneic silencing counter-silencing for synthetic circuit designs and as tightly controlled gene expression systems.

Material and Methods

Bacterial strains and cultivation conditions

All bacterial strains and plasmids used in this project are listed in Table S2, S3 and S4. The strain *C. glutamicum* ATCC 13032 (Kinoshita et al., 1957) served as wild type strain. For cultivations of *C. glutamicum* strains, brain heart infusion (BHI, Difco Laboratories, Detroit, MI, USA) complex medium was inoculated with a single colony from a fresh agar plate and incubated for 8 to 16 hours. For cultivation of $\Delta aceE$ deletion strains as well as cells with dynamically controlled *aceE* expression ($P_{gntK-aceE}$), 85 mM potassium acetate was supplemented to the BHI medium. All cultivation steps were performed at 30°C, 900 rpm and 75% humidity in 96 deep-well plates (VWR, Radnor, PA) in a Microtron incubator shaker (Infors, Bottmingen, Switzerland). For reporter-based assays and analysis of relative *venus* transcript levels driven by the different expression systems, BHI pre-cultures were used to inoculate a second overnight pre-culture in CGXII minimal medium (Keilhauer et al., 1993) supplemented with 25 µg/ml kanamycin and 100 mM gluconate or 100 mM glucose. For growth analysis of $\Delta aceE$ - and $P_{gntK-aceE}$ -based strains, the second overnight preculture was performed in CGXII minimal medium containing 222 mM glucose and 254 mM acetate. Subsequently, the pre-culture was used to inoculate the main culture at a start OD_{600} of one. For reporter-based assays, the main cultures were grown in CGXII medium with 25 µg/ml kanamycin and 100 mM gluconate, 100/111 mM glucose without or with varying supplemented amounts of gluconate (0, 1, 10, 50, 100 mM), 100 mM glucose with either 100 µM IPTG (induction of P_{tac}) or 235 nM ATc (induction of P_{tet}). Growth analysis of the $\Delta aceE$ deletion strains as well as cells with dynamically controlled *aceE* expression ($P_{gntK-aceE}$) was performed in CGXII minimal medium supplemented with either 222 mM glucose and 254 mM acetate, 222 mM glucose, 222 mM glucose and 51 mM gluconate or 51 mM gluconate. *E. coli* DH5 α were used for plasmid amplification and cells were cultivated in Lysogeny Broth (LB) medium or on LB agar plates at 37°C. If needed, 50 µg/ml kanamycin was added.

Microtiter cultivation to monitor cell growth and fluorescence

Analysis of cell growth and reporter-based assays were performed in microliter scale in the BioLector® microcultivation system (m2p-labs, Aachen, Germany) (Kensy et al., 2009). Main cultures with a volume of 750 µl (see Bacterial strains and cultivation conditions) were incubated in 48-well FlowerPlates® (m2p-labs, Aachen, Germany) at 30°C and 1200 rpm. If needed, Venus fluorescence was measured with an excitation wavelength of 508 nm and emission wavelength of 532 nm (signal gain factor 60). Biomass production was measured as backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor 20). All samples were measured at 15 min intervals. Arbitrary units (a.u.) of specific fluorescence were calculated by dividing the Venus signal by the backscatter

signal per time point (Kensy et al., 2009). Growth rates were determined for the exponential growth phase based on the backscatter signal.

Microtiter cultivation of L-valine production strains

For comparison of the productivity of the L-valine production strains $\Delta aceE$ and $P_{gntK}-aceE$ harbouring different plasmids, per strain, three single colonies from a fresh agar plate were used to inoculate 4 ml BHI supplemented with 85 mM potassium acetate and 25 $\mu\text{g/ml}$ kanamycin. After 10 hours of cultivation at 30 °C and 160 rpm, 2 ml of the first preculture were transferred into 25 ml CGXII minimal medium supplemented with 222 mM glucose, 154 mM potassium acetate and 25 $\mu\text{g/ml}$ kanamycin. Cultivation of the second preculture was performed at 30 °C and 120 rpm for 13 hours. Subsequently, cells were washed with 20 ml 0.9% NaCl solution and used to inoculate 25 ml CGXII containing 222 mM glucose, 154 mM potassium acetate and 25 $\mu\text{g/ml}$ kanamycin. Cultivation was performed in 96 deep-well plates (VWR, Radnor, PA). Therefore, 15 * 1.5 ml aliquots were transferred into the plates and incubated at 30 °C, 900 rpm and 75% humidity in a Microtron incubator shaker (Infors, Bottmingen, Switzerland). Per sample time point and clone, the 1.5 ml culture volume of one well was transferred into a 2 ml reaction tube. Separation of cells and supernatant was performed by centrifugation for 5 min at 5000 rpm and 4 °C. The supernatant was transferred into a fresh 1.5 ml tube and stored at -20 °C before it was used for quantification of L-valine, L-alanine and glucose concentrations.

Cultivation in microfluidic chip devices

For the characterization of reversibility and dynamics of the fluorescence-based GntR-dependent toggle (pJC1- $P_{lys_CS_0-venus-T-P_{gntK}-e2-crimson}$) (Wiechert et al., 2020), *C. glutamicum* wild type cells harbouring the plasmid-based construct were cultivated in an in-house developed microfluidic platform (Grünberger et al., 2012; Grünberger et al., 2015). The experimental setup and chip design was performed as previously described (Grünberger et al., 2013). Venus and E2-Crimson fluorescence as well as phase contrast were imaged at 15 min intervals by fully motorized inverted Nikon Eclipse Ti microscope (Nikon GmbH, Düsseldorf, Germany) as described before (Grünberger et al., 2012; Grünberger et al., 2015; Helfrich et al., 2015). The exposure times for phase contrast was 100 ms, for Venus 200 ms and for E2-Crimson 300 ms. In the microfluidic chip system, cells were cultivated in CGXII medium supplemented with 25 $\mu\text{g/ml}$ kanamycin and either 100 mM gluconate or 100 mM glucose. A high-precision syringe pump system (neMESYS, Cetoni GmbH, Korbussen, Germany) and disposable syringes (Omniflix-F Tuberculin, 1 ml; B. Braun Melsungen AG, Melsungen, Germany) were used to achieve continuous medium supply (flow rate of 200 nl/min) and waste removal during the cultivation. The switches of carbon source supply between gluconate and glucose were performed by changing the syringes and the connecting tubing to ensure an immediate medium switch. The temperature was

set to 30°C using an incubator system (PeCon GmbH, Erbach, Germany). Data analysis on colony level was performed using the image-processing package Fiji (Schindelin et al., 2012) which is based on ImageJ (Rueden et al., 2017). Measured fluorescence data were background normalized and plotted with GraphPad prism 7.00 (GraphPad Software, La Jolla. CA. USA).

Recombinant DNA work

All standard molecular methods such as DNA restriction, PCR and Gibson assembly were performed according to manufacturer instructions or following previously described standard protocols (Gibson et al., 2009; Sambrook and Russel, 2001). Details on plasmid construction by Gibson assembly are provided in Table S4. DNA sequencing and synthesis of oligonucleotides used for amplification of DNA fragments for plasmid construction (Table S5) and sequencing (Table S6 and S7) were performed by Eurofins Genomics (Ebersberg, Germany). Chromosomal DNA of *C. glutamicum* ATCC 13032 used as PCR template was prepared as described previously (Eikmanns et al., 1994). P_{priP} -based counter-silencer promoters were constructed as previously described (Wiechert et al., 2020).

Construction of strains $\Delta gntK$ and $P_{gntK}\text{-}aceE$

For the deletion of the gene *gntK* encoding the gluconate kinase GntK, *C. glutamicum* ATCC 13032 wild type cells (Kinoshita et al., 1957) were transformed with the pK19mobsacB- $\Delta gntK$ deletion plasmid (Table S4). The plasmid pK19-mobsacB- $\Delta P_{aceE}\text{-}aceE$ was used to delete the *aceE* gene and its promoter before the plasmid pK19mobsacB- $P_{gntK}\text{-}aceE$ was used for the re-integration of the *aceE* gene under control of the GntR target promoter P_{gntK} within its native locus. Subsequently, two step homologous recombination and selection was performed as described previously (Niebisch and Bott, 2001). Successful integration was verified by sequencing, all primers are listed in Table S7.

Quantitative Real-time PCR (qRT-PCR)

For the comparison of promoter strength of P_{gntK} , $P_{lys_CS_0}$ and $P_{priP_CS_0}$ with the established promoter systems P_{tet} and P_{tac} on transcript levels, promoter sequences were cloned into the plasmid pJC1 and fused to the reporter gene *venus* via a consistent linker containing the ribosomal binding site (AGGAG (Pfeifer-Sancar et al., 2013)). Since LacI and TetR are natively not encoded in the chromosome of *C. glutamicum*, the corresponding genes were inserted into the respective plasmids. Duplicates of single clones of *C. glutamicum* wild type cells harbouring the plasmid-based constructs or the control plasmid pJC1-*venus*-term (no appropriate promoter in front of *venus* (Baumgart et al., 2013)) were analyzed. The first BHI pre-culture (see Bacterial strains and cultivation conditions) was used to inoculate the second pre-culture in CGXII medium with either 100 mM gluconate or glucose as carbon source (non-inducing conditions). Cells from the second overnight pre-culture were used to inoculate

1.6 ml of the main culture starting with OD₆₀₀ of one. All cultivation was performed in 96 deep-well plates (VWR, Radnor, PA). Cells harbouring different constructs were cultivated under non-inducing and inducing conditions depending on the particular promoter construct: P_{prip_CS_0} and P_{lys_CS_0}: +: 100 mM glucose, -: 100 mM gluconate; P_{gntK}: +: 100 mM gluconate, -: 100 mM glucose; P_{tac}: +: 100 μM IPTG, -: 0 μM IPTG; P_{tet}: +: 235 nM ATc, -: 0 nM ATc. After five hours of incubation, cells were cooled down on ice and harvested by centrifugation (4 °C, 10 min, 11,325g). Cell pellets were snap-frozen in liquid nitrogen and stored at -80 °C until use.

Total RNA was isolated using the Monarch® Total RNA Miniprep Kit (New England Bio Labs, Ipswich, MA, USA) following the manufacturer protocol. qRT-PCR was performed with 5 ng total RNA and primers listed in Table S8 using the Luna® Universal One-Step RT-qPCR Kit (New England Biolabs, Ipswich, USA). Measurements of the relative *venus* expression levels were done in biological and technical duplicates with a qTower 2.2 system (Analytik Jena, Jena, Germany). The chromosomal encoded *ddh* gene served as reference gene. The software qPCR-soft 3.1 (Analytik Jena, Jena, Germany) was used for obtaining the corresponding C_T-values. Means of relative *venus* expression levels and the corresponding range were calculated based on the 2^{Δ(-C_T)} method of Livak and Schmittgen (Livak and Schmittgen, 2001) using the following equations:

$$\Delta C_t = \text{mean CT}(\textit{venus}) - \text{mean CT}(\textit{ddh})$$

$$\text{SD } \Delta C_t = \sqrt{(\text{SD CT}(\textit{venus}))^2 + (\text{SD CT}(\textit{ddh}))^2}$$

$$\text{Relative } \textit{venus} \text{ expression levels} = 2^{-\Delta C_t}$$

Plots in Figure 3C are showing the means of relative *venus* expression levels and error bars their range.

Maximum and minimum of the range was calculated by the following equations:

$$\text{Maximum of range for relative } \textit{venus} \text{ expression levels} = 2^{-(\Delta C_t + \text{SD } \Delta C_t)}$$

$$\text{Minimum of range for relative } \textit{venus} \text{ expression levels} = 2^{-(\Delta C_t - \text{SD } \Delta C_t)}$$

Quantification of amino acid concentrations

The amino acids L-valine and L-alanine were quantified as ortho-phthaldialdehyde derivatives by using ultra-high performance liquid chromatography (uHPLC) or high performance liquid chromatography (HPLC) with an automatic pre-column derivatization. Derivatives were separated by reverse-phase chromatography using an Agilent 1290 Infinity LC ChemStation or an Agilent 1260 Infinity II LC system (Agilent, Santa Clara, USA) equipped with a fluorescence detector. A gradient of sodium borate buffer (10 mM Na₂HPO₄, 10 mM Na₂B₄O₇, pH 8.2) and methanol was applied as eluent for the Zorbax Eclipse AAA 3.5 micron 4.6 x 7.5 mm column (Agilent, Santa Clara, USA). Prior to analysis, samples were centrifuged for 5 min at 16,000 x g and were either measured directly or as dilutions of 1:100, 1:400 or 1:1000 in ddH₂O.

Determination of the glucose concentration in the culture supernatant

Glucose concentrations in the supernatant were measured with the D-Glucose UV-Test Kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions (absorption was measured at 340 nm) with the modification that measurements were performed with only half of the indicated reaction volume. Glucose concentrations were calculated as described in the manufacturer manual.

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3.3. Impact of CO₂/HCO₃⁻ availability on anaplerotic flux in pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum* strains.

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Contributor Role	Contributor
Conceptualization	AK 20 %, JW 30 %, RM 20 %, JF 30 %
Formal Analysis	AK 75 %, RM 20 %, TP 5 %
Investigation/Experiments	AK 80 %, CG 10 %, RM 10 %
Methodology	AK 25 %, JW 25 %, GC 10 %, RM 15 %, JF 25 %
Project Administration	JW 70 %, JF 30 %
Software	TP 100 %
Supervision	JW 70 %, JF 30 %
Visualization	AK 100 %
Writing – Original Draft Preparation	AK 10 %, JW 10 %, JF 80 %
Writing – Reviewing & Editing	AK 20 %, JW 10 %, JF 70 %

Overall contribution JW: 30 %

JW was involved in planning of most of the experimental work. Experiments were mainly conducted by AK during her Master thesis time which was supervised by JW.



Impact of CO₂/HCO₃⁻ Availability on Anaplerotic Flux in Pyruvate Dehydrogenase Complex-Deficient *Corynebacterium glutamicum* Strains

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ABSTRACT The pyruvate dehydrogenase complex (PDHC) catalyzes the oxidative decarboxylation of pyruvate, yielding acetyl coenzyme A (acetyl-CoA) and CO₂. The PDHC-deficient *Corynebacterium glutamicum* $\Delta aceE$ strain therefore lacks an important decarboxylation step in its central metabolism. Additional inactivation of *pyc*, encoding pyruvate carboxylase, resulted in a >15-h lag phase in the presence of glucose, while no growth defect was observed on gluconeogenic substrates, such as acetate. Growth was successfully restored by deletion of *ptsG*, encoding the glucose-specific permease of the phosphotransferase system (PTS), thereby linking the observed phenotype to the increased sensitivity of the $\Delta aceE \Delta pyc$ strain to glucose catabolism. In this work, the $\Delta aceE \Delta pyc$ strain was used to systematically study the impact of perturbations of the intracellular CO₂/HCO₃⁻ pool on growth and anaplerotic flux. Remarkably, all measures leading to enhanced CO₂/HCO₃⁻ levels, such as external addition of HCO₃⁻, increasing the pH, or rerouting metabolic flux via the pentose phosphate pathway, at least partially eliminated the lag phase of the $\Delta aceE \Delta pyc$ strain on glucose medium. In accordance with these results, inactivation of the urease enzyme, lowering the intracellular CO₂/HCO₃⁻ pool, led to an even longer lag phase, accompanied by the excretion of L-valine and L-alanine. Transcriptome analysis, as well as an adaptive laboratory evolution experiment with the $\Delta aceE \Delta pyc$ strain, revealed the reduction of glucose uptake as a key adaptive measure to enhance growth on glucose-acetate mixtures. Taken together, our results highlight the significant impact of the intracellular CO₂/HCO₃⁻ pool on metabolic flux distribution, which becomes especially evident in engineered strains exhibiting low endogenous CO₂ production rates, as exemplified by PDHC-deficient strains.

IMPORTANCE CO₂ is a ubiquitous product of cellular metabolism and an essential substrate for carboxylation reactions. The pyruvate dehydrogenase complex (PDHC) catalyzes a central metabolic reaction contributing to the intracellular CO₂/HCO₃⁻ pool in many organisms. In this study, we used a PDHC-deficient strain of *Corynebacterium glutamicum*, which additionally lacked pyruvate carboxylase ($\Delta aceE \Delta pyc$). This strain featured a >15-h lag phase during growth on glucose-acetate mixtures. We used this strain to systematically assess the impact of alterations in the intracellular CO₂/HCO₃⁻ pool on growth in glucose-acetate medium. Remarkably, all measures enhancing CO₂/HCO₃⁻ levels successfully restored growth. These results emphasize the strong impact of the intracellular CO₂/HCO₃⁻ pool on metabolic flux, especially in strains exhibiting low endogenous CO₂ production rates.

KEYWORDS *Corynebacterium glutamicum*, L-valine, anaplerosis, carbon dioxide, pyruvate carboxylase, pyruvate dehydrogenase complex

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CO_2 is an inevitable product and, at the same time, an essential substrate of microbial metabolism. In water, CO_2 is in equilibrium with bicarbonate (HCO_3^-) and CO_3^{2-} , and this equilibrium is influenced by the pH of the medium (1). As products of decarboxylating reactions and substrates for carboxylation, CO_2 and HCO_3^- are involved in various metabolic processes. Consequently, the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool has a significant impact on metabolic fluxes, such as anaplerosis, especially in the early phase of cultivation, when the cell density is low (2).

During aerobic growth, the pyruvate dehydrogenase complex (PDHC), which is conserved in various microbial species, catalyzes an important reaction contributing to the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool (3, 4). The PDHC is a multienzyme complex belonging to the family of 2-oxo acid dehydrogenase complexes, which also include the α -ketoglutarate dehydrogenase complex and the branched-chain 2-keto acid dehydrogenase (3, 5). In particular, the PDHC catalyzes the oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA) and CO_2 .

The Gram-positive actinobacterium *Corynebacterium glutamicum* represents an important platform strain used in industrial biotechnology for the production of amino acids, proteins, and various other value-added products (6–10). In this organism, the PDHC represents a central target for engineering the metabolic pathways of pyruvate-derived products, including L-valine, isobutanol, ketoisovalerate, and L-lysine. To this end, various studies have focused on the reduction or complete abolishment of PDHC activity in order to improve precursor availability (4, 11–13). Due to the deficiency of PDHC activity, however, cells are not able to grow on glucose as a single carbon source, a difficulty that can be circumvented by the addition of acetyl-CoA refueling substrates, such as acetate. In the presence of both carbon sources—glucose and acetate—PDHC-deficient strains initially form biomass from acetate and subsequently convert glucose into products (e.g., L-valine or L-alanine) in the stationary phase (12). In contrast to *Escherichia coli* or *Bacillus subtilis*, *C. glutamicum* does not normally show the typical diauxic growth behavior but prefers cometabolization of many carbon sources (10, 14). Couitilization of glucose and acetate has been studied in detail, showing that while the consumption rates of both carbon sources decrease, total carbon consumption is comparable to that with growth on either carbon source alone, which is regulated by SugR activity (15). Further examples of sugars or organic acids with which glucose is cometabolized include fructose (16), lactate (17), pyruvate (14), and gluconate (18). It has been shown that upon growth on a mixture of glucose and acetate, the glyoxylate shunt is active and is required to fuel the oxaloacetate pool in wild-type (WT) *C. glutamicum* (15). Here it should be noted that the glyoxylate shunt is active in the presence of acetate but is repressed by glucose (19).

For growth on glycolytic carbon sources, organisms depend on tricarboxylic acid (TCA) cycle-replenishing reactions constituting the anaplerotic node (or phosphoenolpyruvate [PEP]-pyruvate-oxaloacetate node), comprising different carboxylating and decarboxylating reactions (20). Consequently, flux via these reactions is influenced by the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool. In contrast to most other organisms, *C. glutamicum* possesses both anaplerotic carboxylases, namely, the PEP carboxylase (PEPCx) (EC 4.1.1.31) encoded by the *ppc* gene (21, 22) and the pyruvate carboxylase (PCx) (EC 6.4.1.1) encoded by *pyc* (20, 23). These two C_3 -carboxylating enzymes catalyze bicarbonate-dependent reactions yielding oxaloacetate from PEP or pyruvate, respectively. It has been shown that in *C. glutamicum*, these two enzymes can replace each other to a certain extent, depending on the intracellular concentrations of the respective effectors for each enzyme. However, under standard conditions during growth on glucose, the biotin-containing PCx contributes 90% of the main anaplerotic activity, in contrast to PEPCx (23). Remarkably, the Michaelis-Menten constants of both carboxylases are about 30-fold higher than those in *Escherichia coli* PEPCx (24). Apparently, a low $\text{CO}_2/\text{HCO}_3^-$ pool may thus limit anaplerotic flux, which is of special relevance in early phases, when the biomass concentration is low but high aeration may strip dissolved CO_2 from the medium.

Several studies have revealed the inhibitory effect of low partial CO_2 pressure ($p\text{CO}_2$)

on microbial growth (2, 25, 26). In the case of *C. glutamicum*, low $p\text{CO}_2$ led to a significant drop in the growth rate in turbidostatic continuous cultures (27) as well as in batch cultures (28). It was further demonstrated that the reduced flux through anaplerotic reactions under low $\text{CO}_2/\text{HCO}_3^-$ levels led to increased production of the pyruvate-derived amino acids L-alanine and L-valine (28).

While several previous studies have focused on the impact of CO_2 by altering the partial CO_2 pressure in the process, we applied targeted genetic perturbations to systematically assess the impact of the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool on the anaplerotic flux and growth of *C. glutamicum*. In this study, we focused on PDHC-deficient strains exhibiting a reduced intracellular $\text{CO}_2/\text{HCO}_3^-$ pool during growth on glucose-acetate mixtures. The effect of low $\text{CO}_2/\text{HCO}_3^-$ levels became even more evident upon the additional deletion of the *pyc* gene, encoding the dominant anaplerotic enzyme, leading to a drastically elongated lag phase for a *C. glutamicum* $\Delta aceE \Delta pyc$ strain during growth on glucose-acetate mixtures. This effect could be attributed to reduced tolerance of glucose by these strains and was successfully complemented by the deletion of *ptsG*, encoding the glucose-specific permease of the phosphotransferase system (PTS). Remarkably, growth was successfully restored by increasing the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool by the addition of bicarbonate, by increasing the pH, by rerouting metabolic flux over the pentose phosphate pathway (PPP), or by refueling the oxaloacetate pool by the addition of TCA cycle intermediates to the growth medium. Finally, adaptive laboratory evolution (ALE) of the *C. glutamicum* $\Delta aceE \Delta pyc$ strain on minimal medium containing glucose and acetate revealed the abolishment of glucose uptake as a key adaptive strategy to allow for growth on acetate.

RESULTS

A lowered intracellular $\text{CO}_2/\text{HCO}_3^-$ pool impacts the growth of PDHC-deficient strains. The pyruvate dehydrogenase complex (PDHC) contributes to a key metabolic reaction in the central metabolism by catalyzing the oxidative decarboxylation of pyruvate to acetyl-CoA (Fig. 1), thereby producing 1 molecule of CO_2 and 1 molecule of NADH per molecule of pyruvate. Previous studies have already revealed that PDHC-deficient strains feature ~2-fold-decreased excretion of CO_2 during exponential growth (29). It is therefore reasonable to assume that a reduction in the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool has an impact on metabolic flux distribution in PDHC-deficient strains. A simple growth comparison of wild-type *C. glutamicum* and a strain lacking the *aceE* gene (and thus PDHC deficient) revealed a slightly increased lag phase for the $\Delta aceE$ strain, which became especially evident when cells were grown in microtiter plates, while the maximal growth rate appeared to be unaffected ($0.40 \pm 0.01 \text{ h}^{-1}$ for the $\Delta aceE$ strain and $0.41 \pm 0.01 \text{ h}^{-1}$ for the WT) (Fig. 2A). Remarkably, this lag phase was completely eliminated by the addition of 100 mM HCO_3^- , suggesting that the effect is attributable to the lowered intracellular $\text{CO}_2/\text{HCO}_3^-$ pool of this mutant (Fig. 2B).

In *C. glutamicum*, the pyruvate carboxylase (PCx) encoded by *pyc* is the dominating HCO_3^- -dependent anaplerotic enzyme required for refueling the TCA cycle via oxaloacetate during growth on glycolytic carbon sources (23). In the next experiment, we deleted *pyc* in the $\Delta aceE$ background in order to examine the influence of different $\text{CO}_2/\text{HCO}_3^-$ levels on bicarbonate-dependent anaplerosis. Remarkably, the additional deletion of the *pyc* gene resulted in an elongated lag phase of about 20 h and a reduced growth rate for the $\Delta aceE \Delta pyc$ strain (maximum growth rate [μ_{max}], $0.27 \pm 0.02 \text{ h}^{-1}$, in contrast to $0.40 \pm 0.01 \text{ h}^{-1}$ for the $\Delta aceE$ strain) during cultivation in CGXII minimal medium containing glucose and acetate (Fig. 2A). An extended lag phase was also observed for the Δpyc single mutant, but the lag phase was significantly less prominent than that for the PDHC-deficient $\Delta aceE \Delta pyc$ variant (Fig. 2C). The growth defects of both strains were successfully complemented by reintroducing the *pyc* gene on plasmid pJC1 under the control of its own promoter (Fig. 2C). Remarkably, this extended lag phase was observed only in the presence of glucose, not during cultivation on minimal medium containing acetate as the sole carbon source (Fig. 2A).

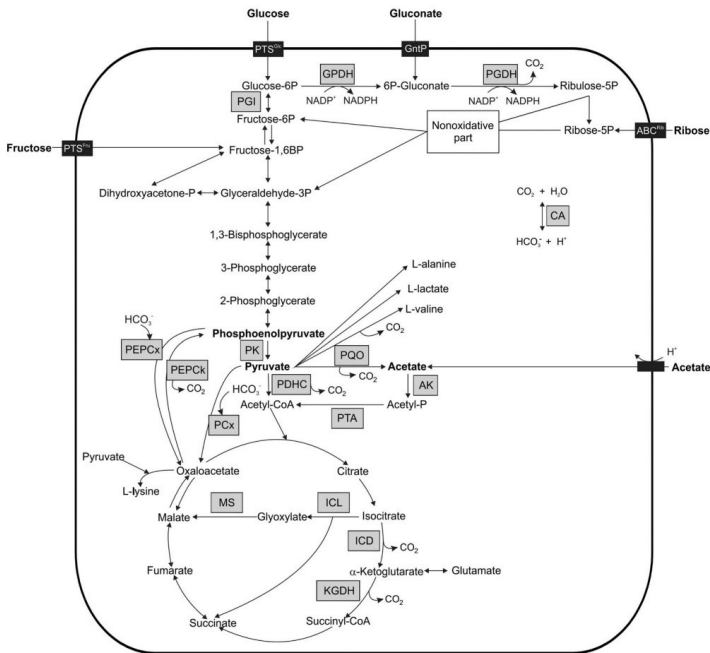


FIG 1 Schematic overview of the central carbon metabolism of *C. glutamicum*. The main aspects of glycolysis, gluconeogenesis, the pentose phosphate pathway, the TCA cycle, and anaplerosis are shown. Shaded boxes represent relevant enzymes. Carboxylation as well as decarboxylation steps are given. ABC^{Rib}, ATP-binding cassette transporter for ribose; AK, acetate kinase; CA, carbonic anhydrase; GntP, gluconate permease; GPDH, glucose-6P dehydrogenase; ICD, isocitrate dehydrogenase; ICL, isocitrate lyase; KGDH, α -ketoglutarate dehydrogenase complex; MS, malate synthase; PCx, pyruvate carboxylase; PDHC, pyruvate dehydrogenase complex; PEPCK, PEP carboxylase; PEPCK, PEP carboxylase; PGDH, 6-phosphogluconate dehydrogenase; PGI, phosphoglucose isomerase; PK, pyruvate kinase; PGO, pyruvate:quinone oxidoreductase; PTA, phosphotransacetylase; PTS^{Glc}, permease of phosphotransferase system for glucose; PTS ^{Fru}, permease of phosphotransferase system for fructose.

Glucose uptake results in strongly retarded growth in strains lacking pyruvate carboxylase. In order to determine whether glucose uptake caused the retarded growth, the $\Delta aceE \Delta pyc$ strain was cultivated in the presence of 154 mM acetate and increasing amounts of glucose (100 to 250 mM). In line with our hypothesis, the lag phase showed a stepwise increase as larger amounts of glucose were added to the medium (Fig. 3A). Accordingly, the $\Delta aceE \Delta pyc$ strain also showed significantly elongated lag phases on the PTS sugars fructose and sucrose (see Fig. S1 in the supplemental material). In contrast, the glucose concentration did not significantly affect the growth of the $\Delta aceE$ strain (Fig. 3A), in which the major anaplerotic enzyme PCx still replenishes the oxaloacetate pool.

To link the observed growth phenotype to the uptake of glucose, we deleted the *ptsG* gene, encoding the glucose-specific permease of the PTS, in the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strain backgrounds. The growth and glucose consumption rates of the resulting strains were analyzed during growth on glucose and acetate. Remarkably, deletion of *ptsG* fully restored the growth of the $\Delta aceE \Delta pyc$ strain (growth rates were 0.27 ± 0.02

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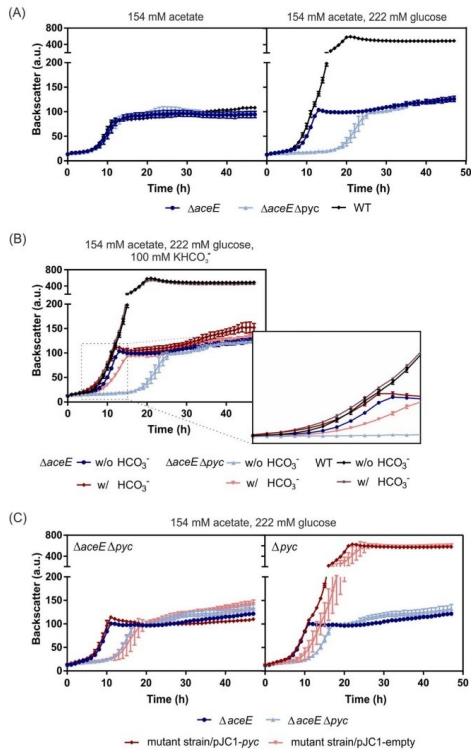


FIG 2 Impact of glucose on *aceE* and *pyc* mutant strains. The growth curves shown are based on the backscatter measurements (expressed in arbitrary units [a.u.]) in a microtiter cultivation system inoculated at an OD_{600} of 1. Data represent the averages for three biological replicates; error bars, standard deviations. (A) The *C. glutamicum* $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains, as well as the wild type, were inoculated in CGXII medium containing 154 mM acetate (left) or 154 mM acetate plus 222 mM glucose (right). (B) The $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains, as well as WT cells, were cultivated in CGXII medium containing 154 mM acetate and 222 mM glucose, either with (w/) or without (w/o) 100 mM KHCO_3^- . The box zooms in on the time interval between 4 h and 15 h. (C) The $\Delta aceE \Delta pyc$ (left) and Δpyc (right) strains were transformed with the pJC1-*pyc* plasmid for complementation of the *pyc* deletion or with the pJC1-empty vector as a control. Cultures were inoculated in CGXII medium containing 154 mM acetate, 222 mM glucose, and 25 $\mu\text{g}/\text{ml}$ kanamycin.

h^{-1} for the $\Delta aceE \Delta pyc$ parental strain and $0.39 \pm 0.02 \text{ h}^{-1}$ for the $\Delta aceE \Delta pyc \Delta ptsG$ strain) but resulted in reduced final backscatter values, comparable to those with growth on acetate alone (Fig. 3B). Strains lacking the *ptsG* gene consumed only minor amounts of glucose, while the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains consumed glucose after entering the stationary phase (see Fig. S2 in the supplemental material). The glucose uptake rate of the $\Delta aceE \Delta pyc$ strain, $10.87 \pm 4.95 \text{ nmol min}^{-1} \text{ g}^{-1}$, was significantly lower than that of the $\Delta aceE$ strain, $16.81 \pm 3.01 \text{ nmol min}^{-1} \text{ g}^{-1}$ (Table 1). In contrast, deletion of *ptsG* did not restore growth on either the PTS substrate fructose (see Fig.

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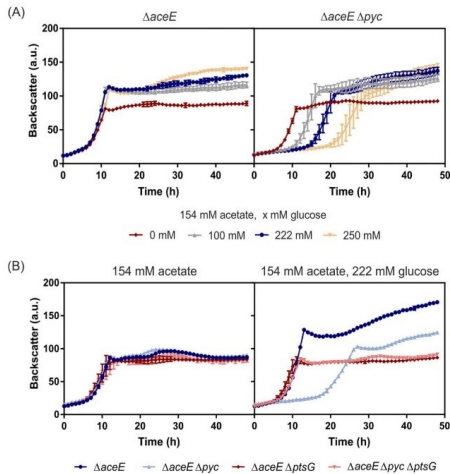


FIG 3 Influence of glucose consumption on the growth of *C. glutamicum* $\Delta aceE$ and *C. glutamicum* $\Delta aceE \Delta pyc$. The growth curves shown are based on the backscatter measurements in a microtiter cultivation system, inoculated at an OD_{600} of 1. (A) The $\Delta aceE$ (left) and $\Delta aceE \Delta pyc$ (right) strains were inoculated to an OD_{600} of 1 in CGXII medium containing 154 mM acetate and either no glucose or 100 mM, 222 mM, or 250 mM glucose. (B) Deletion of the *ptsG* gene in the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains restored growth on glucose-acetate mixtures relative to that for the $\Delta aceE$ and $\Delta aceE \Delta pyc$ parental strains. Data represent the averages for three biological replicates; error bars, standard deviations.

S3), which is imported mainly by the permease encoded by *ptsF* (30, 31), or the non-PTS sugar ribose (see Fig. S4). Taken together, these findings clearly link the observed growth defect of the $\Delta aceE \Delta pyc$ strain on glucose-acetate mixtures to the uptake of residual amounts of glucose.

Increased HCO_3^- availability improves anaplerotic flux in *pyc* mutants. Next, we used different strategies to alter the intracellular CO_2/HCO_3^- pool. This was achieved by (i) supplementing the medium with HCO_3^- , (ii) mutating the endogenous urease gene producing CO_2 from urea in the early growth phase, (iii) increasing the pH of the growth medium, or (iv) rerouting metabolic flux via the pentose phosphate pathway (Fig. 4).

As with the $\Delta aceE$ parental strain, the addition of 100 mM HCO_3^- eliminated the lag phase for PDHC-deficient strains lacking the *pyc* gene (Fig. 2B) during microtiter plate cultivation on glucose-acetate mixtures. Restoration of growth was not possible in a

TABLE 1 Glucose consumption rates of PDHC-deficient *C. glutamicum* strains in the exponential-growth phase^a

Mutation(s)	Glucose consumption rate (nmol min ⁻¹ g ⁻¹) ^b
$\Delta aceE$	16.81 ± 3.01
$\Delta aceE \Delta pyc$	10.87 ± 4.95
$\Delta aceE \Delta ptsG$	7.24 ± 3.97
$\Delta aceE \Delta pyc \Delta ptsG$	7.32 ± 1.88

^aFor an overview of glucose consumption over the entire time course of the experiment, including the stationary phase, see Fig. S2 in the supplemental material.

^bValues are means ± standard deviations for three independent biological replicates.

Influence of $\text{CO}_2/\text{HCO}_3^-$ on Anaplerotic Flux

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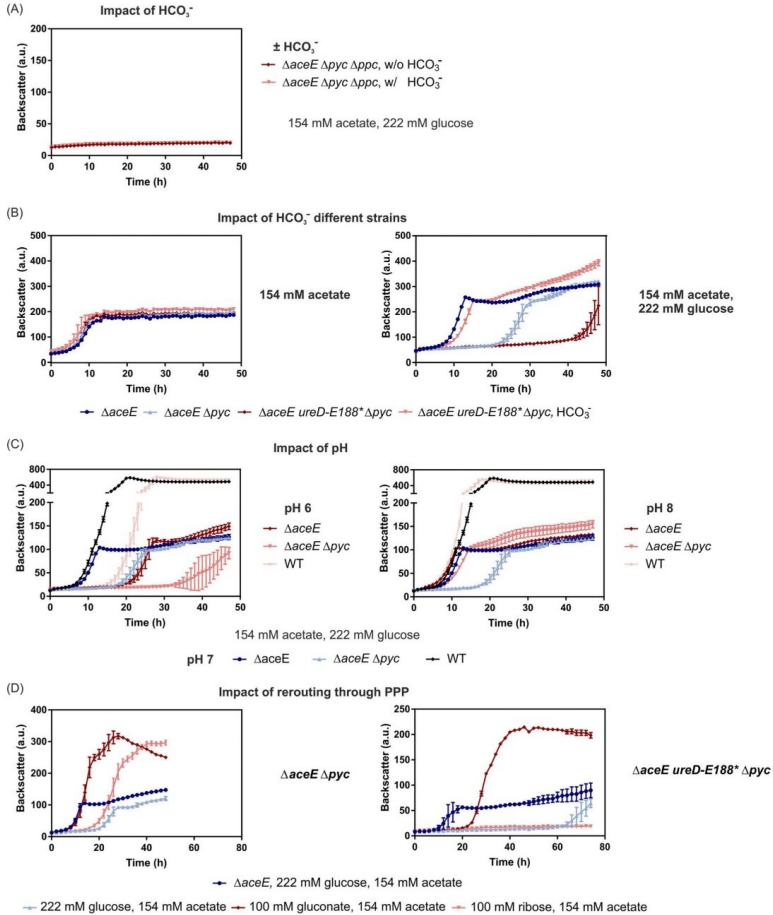


FIG 4 Increased availability of HCO_3^- improved anaplerotic flux via PEPcX in *pyc*-deficient *C. glutamicum* strains. The growth curves shown are based on the backscatter measurements in a microtiter cultivation system, inoculated at an OD_{600} of 1. Symbols represent the backscatter means for biological triplicates ($n = 3$). (A) The $\Delta aceE \Delta pyc \Delta ppc$ strain was inoculated in CGXII medium containing 154 mM acetate and 222 mM glucose (w/o HCO_3^-), and if indicated, 100 mM KHCO_3 was added (w/ HCO_3^-). (B) The $\Delta aceE$, $\Delta aceE \Delta pyc$, and $\Delta aceE ureD-E188^* \Delta pyc$ strains were cultivated in CGXII medium containing 154 mM acetate (left) or 154 mM acetate and 222 mM glucose (right) with or without the addition of KHCO_3 . (C) The $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains, as well as the WT, were cultivated in CGXII medium containing 154 mM acetate and 222 mM glucose adjusted to a pH of 6 (left) or 8 (right), and growth was compared to that with cultivation at pH 7. (D) The $\Delta aceE$ strain and the $\Delta aceE \Delta pyc$ (left) or $\Delta aceE ureD-E188^* \Delta pyc$ (right) strain were inoculated in different growth media containing 154 mM acetate and either 222 mM glucose, 100 mM gluconate, or 100 mM ribose.

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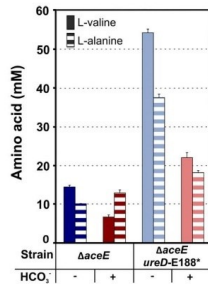


FIG 5 The intracellular CO₂/HCO₃⁻ pool significantly affects L-valine production. The influence of carbonate on the L-alanine and L-valine production of *C. glutamicum* Δ*aceE* and *C. glutamicum* Δ*aceE ureD-E188** was investigated. Shown are L-valine and L-alanine titers in the supernatant after 28 h of cultivation in the microtiter cultivation system. Data represent average values from three independent biological replicates using uHPLC.

strain lacking both anaplerotic enzymes PCx and PEPcX, indicating that the positive effect of HCO₃⁻ on the Δ*aceE* Δ*pyc* strain depends on the increased flux over PEPcX (Fig. 4A; see also Fig. S5 in the supplemental material). In contrast to the finding for PCx-deficient strains, no negative impact of glucose on the growth of the Δ*aceE* Δ*pyc* strain was observed (see Fig. S6), confirming again the superior role of PCx in *C. glutamicum* (23, 32). Interestingly, we also observed a significant impact of the culture volume on the growth of the Δ*aceE* Δ*pyc* strain. Since efficient liquid-air mixing continuously removes CO₂ from the culture medium, the lag phase of the Δ*aceE* Δ*pyc* strain was much more prominent during microtiter plate cultivation (~17 h) than in cultures in shake flasks (a ~7-h lag phase in 100-ml cultures and a ~13-h lag phase in 50 ml) (see Fig. S7).

The urease enzyme, catalyzing the degradation of urea to ammonium and carbon dioxide, represents a further important contributor to the intracellular CO₂/HCO₃⁻ pool, especially in the early exponential phase, when cell densities and decarboxylation reaction rates are low. In a previous study, the *ureD-E188** mutation was found to abolish urease activity in *C. glutamicum* and to enhance L-valine production by PDHC-deficient strains (33). In this study, we introduced the *ureD-E188** mutation into the Δ*aceE* Δ*pyc* background so as to examine the influence of an even lower CO₂/HCO₃⁻ level on anaplerosis in this strain background. The additional inactivation of the urease enzyme resulted in a significantly elongated lag phase of about 46 h, which could also be complemented by the addition of HCO₃⁻ (Fig. 4B), demonstrating the importance of the CO₂/HCO₃⁻ pool during the initial growth phase. Furthermore, we assessed the impact of urease mutation on the production of L-valine and L-alanine by the Δ*aceE* and Δ*aceE ureD-E188** strains and the effect of complementation via HCO₃⁻ addition. Within 28 h of cultivation, the Δ*aceE* strain produced 15 mM L-valine and 10 mM L-alanine as by-products, while the Δ*aceE ureD-E188** strain accumulated 54 mM L-valine and 38 mM L-alanine (Fig. 5). The addition of HCO₃⁻, however, significantly reduced L-valine production, by 54% for the Δ*aceE* strain and 59% for the Δ*aceE ureD-E188** strain. The concentration of the by-product L-alanine increased by 22% for the Δ*aceE* strain and decreased by 51% for the Δ*aceE ureD-E188** strain (Fig. 5). The examples of L-valine and L-alanine production illustrate the important role of the intracellular CO₂/HCO₃⁻ levels on metabolic flux in PDHC-deficient strains.

Another approach to influencing the extracellular HCO₃⁻ availability was to change the pH conditions (2). According to the Bjerrum plot, CO₂ is predominant under acidic conditions, while CO₃²⁻ is the dominant form under alkaline conditions. At a pH of

about 8, HCO_3^- is the prevalent form. Since HCO_3^- is the substrate of the anaplerotic enzymes PCx and PEPCx, the pH in the cultivation medium was adjusted to 8 using KOH, leading to an equilibrium shift toward higher HCO_3^- availability. As a negative control, the pH was adjusted to 6 using HCl. While the growth of both strains tested was retarded at pH 6, it was significantly enhanced at pH 8 (Fig. 4C). Thus, elevation of the culture pH improved the growth of PDHC-deficient strains, likely by increasing anaplerotic flux.

The PPP includes another decarboxylation reaction in the central carbon metabolism, the reaction catalyzed by 6-phosphogluconate dehydrogenase (6PGDH), that contributes to the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool. Remarkably, the lag phases of the $\Delta aceE \Delta pyc$ and $\Delta aceE ureD-E188^* \Delta pyc$ strains were mostly complemented during growth on acetate and gluconate—the latter entering the PPP via gluconate-6-phosphate (Fig. 4D). Growth on ribose, which enters the PPP via ribulose-5-phosphate and thereby bypasses the decarboxylation catalyzed by 6PGDH, showed a significantly elongated lag phase in *pyc*-deficient strains (Fig. 4D). In a further experiment, metabolic/glycolytic flux was rerouted through the PPP by deleting *pgi*, encoding glucose-6-phosphate isomerase. However, deletion of *pgi* resulted in a severe growth defect in the $\Delta aceE$ background. In this context, the growth of the $\Delta aceE \Delta pyc$ strain featured only minor, nonsignificant improvement (see Fig. S8 in the supplemental material).

Taken together, our findings highlight the important impact of the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool on metabolic flux in the central carbon metabolism. This is especially evident in the $\Delta aceE \Delta pyc$ strain, which lacks a central decarboxylation reaction and the key carboxylase PCx in *C. glutamicum*.

Refueling the TCA cycle improves the growth of *pyc* mutants. Glucose catabolism requires sufficient anaplerotic flux to replenish TCA cycle intermediates, providing precursors for various anabolic pathways. Therefore, we tested whether the addition of TCA cycle intermediates would complement the negative impact of glucose on the growth of the $\Delta aceE \Delta pyc$ strain. Remarkably, all TCA cycle intermediates tested (succinate, malate, citrate, and the glutamate-containing dipeptide Glu-Ala) reduced the extended lag phase of the $\Delta aceE \Delta pyc$ strain during growth on glucose-acetate mixtures (Fig. 6A). The value of Δt was defined to give the percentage change of lag phase time compared to the first doubling time of the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains on glucose-acetate mixtures (100%). The dipeptide Glu-Ala and succinate reduced the elongated lag phase to high extents, by 90% ($\Delta t = 10\%$) and 85% ($\Delta t = 15\%$), respectively, while the effects of citrate and malate were weaker (Δt , 40% and 38%, respectively) (Table 2).

Based on previous studies, it was known that the glyoxylate shunt might be switched off during growth on glucose-acetate mixtures, due to the inhibitory effect of glucose (34). In this study, overexpression of the *aceA* and *aceB* genes, encoding the glyoxylate shunt enzymes isocitrate lyase and malate synthase, resulted in a significantly shorter lag phase for the $\Delta aceE \Delta pyc$ strain (Fig. 6B). These genes were overexpressed by use of the pJC1 vector harboring an inducible P_{tac} promoter in front of either of two synthetic operon variants (pJC1- P_{tac} -*aceA-aceB* and pJC1- P_{tac} -*aceB-aceA*). However, it must be noted that for the $\Delta aceE$ strain, overexpression of *aceA* and *aceB* led to lag phases slightly longer than those with the empty-vector control (Fig. 6B). Expression from the leaky P_{tac} promoter yielded the best result, while induction with isopropyl- β -D-thiogalactopyranoside (IPTG) resulted in severe growth defects (data not shown).

Unusual intermediate accumulation or depletion can provide valuable information regarding intracellular flux imbalances causing the observed growth defects. Gas chromatography–time of flight (GC-ToF) experiments were performed for analysis of the metabolic states by comparing samples of the $\Delta aceE$ strain during the early-exponential phase, the $\Delta aceE \Delta pyc$ strain at the same time point during the lag phase, and the $\Delta aceE \Delta pyc$ strain during the early exponential phase (Fig. S9 in the supplemental material shows the sampling scheme). Additionally, samples of both PDHC-

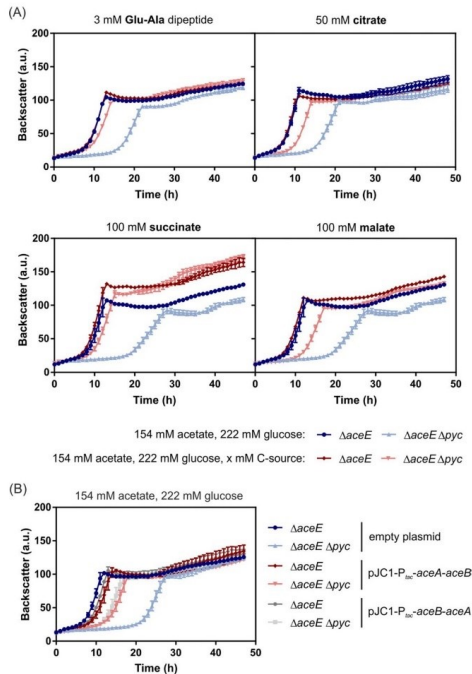


FIG 6 Refueling the TCA cycle to complement the glucose intolerance of the $\Delta aceE \Delta pyc$ strain. The growth curves shown are based on the backscatter measurements in a microtiter cultivation system, while symbols represent the backscatter means for biological triplicates ($n = 3$). The $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains were inoculated to an OD_{600} of 1 into CGXII medium with 154 mM acetate and 222 mM glucose. (A) The TCA cycle-fueling carbon source glutamate (3 mM Glu-Ala dipeptide), citrate (50 mM), succinate (100 mM), or malate (100 mM) was added to the medium in order to analyze the effect on lag phase complementation (shades of red). As a control, growth on 154 mM acetate and 222 mM glucose is shown for the $\Delta aceE$ (dark blue) and $\Delta aceE \Delta pyc$ (light blue) strains in each experiment. (B) The $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains were transformed with the pJC1- P_{aceA} - $aceA$ - $aceB$ or pJC1- P_{aceB} - $aceB$ - $aceA$ plasmid for simultaneous overexpression of the glyoxylate shunt enzymes isocitrate lyase (encoded by $aceA$) and malate synthase (encoded by $aceB$), while the $\Delta aceE \Delta pyc$ and Δpyc strains transformed with the empty pJC1 vector served as controls. Cultures were inoculated to an OD_{600} of 1 in CGXII medium containing 154 mM acetate, 222 mM glucose, and 25 $\mu\text{g}/\text{ml}$ kanamycin.

deficient strains cultivated with 100 mM HCO_3^- were measured, and WT cells in exponential phase were used as a reference (see Table S1). No significant intermediate accumulation and no occurrence of unusual compounds was observed in the lag-phase sample of the $\Delta aceE \Delta pyc$ strain relative to all other samples. Although oxaloacetate cannot be measured by this method, complete depletion of oxaloacetate-derived aspartate was detected in $\Delta aceE \Delta pyc$ cells during the lag phase but not during the exponential phase or in HCO_3^- -complemented samples. This result supports the hypothesis that oxaloacetate depletion is the key reason for the elongated lag phase.

In order to identify oxaloacetate depletion as a key growth-limiting factor and to decouple this effect from anaplerosis-dependent replenishment, the Δpyc , $\Delta pyc \Delta ppc$,

TABLE 2 Overview of the lag phases and growth rates of the *C. glutamicum* $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains cultivated with different TCA cycle carbon sources^a

Added carbon source (concn [mM])	<i>C. glutamicum</i> $\Delta aceE$			<i>C. glutamicum</i> $\Delta aceE \Delta pyc$		
	Lag phase ^b	Δt (%) ^c	Growth rate (h ⁻¹)	Lag phase ^b	Δt (%) ^c	Growth rate (h ⁻¹)
None	–	0	0.41 ± 0.01	+	100	0.27 ± 0.02
Glu-Ala (3)	–	0	0.35 ± 0.01	±	10	0.33 ± 0.01
Citrate (50)	–	0	0.39 ± 0.01	±	40	0.35 ± 0.01
Succinate (100)	–	–8	0.36 ± 0.01	±	15	0.34 ± 0.02
Malate (100)	–	–8	0.35 ± 0.01	±	38	0.35 ± 0.02

^aThe strains were cultivated in CGXII medium containing 154 mM acetate and 222 mM glucose with or without a TCA cycle carbon source.

^b+, lag phase greater than or equal to the lag phase observed for the strain on 154 mM acetate with 222 mM glucose; ±, shorter lag phase; –, no lag phase.

^cThe difference between the first doubling time of the $\Delta aceE$ strain and that of the $\Delta aceE \Delta pyc$ strain was defined as 100%.

and $\Delta aceE \Delta pyc \Delta ppc$ strains were tested in the presence of different TCA cycle intermediates. Here, mutants lacking both anaplerotic reactions (PCx and PEPcX)—the $\Delta pyc \Delta ppc$ and $\Delta aceE \Delta pyc \Delta ppc$ strains—were not able to grow on glucose-acetate mixtures at all (Fig. 4A; also Fig. S5 in the supplemental material). Again, all TCA cycle intermediates were able to complement the glucose sensitivity of these *pyc*-deficient strains, and the growth of the $\Delta pyc \Delta ppc$ and $\Delta aceE \Delta pyc \Delta ppc$ strains was effectively restored (Table 3). Nevertheless, slight differences were observed, which might also be caused by differences in the uptake of these carbon sources.

Adaptation of *C. glutamicum* $\Delta aceE \Delta pyc$ to growth on glucose and acetate. In a previous study, Kotte et al. reported on an elongated lag phase of *E. coli* as a result of glucose-gluconeogenic substrate shifts (35). This phenomenon was ascribed to the formation of a small subpopulation that is able to start growing after carbon source switches (35). Usually, the growth of a small subpopulation is obscured by typical bulk measurements but can be visualized by single-cell approaches, such as flow cytometry (FC). To this end, the membranes of $\Delta aceE$ and $\Delta aceE \Delta pyc$ cells were stained using the nontoxic green fluorescent dye PKH67 prior to inoculation. During cellular growth, the amount of fluorescent dye is diluted by membrane synthesis, leading to a decrease in the level of single-cell PKH67 fluorescence (35). Stained $\Delta aceE$ and $\Delta aceE \Delta pyc$ cells were cultivated in minimal medium containing glucose and acetate (Fig. 7A). As a nonproliferating control, $\Delta aceE \Delta pyc$ cells were incubated in glucose as the sole carbon source. Using flow cytometry, membrane staining was analyzed during cultivation at the single-cell level (Fig. 7B). While the mean of the fluorescence of the whole $\Delta aceE$ population decreased from 2.7×10^4 arbitrary units (AU) to 7.5×10^2 AU, the mean of the fluorescence of the $\Delta aceE \Delta pyc$ population incubated in glucose medium alone shifted from 3.1×10^4 AU to 1.3×10^3 AU, resulting from a minor dilution/bleaching of fluorescence intensity during incubation. Remarkably, both the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains featured rather heterogeneous adaptation behavior on glucose-acetate mixtures, which was apparently delayed in the $\Delta aceE \Delta pyc$ strain, reflecting the elongated

TABLE 3 Overview of the lag phases and growth rates of the *C. glutamicum* Δpyc , $\Delta pyc \Delta ppc$, and $\Delta aceE \Delta pyc \Delta ppc$ strains cultivated with different TCA cycle carbon sources^a

Added carbon source (concn [mM])	Result for the <i>C. glutamicum</i> strain with the following mutation(s):					
	Δpyc		$\Delta pyc \Delta ppc$		$\Delta aceE \Delta pyc \Delta ppc$	
	Δt_{lag} (h) ^b	Growth rate (h ⁻¹) ^c	Δt_{lag} (h)	Growth rate (h ⁻¹)	Δt_{lag} (h)	Growth rate (h ⁻¹)
None	6 h	0.17 ± 0.02	No growth		No growth	
Glu-Ala (3)	ND	ND	8	0.21 ± 0.01	30	0.14 ± 0.01
Citrate (50)	3	0.19 ± 0.01	5	0.26 ± 0.01	8	0.20 ± 0.03
Succinate (100)	2	0.2 ± 0.02	3	0.16 ± 0.03	5	0.12 ± 0.03
Malate (100)	3	0.21 ± 0.01	17	0.18 ± 0.01	18	0.24 ± 0.01

^aStrains were cultivated in CGXII medium containing 154 mM acetate and 222 mM glucose with or without a TCA cycle carbon source.

^bDifference in lag phase duration between the $\Delta aceE$ strain growing on a glucose-acetate mixture and the strain of interest growing on the respective carbon source(s). ND, not determined.

^cFor comparison, the growth rate in a glucose-acetate mixture was 0.41 h⁻¹ for the $\Delta aceE$ strain and 0.28 h⁻¹ for the $\Delta aceE \Delta pyc$ strain in this experiment.

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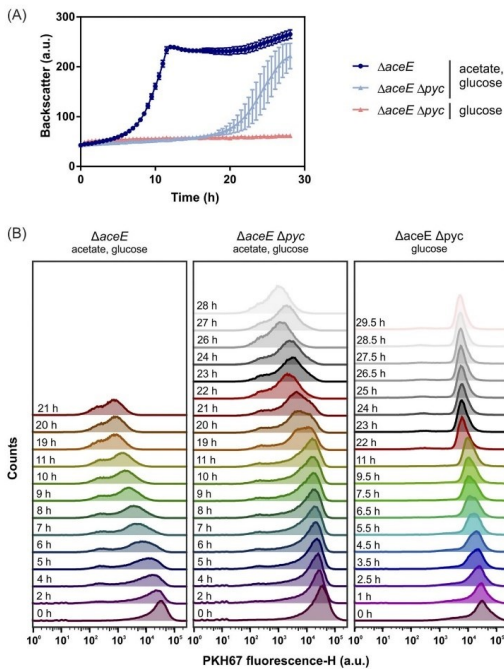


FIG 7 Heterogeneous adaptation of *C. glutamicum* $\Delta aceE$ and *C. glutamicum* $\Delta aceE \Delta pyc$ during growth on glucose and acetate. To identify a potential growing subpopulation, the fluorescent dye PKH67 was used to stain the membranes of $\Delta aceE$ and $\Delta aceE \Delta pyc$ cells prior to cultivation. The cells were cultivated in a microtiter cultivation system in the presence of 154 mM acetate and 222 mM glucose. As a nongrowing control, the $\Delta aceE \Delta pyc$ strain was additionally incubated in CGXII medium containing solely 222 mM glucose. (A) Cell growth was monitored online using a microtiter cultivation system. (B) Staining intensities were measured on the single-cell level during cultivation using flow cytometry (with three biological replicates). Shown are the frequencies of PKH67 staining intensities of one representative culture of each sample over the time course of cultivation. H, height; Counts, cell count.

lag phase. Already in the early phase of cultivation, the populations split into two subpopulations of low and high PKH67 fluorescence, indicating that only a few cells started to proliferate, while a fraction of the population was not able to grow under the same conditions (36–38).

To study the adaptation of the $\Delta aceE \Delta pyc$ strain, comparative transcriptome analyses were performed using DNA microarrays. To this end, $\Delta aceE$ and $\Delta aceE \Delta pyc$ cells were harvested at comparable optical densities (ODs) during early exponential phase (see Fig. S9 in the supplemental material). Both strains were cultivated in CGXII medium with 154 mM acetate and 222 mM glucose. Under the conditions chosen, a total of 354 genes showed significantly altered mRNA levels. While 121 genes were at least 1.7-fold upregulated ($P < 0.05$), 233 genes were 0.7-fold downregulated ($P < 0.05$) in the $\Delta aceE \Delta pyc$ strain (see Table S2). An overview of expression changes of selected genes is shown in Table 4. Among the various changes in the levels of gene expression,

TABLE 4 Comparative transcriptome analysis of *C. glutamicum* $\Delta aceE \Delta pyc$ and *C. glutamicum* $\Delta aceE$ during growth on glucose and acetate^a

Function and gene locus	Gene name	Annotation	mRNA ratio	P
PTS component				
cg1537	<i>ptsG</i>	Glucose-specific enzyme IIBC component of PTS	0.59	0.02
cg2117	<i>ptsI</i>	PEP:sugar phosphotransferase system enzyme I	0.67	0.01
cg2121	<i>ptsH</i>	Phosphocarrier protein HPr of PTS	1.16	0.11
myo-Inositol transport				
cg0223	<i>iolT1</i>	myo-Inositol transporter	1.97	0.04
cg3387	<i>iolT2</i>	myo-Inositol transporter	1.41	0.19
Transcriptional regulation of <i>ptsG</i>				
cg2783	<i>gntR1</i>	Transcriptional regulator, GntR family	0.76	0.05
cg1935	<i>gntR2</i>	Transcriptional regulator, GntR family	0.97	0.46
cg2115	<i>sugR</i>	DeoR-type transcriptional regulator of <i>ptsG</i> , <i>ptsS</i> , and <i>ptsF</i>	0.79	0.03
Acetate metabolism				
cg3047	<i>ackA</i>	Acetate/propionate kinase	1.99	0.01
cg3048	<i>pta</i>	Phosphate acetyltransferase	1.34	0.04
cg2560	<i>aceA</i>	Isocitrate lyase	1.70	0.12
cg2559	<i>aceB</i>	Malate synthase	1.88	0.02
Anaplerosis				
cg1787	<i>ppc</i>	Phosphoenolpyruvate carboxylase	0.91	0.32
cg3169	<i>pck</i>	Phosphoenolpyruvate carboxykinase (GTP)	0.72	0.03
cg1458	<i>odx</i>	Oxaloacetate decarboxylase	1.07	0.32
cg3335	<i>malE</i>	Malic enzyme	<u>0.48</u>	0.26
Other functions in the central carbon metabolism				
cg0973	<i>pgi</i>	Glucose-6-phosphate isomerase	<u>0.44</u>	0.03
cg2291	<i>pyk</i>	Pyruvate kinase	<u>0.59</u>	0.01
cg2891	<i>pqo</i>	Pyruvate:quinone oxidoreductase	<u>0.47</u>	0.02

^aThe expression of selected genes encoding central metabolic enzymes or regulators is given as the ratio of the mRNA level in the $\Delta aceE \Delta pyc$ strain to that in the $\Delta aceE$ strain. mRNA ratios of ≤ 0.70 are underlined, and mRNA ratios of ≥ 1.70 are shown in boldface. P values of > 0.05 (from three biological replicates) are italicized. For the complete data set, see Table S4 in the supplemental material.

we observed 0.59-fold and 0.67-fold downregulation of the PTS components *ptsG* and *ptsI*, respectively, in the $\Delta aceE \Delta pyc$ strain, in line with the decreased glucose uptake rates of this strain (Table 1). In contrast, *iolT1*, encoding the myo-inositol transporter, which is responsible, *inter alia*, for PTS-independent glucose uptake, was upregulated (39, 40). This is in line with the fact that the *ptsG*-deficient strains still consumed minor amounts of glucose. Interestingly, genes involved in acetate metabolism, including *aceA* and *aceB*, constituting the glyoxylate shunt, displayed increased mRNA levels in the $\Delta aceE \Delta pyc$ strain. This may also represent an important adaptive mechanism, since the glyoxylate shunt needs to be active in the $\Delta aceE \Delta pyc$ strain to compensate for the loss of TCA cycle intermediates during coconsumption of acetate and glucose, as demonstrated by the effect of *aceA-aceB* overexpression in this study (Fig. 6B). While the mRNA levels of genes encoding anaplerotic enzymes did not show significant changes, the glycolytic genes *pgi* and *pyk* featured ~2-fold-reduced levels in the $\Delta aceE \Delta pyc$ mutant. This might well reflect an adaptive mechanism, since downregulation of *pyk* would reduce flux from PEP to pyruvate, and reduced expression of *pgi* would probably lead to increased flux through the PPP, contributing an additional decarboxylation step.

Less is more: ALE of the $\Delta aceE \Delta pyc$ strain reveals rapid inactivation of glucose uptake. In order to identify key mutations abolishing the lag phase of the $\Delta aceE \Delta pyc$ strain, an adaptive laboratory evolution (ALE) experiment was performed. In this ALE experiment, *C. glutamicum* $\Delta aceE \Delta pyc$ was grown in CGXII minimal medium containing 154 mM acetate and 222 mM glucose in 16 repetitive-batch cultures overall. Remarkably, after 8 inoculations, a significantly shortened lag phase was already observed; after 10 inoculations, the $\Delta aceE \Delta pyc$ strain featured a lag phase similar to that of the

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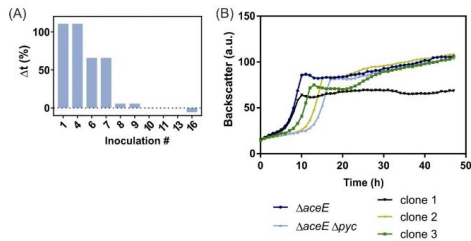


FIG 8 Adaptive laboratory evolution of *C. glutamicum* $\Delta aceE \Delta pyc$. Growth was analyzed for single inoculation steps obtained from the adaptive laboratory evolution (ALE) approach, which was performed with the $\Delta aceE \Delta pyc$ strain in the presence of glucose and acetate. Growth curves are shown based on the backscatter measurements in a microtiter cultivation system. Symbols represent the backscatter means. (A) For growth analysis on the population level, glycerol stocks prepared during the ALE experiment were used for the inoculation of a first preculture in BHI medium supplemented with 51 mM acetate. The second preculture, in CGXII medium containing 154 mM acetate, was then used for inoculation of the main culture in CGXII medium containing 154 mM acetate and 222 mM glucose (starting $OD_{600} = 1$). Shown is the development of the relative lag phase duration after repetitive inoculations of the $\Delta aceE \Delta pyc$ strain in media containing glucose and acetate. The difference in the first doubling time between the $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains was set to 100%. For each inoculation, the difference in the lag phase from that for the $\Delta aceE$ strain was calculated and is given as a percentage. (B) Single clones were isolated from batches 7 (clone 1), 8 (clone 2), and 10 (green) and were cultivated as described above. The $\Delta aceE$ and $\Delta aceE \Delta pyc$ strains served as controls. These single clones were further analyzed by genome sequencing (Table 5).

$\Delta aceE$ strain (Fig. 8A). In contrast, serial cultivation transfers in CGXII medium containing solely acetate did not lead to improved growth in a glucose-acetate medium, indicating that glucose exerted evolutionary selection pressure (see Fig. S10 in the supplemental material). Genome resequencing of the three selected glucose-acetate clones for which data are presented in Fig. 8B revealed mutations in the *ptsI* gene, encoding the EI enzyme of the PTS, in two independently evolved clones (Table 5). This is in agreement with the reduced biomass formation of both clones, in which glucose uptake was apparently abolished to optimize growth on acetate. The third clone showed only slightly accelerated growth, but biomass formation was not impaired. Here, sequencing revealed a mutation in the *rpsC* gene, encoding the ribosomal S3 protein.

In summary, it was indeed possible to eliminate the observed lag phase of the $\Delta aceE \Delta pyc$ strain ascribed to glucose sensitivity by an adaptive laboratory evolution approach. Especially, the mutations identified in the *ptsI* gene are in agreement with other experiments emphasizing the high selective pressure exerted by glucose on PCx-deficient strains when the intracellular HCO_3^- levels are limiting.

DISCUSSION

During growth on glucose, the pyruvate dehydrogenase complex catalyzes a central metabolic reaction contributing to the intracellular CO_2/HCO_3^- pool. In this study, we systematically perturbed the intracellular CO_2/HCO_3^- pool of PDHC-deficient *C. glutamicum* strains in order to monitor the impact on growth and anaerobic flux. Even the PDHC-deficient strain *C. glutamicum* $\Delta aceE$ features a slightly elongated lag phase on glucose-acetate mixtures relative to that of the wild type, while no difference in growth is observed when strains are growing on gluconeogenic substrates, such as acetate.

TABLE 5 Key mutations identified in an adaptive laboratory evolution experiment with *C. glutamicum* $\Delta aceE \Delta pyc$

Clone no.	Batch no.	Gene locus	Gene name	Annotation	Mutation
1	7	cg2117	<i>ptsI</i>	EI enzyme, general component of PTS (EC 2.7.3.9)	Exchange, R132H
2	8	cg0601	<i>rpsC</i>	30S ribosomal protein S3	Exchange, R225H
3	10	cg2117	<i>ptsI</i>	EI enzyme, general component of PTS (EC 2.7.3.9)	Exchange, G155

This is remarkable, since this strain catabolizes only a minor fraction of the glucose provided in the exponential-growth phase but turns to the production of L-valine and L-alanine from glucose in the stationary phase (11, 12). The growth defect of *C. glutamicum* $\Delta aceE$ was, however, successfully complemented by the addition of HCO_3^- , which had no significant effect on the growth of wild-type cells, thus hinting at problems caused by impaired anaplerotic flux in this strain background. By comparing the CO_2 production rates of the *C. glutamicum* $\Delta aceE$ strain and the wild type, Bartek et al. were able to show in a previous study that the $\Delta aceE$ strain excretes only around $0.83 \text{ mmol of CO}_2 \text{ g}^{-1} \text{ h}^{-1}$, while the wild type excretes ca. $1.65 \text{ mmol of CO}_2 \text{ g}^{-1} \text{ h}^{-1}$ (29). The lower CO_2 production rates of the PDHC-deficient strain already indicate a significant impact on the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool affecting metabolic flux distribution, especially at low cell densities, when $\text{CO}_2/\text{HCO}_3^-$ is limiting.

The anaplerotic node comprises the essential link between glycolysis/gluconeogenesis and the TCA cycle (20). In contrast to most other organisms, *C. glutamicum* possesses both anaplerotic carboxylases, PEPCx (encoded by *ppc*) and PCx (encoded by *pyc*), catalyzing the anaplerotic bicarbonate-dependent reactions to yield oxaloacetate from PEP or pyruvate, respectively. It was shown that in *C. glutamicum*, these two enzymes can replace each other to a certain extent, depending on the intracellular concentrations of the respective effectors for each enzyme. However, during growth on glucose, the biotin-containing PCx contributes 90% to the main anaplerotic activity, in contrast to PEPCx (23, 32). In our study, deletion of the *pyc* gene resulted in a severely elongated lag phase (>15 h) of the $\Delta aceE \Delta pyc$ strain when glucose was present in the medium. Retarded growth of Δpyc strains has been observed previously, and in line with our data, other studies also reported a severe growth defect of a Δpyc strain under low $\text{CO}_2/\text{HCO}_3^-$ levels (23, 28). This effect appeared to be even more severe when the cells were grown in microtiter plates under CO_2 -stripping conditions (low culture volume, high air mixing) than when they were grown in higher culture volumes (see Fig. S7 in the supplemental material). The PDHC-deficient mutant strain *C. glutamicum* $\Delta aceE \Delta pyc$ was found to react very sensitively to small changes in intracellular bicarbonate availability and was therefore used as a test platform with which to assess systematically the impact of perturbations affecting the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool. While addition of HCO_3^- , an increase in the pH, and higher culture volumes rescued the strain, mutation of the urease accessory protein (*ureD-E188**), which lowers the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool, resulted in an even more severe phenotype. It was not possible to restore growth if the second anaplerotic gene, *ppc*, was deleted as well, confirming that the measures mentioned above fostered the flux over the remaining anaplerotic reaction catalyzed by PEPCx.

The mutation in *ureD* was revealed by a previous biosensor-driven evolution approach selecting for mutations enhancing L-valine production in *C. glutamicum* (33). In that study, inactivation of the urease enzyme by the *ureD-E188** mutation reduced the anaplerotic flux via PCx, resulting in increased precursor availability of pyruvate-derived products, such as L-valine and L-alanine (33). In the present study, we confirmed this finding by complementation with HCO_3^- , counteracting the effect of the *ureD-E188** mutation. In another study, by Blombach et al., lower $\text{CO}_2/\text{HCO}_3^-$ levels also triggered enhanced production of L-valine and L-alanine by *C. glutamicum* during shake flask cultivation (28). However, a positive impact of anaplerotic reactions has been confirmed for other products, for example, oxaloacetate-derived products, such as L-lysine. In those studies, attempts to overexpress *pyc* or the introduction of deregulated variants significantly improved L-lysine production (41, 42). However, in spite of great efforts and success in the development of L-lysine production strains, the impact of altered $\text{CO}_2/\text{HCO}_3^-$ levels has not been systematically assessed so far.

Residual glucose consumption by PDHC-deficient *C. glutamicum* strains has already been reported in previous studies (11, 12, 29). The results of this work, however, emphasize that mutants lacking the major anaplerotic route via PCx (Δpyc mutants) are under strong evolutionary pressure in the presence of glucose. Although the strains would be able to grow on the acetate supplied in the growth medium, increasing levels

of glucose resulted in a severely elongated lag phase (as long as >40 h with 250 mM) for *C. glutamicum* $\Delta aceE \Delta pyc$. Besides the above-mentioned efforts to increase the CO_2/HCO_3^- pool, the deletion of the *ptsG* gene itself effectively restored growth on glucose but not on other PTS substrates and not on ribose. These results indicate that growth retardation is not the result of PtsG-dependent regulation as known for *E. coli* (43, 44).

Furthermore, among the three key mutations identified in the ALE experiment, two single nucleotide polymorphisms (SNPs) obtained from independent cell lines were located within the *ptsI* gene, encoding the EI component of the PTS. These findings clearly highlight the problems of residual glucose consumption in strains featuring impaired anaerobic flux. One reason for the observed growth phenotype might be stress resulting from the intracellular accumulation of sugar phosphates. In *Escherichia coli*, sugar phosphate stress can be caused by the accumulation of any sugar phosphates due to a block in glycolysis (45), e.g., by mutations in *pgi* or *pfkA* (46), or due to excessive glucose uptake caused, e.g., by overexpression of *uhpT*, encoding a sugar phosphate permease (47). The resulting metabolic imbalance causes growth inhibition. For example, in *C. glutamicum*, accumulation of several sugar phosphates, such as fructose-1,6-bisphosphate or PEP, inhibits the key enzyme of the glyoxylate shunt, isocitrate lyase, already at low concentrations (34). To counteract this stress, *E. coli* triggers SgrR, which, in turn, activates SgrS by an unknown signal. The transcription factor SgrS then prevents further uptake of glucose by downregulation of *ptsG* (45, 48, 49). This is in line with the finding of our study showing a slight downregulation of *ptsG* upon resumed growth of the $\Delta aceE \Delta pyc$ strain (Table 4). This reduction of glucose uptake via the PTS, which converts PEP to pyruvate during glucose transport, also contributes to increased PEP availability for the remaining anaerobic reaction catalyzed by PEPCx. Further, this is in line with an upregulation of *iolT1*, which ensures minor usage of glucose without PEP depletion (39, 40). In this context, the downregulation of the *pyk* gene in the $\Delta aceE \Delta pyc$ strain can also be interpreted as a potential adaptation to increase the PEP pool, fostering anaerobic flux. All these strategies counteracting PEP depletion underline the fact that under these conditions, the anaerobic enzyme PEPCx is not able to deliver appropriate amounts of oxaloacetate in the $\Delta aceE \Delta pyc$ strain.

During growth on gluconeogenic carbon sources—such as acetate—the transcriptional repressor SugR represses the expression of the PTS genes in *C. glutamicum*, including the glucose-specific *ptsG* gene but also *ptsF*, *ptsS*, and general components, such as *ptsI* and *ptsH* (50–52). Derepression appears to be triggered by the accumulation of fructose-6-phosphate (F6P), which is generated from glucose-6-phosphate entering glycolysis. Consequently, F6P accumulation leads to an increase in glucose consumption rates, resulting in parallel catabolization of glucose and acetate (15). The fact that the PDHC-deficient strains are also subject to regulation by SugR has been demonstrated by studies showing that the $\Delta aceE \Delta sugR$ strain leads to glucose consumption rates as much as 4-fold higher (53).

In aerobic glucose-based bioprocesses, the endogenous production of CO_2 is typically sufficiently high to promote microbial growth even at low cell densities. However, it is well known that the anaerobic growth of some bacteria, such as *E. coli*, requires an external supply of CO_2/HCO_3^- to avoid long lag phases (25, 54, 55). The critical impact of CO_2/HCO_3^- levels may become especially evident under conditions where the endogenous CO_2 production rate becomes limiting. This was nicely demonstrated by a recent study of Bracher et al., who showed that long lag phases of engineered, but non-evolved, *Saccharomyces cerevisiae* strains during xylose fermentation could be avoided by sparging the bioreactor cultures with CO_2-N_2 mixtures (56). Alternatively, the addition of L-aspartate, whose transamination provides oxaloacetate, which refuels the TCA cycle, completely abolished the long adaptation phase of the respective yeast strains. In line with these findings, a recent ^{13}C flux analysis with *E. coli* revealed that considerable turnover of lipids via β -oxidation appears to be required for growth on xylose to enhance the intracellular CO_2/HCO_3^- pool to a growth-promoting level that

enables anaplerotic flux (57). These findings are in good agreement with our demonstration in this study that the elongated lag phase of the *C. glutamicum* $\Delta aceE \Delta pyc$ strain can be eliminated by various measures, either by increasing the intracellular $\text{CO}_2/\text{HCO}_3^-$ level or by refueling the TCA cycle with various intermediates, such as citrate, malate, or succinate.

Overall, oxaloacetate depletion appeared to represent a key issue causing the delayed growth of the $\Delta aceE \Delta pyc$ strain. Our data revealed that this is caused by impaired anaplerotic flux on glucose due to low intracellular $\text{CO}_2/\text{HCO}_3^-$ levels as well as reduced activities of glyoxylate cycle enzymes. As known from several organisms, the activity of glyoxylate shunt enzymes is tightly repressed during growth on glucose. In *C. glutamicum*, the transcription of *aceA* (encoding isocitrate lyase) and *aceB* (encoding malate synthase) is repressed by RamB in the presence of glucose (58) and is activated by RamA in the presence of acetate (59). The PDHC-deficient strains in this study were grown on glucose-acetate mixtures. In an earlier study by Wendisch et al., it was emphasized that the anaplerotic function is entirely achieved by the glyoxylate cycle enzymes when wild-type *C. glutamicum* is grown on glucose plus acetate (15). They further revealed that this function can be partly complemented by PEPCx or PCx (15). However, the possibility remains that the glyoxylate cycle enzymes, especially isocitrate lyase, are repressed in the PDHC-deficient mutants due to accumulation of, e.g., sugar phosphates caused by the PDHC and/or *pyc* inactivation. Reinscheid et al. (1994) claimed that the sugar phosphates 3-phosphoglycerate, 6-phosphogluconate, and fructose 1,6-bisphosphate cause inhibition of the isocitrate lyase in *C. glutamicum* (34). Although transcriptional regulation of the glyoxylate shunt is different, isocitrate lyases of *E. coli* are also repressed by glucose catabolites (60–62). Consequently, a repressed glyoxylate shunt due to glucose catabolite accumulation probably also accounts for oxaloacetate depletion in PDHC-deficient strains; this is especially problematic for the $\Delta aceE \Delta pyc$ strain, which cannot complement this depletion by anaplerotic pathways due to lower intracellular $\text{CO}_2/\text{HCO}_3^-$ levels.

Taken together, these results emphasize the important impact of the intracellular $\text{CO}_2/\text{HCO}_3^-$ pool on metabolic flux distribution, which is especially relevant in engineered strains exhibiting lower endogenous CO_2 production rates, as exemplified by PDHC-deficient strains in this study, but also by the performance of pentose-fermenting yeast and *E. coli* strains (56, 57). Consequently, the lack of an important by-product, such as CO_2 released by the PDHC, may have a significant impact on cellular metabolism and growth, especially on glycolytic substrates demanding high flux via anaplerotic reactions.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Tables 6 and 7, respectively. Mutant strains are based on the wild-type *Corynebacterium glutamicum* strain ATCC 13032 (63).

Standard cultivation of *C. glutamicum* $\Delta aceE$ cells and derivatives was performed on brain heart infusion (BHI; Difco; BD, Heidelberg, Germany) agar plates containing 51 mM potassium acetate (referred to here as acetate) (ChemSolute; Th. Geyer, Stuttgart, Germany) at 30°C for 2 days. One single colony was picked and was incubated for 8 to 10 h at 30°C in either 4.5 ml or 1 ml BHI containing 154 mM acetate in reaction tubes or deep-well plates (VWR International, PA, USA), respectively. First precultures were used to inoculate second precultures in CGXII minimal medium (64) supplemented with 154 mM acetate either as 10-ml cultures in shake flasks or in 1-ml volumes in deep-well plates. After overnight growth, a main culture was inoculated at an optical density at 600 nm (OD_{600}) of 1 in CGXII medium containing 154 mM acetate and either 222 mM D-(+)-glucose monohydrate (Riedel-de Haën, Seelze, Germany) (referred to here as glucose) or any other carbon source as stated, e.g., D-(-)-fructose (Sigma-Aldrich, Taufkirchen, Germany) (referred to as fructose), D-(+)-sucrose (Roth, Karlsruhe, Germany) (referred to as sucrose), D-gluconic acid sodium salt (Sigma-Aldrich, Taufkirchen, Germany) (referred to as gluconate), D-(-)-ribose (Sigma-Aldrich, Taufkirchen, Germany) (referred to as ribose), citric acid monohydrate (Merck Millipore, Darmstadt, Germany) (referred to as citrate), H-Glu-Ala-OH (Bachem AG, Bubendorf, Switzerland) (referred to as Glu-Ala), succinic acid (Sigma-Aldrich, Taufkirchen, Germany) (referred to as succinate), or L-malic acid (Merck Millipore, Darmstadt, Germany) (referred to as malate). To increase the extracellular availability of bicarbonate, 100 mM potassium HCO_3^- (Merck Millipore, Darmstadt, Germany) was added to the basic CGXII solution. The pH was adjusted to 7, and subsequently, the medium was passed through a sterile filter before inoculation. In order to analyze the effects of different pH levels, either HCl (to lower the pH) or KOH (to increase the pH) was added to the CGXII basis, which was sterile

TABLE 6 Bacterial strains used in this study

Strain	Characteristics	Source or reference
<i>C. glutamicum</i>		
ATCC 13032	Wild type, biotin auxotroph	S. Kinoshita et al. (77)
$\Delta aceE$ strain	ATCC 13032 derivative with deletion of <i>aceE</i>	M. E. Schreiner et al. (5)
$\Delta aceE \Delta pyc$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>pyc</i>	This work
$\Delta aceE \Delta ptsG$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>ptsG</i>	This work
$\Delta aceE \Delta pyc \Delta ptsG$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> , <i>pyc</i> , and <i>ptsG</i>	This work
$\Delta aceE \Delta pgi$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>pgi</i>	This work
$\Delta aceE \Delta pyc \Delta pgi$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> , <i>pyc</i> , and <i>pgi</i>	This work
$\Delta aceE \Delta ppc$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>ppc</i>	This work
$\Delta aceE \Delta pyc \Delta ppc$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> , <i>pyc</i> , and <i>ppc</i>	This work
$\Delta aceE ureD-E188^*$ strain	ATCC 13032 derivative with deletion of <i>aceE</i> and with <i>ureD-E188^*</i> (Glu188 replaced by the stop codon)	R. Mahr et al. (33)
$\Delta aceE ureD-E188^* \Delta pyc$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>pyc</i> and with <i>ureD-E188^*</i> (Glu188 replaced by the stop codon)	This work
Δpyc strain	ATCC 13032 derivative with deletion of <i>pyc</i>	P. G. Peters-Wendisch et al. (23)
$\Delta pyc \Delta ppc$ strain	ATCC 13032 derivative with deletions of <i>aceE</i> and <i>ppc</i>	A. Schwentner et al. (78)
<i>E. coli</i> DH5 α		
	F ⁻ ϕ 80lacZDM15 $\Delta(lacZYA-argF)U169 recA1 endA1 hsdR17(r_K^- m_K^-)$ <i>phoA supE44 thi-1 gyrA96 relA1 λ^-</i> ; for general cloning purposes	Invitrogen

filtered afterwards. For cultivations in the presence of TCA cycle-filling/refueling substrates, 50 mM citric acid monohydrate (citrate), 3 mM H-Glu-Ala-OH dipeptide (Glu-Ala), 100 mM succinic acid (succinate), or 100 mM L-malic acid (malate) was used. In experiments where gluconate or ribose was used as the carbon source, 100 mM D-gluconic acid sodium salt or 100 mM D-ribose was added, respectively. Biomass formation was monitored during cultivation in shake flasks by measuring the OD₆₀₀ or by measuring backscatter values during microtiter plate cultivation. Where necessary, the medium was also supplemented with 25 μ g/ml kanamycin.

Escherichia coli DH5 α was used for cloning and plasmid isolation. Cells were directly inoculated from a glycerol stock or from a lysogeny broth (LB) agar plate and were grown in shake flasks in LB medium at 37°C. If necessary for selection, the medium was also supplemented with 50 μ g/ml kanamycin.

TABLE 7 Plasmids used in this study

Plasmid	Characteristics	Source or reference
pK19mobsacB	Contains negative (<i>sacB</i>) and positive (Kan ^r) selection markers for genomic integration and deletion	A. Schäfer et al. (70)
pK19mobsacB- Δpyc	Kan ^r ; derivative of pK19mobsacB for partial <i>pyc</i> gene deletion, containing only the first 499 bp and last 493 bp of <i>pyc</i> , with the deletion of 2,429 bp in the middle	P. G. Peters-Wendisch et al. (23)
pK19mobsacB- $\Delta ptsG$	Kan ^r ; derivative of pK19mobsacB for partial <i>ptsG</i> gene deletion, containing only the last 491 bp of <i>ptsG</i> , with the deletion of the first 1,561 bp	This work
pK19mobsacB- Δpgi	Kan ^r ; derivative of pK19mobsacB for <i>pgi</i> gene deletion, containing only the last 537 bp of <i>pgi</i> , with the deletion of the first 1,086 bp	This work
pK19mobsacB- Δppc	Kan ^r ; derivative of pK19mobsacB for partial <i>ppc</i> gene deletion, containing only the first 391 bp and last 447 bp of <i>ppc</i> , with the deletion of 1,922 bp in the middle	J. Buchholz et al. (13)
pK19mobsacB- <i>ureD-E188^*</i>	Kan ^r ; derivative of pK19mobsacB for <i>ureD</i> gene truncation by replacing Glu188 in <i>ureD</i> with the stop codon	R. Mahr et al. (33)
pJC1	Kan ^r <i>oriV_{EC}</i> <i>oriV_{col}</i> ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector	J. Cremer et al. (79)
pJC1- <i>venus-term-BS</i>	Kan ^r ; derivative of pJC1, containing the terminator sequence of <i>Bacillus subtilis</i> behind <i>venus</i>	M. Baumgart et al. (74)
pJC1- <i>pyc</i>	Kan ^r ; derivative of pJC1- <i>venus-term-BS</i> , containing <i>pyc</i> under the control of its native promoter <i>P_{pyc}</i>	This work
pJC1- <i>P_{lac}-aceA-aceB</i>	Kan ^r ; derivative of pJC1- <i>venus-term-BS</i> , containing <i>lacI</i> and <i>aceA</i> followed by a linking sequence (5'-ACTAGAAATAATTTGTTTAACTTTAAGAAGGAGATATACAT-3') and <i>aceB</i> under the control of the inducible promoter <i>P_{lac}</i>	This work
pJC1- <i>P_{lac}-aceB-aceA</i>	Kan ^r ; derivative of pJC1- <i>venus-term-BS</i> , containing <i>lacI</i> and <i>aceB</i> followed by a linking sequence (5'-ACTAGAAATAATTTGTTTAACTTTAAGAAGGAGATATACAT-3') and <i>aceA</i> under the control of the inducible promoter <i>P_{lac}</i>	This work

Microtiter plate cultivation. Online monitoring of growth and/or pH was performed in 48-well microtiter FlowerPlates (m2p-labs GmbH, Baesweiler, Germany) sealed with sterile breathable rayon film (VWR International, PA) in a BioLector microtiter cultivation system (m2p-labs GmbH, Baesweiler, Germany) (65). The cultivation conditions were adjusted as described previously (66), and biomass formation was recorded every 15 min as the backscattered light intensity (light wavelength, 620 nm; signal gain factor of 20) for 24 to 72 h at 30°C and 1,200 rpm. pH measurements were performed with 48-well microtiter FlowerPlates equipped with pH optodes. The data obtained were evaluated using BioLector (m2p-labs, Baesweiler, Germany) and GraphPad Prism 7 (GraphPad Software, Inc., San Diego, CA) software.

ALE. Adaptive laboratory evolution (ALE) of *C. glutamicum ΔaceE* and *C. glutamicum ΔaceE Δpyc* was performed in deep-well plates (VWR International, PA) in a main culture of 1 ml CGXII medium containing either 154 mM acetate and 222 mM glucose or solely 154 mM acetate (as a control without selection pressure) adjusted to an OD₆₀₀ of 1. Cells were cultivated for 2 to 3 days before the next generation was inoculated, starting at an OD₆₀₀ of 1, and cultivated again for 2 to 3 days. After each generation step, glycerol stocks of cultures were prepared (20% glycerol) and were stored at -80°C, allowing growth analysis and DNA sequencing of cultures from individual inoculations. In total, 16 serial transfers were analyzed.

Cloning techniques and recombinant DNA work. Standard molecular biology methods were performed according to J. Sambrook and D. W. Russell (67). *C. glutamicum* ATCC 13032 chromosomal DNA was used as the template for PCR amplification of DNA fragments and was prepared as described previously (68). DNA fragment and plasmid sequencing, as well as synthesis of oligonucleotides, was performed by Eurofins Genomics (Ebersberg, Germany).

For the construction of plasmids (see Table S3 in the supplemental material), DNA fragments were amplified using the respective oligonucleotides (see Table S4) and were enzymatically assembled into a vector backbone according to reference 69.

To achieve genomic deletion of *pyc*, *ptsG*, *ppc*, and *pgi*, two-step homologous recombination using the pK19 Δ mob Δ system (70) was implemented. The suicide plasmids (compare Table 7 with Table S2 in the supplemental material) were isolated from *E. coli* cells using the QIAprep spin miniprep kit (Qiagen, Hilden, Germany). Electrocompetent *C. glutamicum ΔaceE* and *C. glutamicum ΔaceE Δpyc* cells were transformed with these plasmids by electroporation (71). The first and second recombination events were performed and verified as described in previous studies (72). The deletion of *pyc*, *ptsG*, *ppc*, and *pgi* was reviewed by amplification and sequencing using primers shown in Table S1.

Measurement of glucose concentrations. To measure the glucose concentration of the culture medium at different time points, cultivation was performed in 50 ml in shaking flasks. During cultivation, 0.5-ml samples were taken every 3 h and were centrifuged (16,000 × g). The supernatant was collected and was stored at -20°C until use.

The actual glucose concentration was measured using a D-glucose UV test kit (R-Biopharm, Darmstadt, Germany), and calculations were done, according to the manufacturer's instructions. Absorption was measured at 340 nm.

Further calculations of the glucose uptake rates could be done based on the glucose concentrations and OD₆₀₀ values obtained. According to reference 18, the following equation was used to determine the glucose concentration in nanomoles per gram (dry weight) per hour:

$$\left(\frac{S}{M}\right) \cdot \mu \left[\left(\frac{\text{nmol} \cdot \text{liter}^{-1} \cdot \text{OD}_{600}^{-1}}{\text{gDW} \cdot \text{liter}^{-1} \cdot \text{OD}_{600}^{-1}} \right) \cdot \text{h}^{-1} \right] = \left[\frac{\text{nmol}}{\text{gDW} \cdot \text{h}} \right]$$

where S represents the slope of the glucose concentration versus the OD₆₀₀ (expressed as nanomoles per liter per OD₆₀₀ unit), M is the correlation between the dry weight and the OD₆₀₀ (expressed in grams (dry weight) per liter per OD₆₀₀ unit), and μ is the growth rate per hour. According to A. Kabus et al. (73), an OD₆₀₀ of 1 corresponds to 0.25 g (dry weight) liter⁻¹, so this value was used as M throughout these calculations.

Quantification of amino acid production. Using ultrahigh-performance liquid chromatography (uHPLC), amino acids were quantified as *ortho*-phthalaldehyde derivatives by automatic precolumn derivatization. Derivatives were separated by reverse-phase chromatography on an Agilent (Santa Clara, CA) 1290 Infinity LC ChemStation equipped with a fluorescence detector. As the eluent for the Zorbax Eclipse amino acid analysis (AAA) column (particle size, 3.5 μm; inside diameter, 4.6 mm; length, 75 mm; Agilent, Santa Clara, CA), a gradient of Na-borate buffer (10 mM Na₂HPO₄, 10 mM Na₂B₄O₇ [pH 8.2]; adapted to operator's guide) and methanol was applied. Prior to analysis, samples were centrifuged for 10 min at 16,000 × g and 4°C and were diluted 1:100.

Monitoring of cellular proliferation by cell staining. For the staining of proliferating cells, the PKH67 green fluorescent cell linker kit for general cell membrane labeling (Sigma-Aldrich, Munich, Germany) was used, and the protocol was adapted according to the work of O. Kotte et al. (35). From an exponentially growing preculture in CGXII minimal medium containing 222 mM glucose and 154 mM acetate, 1.5 × 10⁹ cells were harvested by centrifugation for 4 min at 4,000 × g and 4°C. Then the cells were washed again in 5 ml ice-cold CGXII basic solution, without MgSO₄ CaCl₂, biotin, trace elements, or protocatechuic acid. For staining, the cell pellet was resuspended in 500 μl dilution buffer C (Sigma-Aldrich, Munich, Germany) at room temperature, and a freshly prepared mixture of 10 μl PKH67 dye (Sigma-Aldrich, Munich, Germany) and 500 μl dilution buffer C was added. Subsequently, cells were incubated for 3 min at room temperature, and afterwards, 4 ml ice-cold filtered CGXII basic solution containing 1% (wt/vol) bovine serum albumin, 1 mM MgSO₄, and 0.1 mM CaCl₂ was added. Then the cells were centrifuged for 4 min at 4,000 × g and 4°C, and the cell pellet was washed twice. Finally, the cells

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were resuspended in CGXII minimal medium containing 222 mM glucose and 154 mM acetate and were cultivated in a microtiter cultivation system.

Flow cytometry. Flow cytometric (FC) analyses were conducted on a FACSAria II flow cytometer (Becton, Dickinson, San Jose, CA) equipped with a blue solid-state laser (excitation, 488 nm). Forward-scatter characteristics (FSC) and side-scatter characteristics (SSC) were recorded as small-angle and orthogonal scatters of the 488-nm laser, respectively. PKH67 fluorescence was detected using a 502-nm long-pass and 530/30-nm band-pass filter set. FACSDiva software, version 6.0, was used to monitor the measurements. During analyses, thresholding on FSC was applied to remove background noise. For FC analyses, PKH67-stained culture samples were diluted to an OD_{600} of 0.05 in FACSFlow sheath fluid buffer (BD, Heidelberg, Germany). FlowJo (version 10.0.8) analysis software was used to visualize and evaluate the data (Tree Star, Ashland, OR).

DNA microarrays. For analysis of the transcriptome, *C. glutamicum* $\Delta aceE$ and *C. glutamicum* $\Delta aceE \Delta pyc$ were cultivated in triplicate as described above in 50 ml CGXII medium containing 154 mM acetate and 222 mM glucose in shake flasks. After reaching exponential phase at an OD_{600} of ca. 12 to 15, the cell suspension was harvested by centrifugation ($4,256 \times g$, 10 min, 4°C). The resulting pellet was directly frozen in liquid nitrogen and was stored at -80°C . RNA preparation and cDNA synthesis, as well as microchip hybridization, scanning, and evaluation, were performed as described in previous studies (74).

GC-ToF MS analysis. For analysis of the metabolome, samples of *C. glutamicum* $\Delta aceE$ in the exponential phase, as well as samples of *C. glutamicum* $\Delta aceE \Delta pyc$ in the stationary and exponential phases, were taken, and cells were disrupted using hot methanol. Further sample preparation, derivatization, mass spectrometry (MS) operation, and peak identification were accomplished according to the method of N. Paczia et al. (75) in an Agilent (Santa Clara, CA) 6890N gas chromatograph coupled to a Waters (Milford, MA) Micromass GCT Premier high-resolution ToF MS. Known metabolites were identified using the in-house database JuPoD, the commercial database NIST17 (National Institute of Standards and Technology, USA), and the GMD database (Max Planck Institute of Molecular Plant Physiology, Golm, Germany) (76).

Whole-genome sequencing. In order to sequence the whole genome of *C. glutamicum* $\Delta aceE \Delta pyc$ mutants from the ALE experiment using next-generation sequencing (NGS), genomic DNA was prepared using the NucleoSpin microbial DNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. Subsequently, the concentrations of the purified genomic DNA were measured using a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Overall, 4 μg purified genomic DNA was employed for the preparation for genome sequencing using a TruSeq DNA library prep kit and a MiSeq reagent kit, version 1 (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. The sequencing run was performed in a MiSeq system (Illumina, San Diego, CA). Data analysis and base calling were performed with the Illumina instrument software. The resulting FASTQ output files were examined using CLC Genomics Workbench 9 (Qiagen, Aarhus, Denmark).

Accession number(s). The microarray data determined in this study are available at NCBI's Gene Expression Omnibus under accession number GSE134218.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00387-19>.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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4. Appendix

4.1. Evolutionary engineering of *Corynebacterium glutamicum*

Stella, R., **Wiechert, J.**, Noack, S. and Frunzke, J.

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Contributor Role	Contributor
Conceptualization	RS 35 %, JW 10 %, SN 10 %, JF 45 %
Formal Analysis	-
Investigation/Experiments	-
Methodology	-
Project Administration	RS 50 %, JW 5 %, JF 45 %
Software	-
Supervision	RS 50 %, JF 50 %
Visualization	RS 100 %
Writing – Original Draft Preparation	RS 60 %, JW 10 %, SN 10 %, JF 20 %
Writing – Reviewing & Editing	RS 45 %, JW 5 %, SN 5 %, JF 45 %

Overall contribution JW: 15 %

The main contribution of JW to this review was the writing of the chapter “Metabolic Engineering to Guide Evolution”.

Evolutionary engineering of *Corynebacterium glutamicum*

Roberto G. Stella, Johanna Wiechert, Stephan Noack, and Julia Frunzke*

A unique feature of biotechnology is that we can harness the power of evolution to improve process performance. Rational engineering of microbial strains has led to the establishment of a variety of successful bioprocesses, but it is hampered by the overwhelming complexity of biological systems. Evolutionary engineering represents a straightforward approach for fitness-linked phenotypes (e.g., growth or stress tolerance) and is successfully applied to select for strains with improved properties for particular industrial applications. In recent years, synthetic evolution strategies have enabled selection for increased small molecule production by linking metabolic productivity to growth as a selectable trait. This review summarizes the evolutionary engineering strategies performed with the industrial platform organism *Corynebacterium glutamicum*. An increasing number of recent studies highlight the potential of adaptive laboratory evolution (ALE) to improve growth or stress resistance, implement the utilization of alternative carbon sources, or improve small molecule production. Advances in next-generation sequencing and automation technologies will foster the application of ALE strategies to streamline microbial strains for bioproduction and enhance our understanding of biological systems.

1. Introduction

The Gram-positive actinomycete *Corynebacterium glutamicum* has a long-standing history in microbial biotechnology, following its isolation in 1957 by Kinoshita et al.^[1] due to its potential to secrete large amounts of L-glutamate.^[2] In the last few decades, *C. glutamicum* has been engineered for the production of a variety of value-added products, including amino acids, organic acids, polymer precursors, aromatic chemicals, and proteins. These achievements are mainly the result of classical metabolic engineering and have been summarized in a number of recent reviews.^[3–6]

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Systems biology and metabolic engineering take a rational approach at strain development and are aided by the high level of information available for *C. glutamicum*, including a comprehensive overview on the transcriptomic map and detailed information on metabolic pathways and their regulation.^[5,7,8] Strain construction is accelerated by an ever-increasing amount of synthetic biology tools, such as CRISPR/Cpf1^[9] and CRISPRi,^[10] which has been covered by several reviews.^[5,11,12] Transcription factor-based biosensors, which enable the visualization of cellular productivity at the single-cell level, have proven to be a powerful tool for the high-throughput screening of strain or enzyme libraries and for single-cell analysis of producer strains.^[13–16]

While rational approaches are indispensable for the development of industrial platform strains in general, their application is often hampered by the overwhelming complexity of biological systems. Even for well-established model systems, like *C.*

glutamicum or *Escherichia coli*, we are still not able to quantitatively predict responses to environmental changes or genetic perturbations. However, nature itself provides us with the most powerful approach for the optimization of biological systems: evolution. Evolutionary engineering, also known as adaptive laboratory evolution (ALE), has a long-standing history in the development of microbial production strains and has been heavily applied to improve growth rates, stress, or product tolerance.^[17,18] ALE requires microbial growth, which is usually facilitated by repetitive batch cultivations or continuous cultivations, which can be simple (e.g., shake flasks) or more advanced (e.g., pH auxostats).^[17,19] When combined with next-generation sequencing and comprehensive omics analysis, ALE strategies can be used to not only obtain better production strains, but also to identify non-intuitive targets for strain engineering, and ultimately to gain a comprehensive understanding of biological pathway regulation. Furthermore, advances in automation of laboratory workflows and microbial phenotyping are fostering a tremendous increase in throughput and efficiency.

This review gives an overview of evolutionary engineering of *C. glutamicum*, including approaches to improve growth on glucose or alternative carbon sources, stress tolerance, or small molecule production by growth coupling strategies and biosensor-guided evolution (Figure 1, examples summarized in Table 1). We conclude by highlighting the potential of a smart combination of synthetic biology and workflow automation for next-generation evolutionary engineering approaches.

2. Accelerating Growth on Glucose

The time needed for biomass production represents a key factor in the economic success of biotechnological processes. While ALE approaches have been intensively explored to accelerate the growth of *E. coli*,^[37,38] so far, only two recent studies have reported on the evolution of *C. glutamicum* towards higher growth rates on glucose minimal medium (Table 1).^[20,21] Interestingly, these studies revealed similar key targets for enhancing metabolic flux and increasing substrate uptake rates for both species, indicating that microorganisms follow similar principles to adapt to fast growth.

Among the key targets for improving growth on glucose, mutations in the *pyk* gene, encoding the pyruvate kinase (PK), were recently described. Reintroduction of the identified single-nucleotide polymorphism (SNPs) into the parental strain caused a significant decrease in PK activity (T12A, and A20V).^[20] Furthermore, a mutation of alanine 271 to threonine (A271T) was enriched in one cell line. Intriguingly, a PK enzyme carrying this mutation was previously described as being desensitized toward the allosteric activator fructose 1,6-bisphosphate (FBP), resulting in reduced PK activity upon accumulation of the glycolytic intermediate FBP.^[39] Mutations in the *pyk* gene were also identified in the prominent *E. coli* long-term evolution experiment that lasted for more than 25 years,^[40] and in a recent study by LaCroix et al.^[41] where cells were evolved towards fast growth on glucose. It is interesting to note that fast proliferating eukaryotic cells, like embryonic stem cells and cancer cells, also harbor a less active PKM2 isoform of the PK enzyme.^[42,43] From these findings, we can conclude that lowering the PK activity appears to be a conserved strategy of fast-growing cells that need to exploit large amounts of glucose for anabolic pathways.



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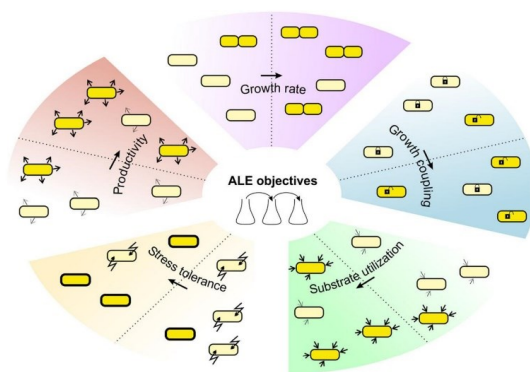


Figure 1. Overview of different objectives in *C. glutamicum* ALE studies. ALE has successfully been applied to increase the growth rate,^[20,21] substrate utilization,^[22] stress tolerance,^[23,24] and small molecule production.^[25] Growth coupling strategies have been used to increase precursor supply and small molecule production.^[26]

Table 1. Overview of ALE studies with *C. glutamicum*.

ALE target	Experimental setup	ALE duration	Phenotype of evolved strain	Sequencing/transcriptomics	Proven causal mutations	References
Growth						
Accelerating growth on glucose minimal media	Repetitive batch in minimal media containing glucose as sole carbon source	Approximately 630 generations	26% higher growth rate ($0.67 \pm 0.01 \text{ h}^{-1}$)	WGS of two independently evolved cell lines, 70 mutations	Mutational hotspots in <i>pyk</i> , <i>fruK</i> , and <i>corA</i>	[20]
Accelerating growth on glucose minimal media	Repetitive batch in minimal media containing glucose as sole carbon source	7 months, approximately 1500 generations	42% higher growth rate ($0.64 \pm 0.01 \text{ h}^{-1}$)	WGS after different ALE times, seven mutations and one genomic deletion (CGP3 element)	<i>gntR1-E70K</i> and <i>ramA-A52V</i>	[21]
Stress tolerance						
Increased growth at high temperatures	Repetitive batch in rich media, a gradual increase in process temperature	65 days	Better performance at suboptimal temperatures, increased T_{max} ($41.5 \text{ }^{\circ}\text{C}$)	WGS of three evolved isolates, deletion of two genomic regions, 295 total point mutations	<i>glmU-E295K</i> and <i>otsA-R328H</i>	[27]
Increased tolerance to inhibitors in corn stover hydrolysate	Repetitive batch in minimal media with corn stover hydrolysate	128 days	Higher degradation rate of several inhibitors, higher growth rate on media containing corn stover hydrolysate	WGS of evolved and parental culture, seven mutations; transcriptome comparison between evolved and parental strain	Not determined	[23]
Increased tolerance to methanol	Repetitive batch in minimal media containing methanol	50 generations	Improved growth rate on minimal media with 500–2000 mM methanol	WGS of single isolate, 29 mutations found; transcriptome analysis using DNA microarrays	<i>metY-A165T</i> and <i>cat-Q342^R</i>	[24]
Increased tolerance to H_2O_2	Chemostat with a dilution rate of 0.15 h^{-1} , growth on minimal media containing glucose, gradual increase of H_2O_2 concentration to 10 mM	1900 h (approximately 411 generations)	Better performance in media containing 10 mM H_2O_2	No sequencing performed; transcriptome analysis using RNAseq	Not determined	[28]
Substrate expansion						
Improved growth on cellobiose	Repetitive batch in minimal media containing cellobiose	25 days	Improved growth on cellobiose	WGS of two differently evolved strains, ten shared mutations identified; transcriptome analysis using DNA microarrays	Not determined	[29]
Improved growth on D-xylose	Automated repetitive batch in minimal media containing D-xylose	13 days (35 generations)	Twofold increase in growth rate on D-xylose ($0.26 \pm 0.02 \text{ h}^{-1}$)	WGS of multiple evolved strains	Upregulation of <i>iofT1</i>	[22,30]
Co-utilization of xylose and methanol	Repetitive batch in minimal media with xylose and methanol	206 days (approximately 27 generations)	20-fold increase in growth rate (0.03 h^{-1})	WGS of three evolved isolates, six missense mutations; ^{13}C flux analysis	Not determined	[31]
Metabolic engineering to guide evolution						
Evolution of a PEP and	Repetitive batch in media containing	13, 15 or 33 days			<i>icd-A94D</i> , <i>icd-R453C</i> ,	[26]

(Continued)

Table 1. (Continued)

ALE target	Experimental setup	ALE duration	Phenotype of evolved strain	Sequencing/transcriptomics	Proven causal mutations	References
pyruvate carboxylase-deficient strain towards growth on glucose	yeast extract and glucose		Improved growth rate to 59% of WT ($0.23 \pm 0.01 \text{ h}^{-1}$)	WGS of three independently evolved mutants	and <i>icd-C407S</i>	
Increasing small molecule production						
Increased fatty acid production	Sequential selection for suppressor mutants on agar plates containing Tween40 or cerulenin	Multiple days	Increased fatty acid production ($322.23 \pm 15.09 \text{ mg L}^{-1}$)	WGS of best-producing isolate, a total of four mutations	<i>fasR-S20D</i> , <i>fasA¹⁰⁰-C63G</i> , <i>fasA-A2623V</i> and <i>accD3-A433T</i>	[32,33]
Increased putrescine production	Repetitive batch in rich media containing putrescine, after random mutagenesis	11 days	Increased putrescine titer ($111.42 \pm 2.56 \text{ mM}$)	WGS of best-producing isolate, 78 SNPs	Not determined	[34]
Increased L-ornithine production	Repetitive batch in media containing glucose and L-ornithine	70 days	20% higher L-ornithine titer ($13.6 \pm 0.5 \text{ g L}^{-1}$)	Not performed	Not determined	[35]
Biosensor-guided evolution						
Increased L-valine production	Repetitive batch in minimal media containing glucose and acetate, selection via biosensor mediated FACS	6 days	Higher growth rate ($0.41 \pm 0.02 \text{ h}^{-1}$) and 63% increased L-valine titer (57 mM)	WGS of evolved culture and two isolates	<i>glvR-T93S</i> , <i>pppD-T201I</i> , <i>rpsP-D30D</i> , and <i>ureD-E188*</i>	[25]
Plasmid-based protein production	Repetitive batch in minimal media containing glucose, selection via biosensor mediated FACS	8 days	Higher plasmid copy number	Sequencing of plasmid backbone	Several inactivating mutations in <i>parB</i>	[36]

Further key targets identified by Pfeifer et al.^[20] were mutations in *fruK*, which encodes for 1-phosphofructokinase (Pfk1). In a previous study, deletion of the *fruK* gene was actually reported to enhance glucose uptake of *C. glutamicum*.^[44] The authors speculated that this was the result of an accumulation of F1P relieving *ptsG* repression via the SugR regulator. However, further analyses are required to understand the impact of Pfk1 on the sugar phosphate pool and its impact on regulatory networks in these strains.

The *corA* gene, coding for a putative $\text{Mg}^{2+}/\text{Co}^{2+}$ transport protein,^[45] represented an unexpected mutational hotspot in the *C. glutamicum* long-term evolution and was also identified, but not discussed, in the *E. coli* experiment of LaCroix et al.^[20,41] Mg^{2+} limitation was previously described to increase glucose consumption rates and metabolic flux of *E. coli* in the stationary phase. Here, the authors assumed that reduced Mg^{2+} levels might cause a block in pyruvate dehydrogenase (PDH) activity, leading to an accumulation of upstream intermediates like 2-phosphoenolpyr-

uvate (PEP), which stimulate glucose uptake.^[46] However, the availability of magnesium ions is known to affect a variety of different enzymes and to have an impact on the stability of polyphosphates.^[45] For example, the biologically active form of ATP is a complex with Mg^{2+} . Based on these findings, we can conclude that mutation of *corA* linked to magnesium limitation appears to be a prime target to enhance glucose uptake, but the underlying mechanism is not fully understood and requires further theoretical and experimental attention.

In another recent ALE experiment, *C. glutamicum* cultures were evolved for about 1500 generations in defined media with glucose.^[21] The evolved cell line was found to lack large parts of the CGP3 element. Deletion of the prophage CGP3 was also revealed as a competitive fitness advantage in the other *C. glutamicum* ALE study, where a competitive growth experiment showed that a prophage-free strain (MB001) outcompetes the wild type.^[20] Besides the lack of the prophage element, the authors found mutations in the *gntR1* gene, encoding a repressor of

gluconate catabolism and an activator of *ptsG*,^[47] and in *ramA*, encoding a LuxR-type regulator, which inherits a global role in the coordination of the central metabolism.^[48] Re-introduction into the wild-type background revealed that both mutations significantly increased the growth rate and glucose consumption rate (Table 1).^[21] Metabolomic flux and transcriptome analysis showed an increased flux through the pentose phosphate pathway but did not indicate significant rewiring of the central metabolic network, which is in agreement with similar studies on *E. coli*.^[49]

3. Improving Performance under Industrial Conditions

Evolutionary engineering provides an efficient means to improve the performance of microbial strains under harsh industrial conditions and/or to realize the utilization of second and third generation feedstock.^[50,51] Recent studies have employed ALE strategies for both aspects, and while the division is not always clear (e.g., in the case of methanol tolerance and utilization), they will be discussed separately in this section (Table 1).

3.1. Improving Stress Tolerance

In an economically optimized bioprocess, microorganisms should be able to grow on low-purity feedstock (lower substrate costs) and at favorable process conditions (e.g., at a higher temperature, to reduce cooling costs). An improved heat tolerance was reported for *C. glutamicum* after a repetitive batch ALE (rbALE) of 65 days, in which the temperature was gradually increased from 38 °C to 41.5 °C.^[27] Sequencing of three evolved isolates revealed a surprisingly high amount of 295 point mutations and two genomic deletions in total. This was caused by an acquired mutator phenotype, which resulted in a mutation rate 40- to 80-fold higher than that of the parental strain. A genetic basis for this phenotype was not found. However, the combined reengineering of the mutations *glmU* (E295K) and *otsA* (R328H) resulted in an improved heat tolerance and increased the maximum specific growth rate at 40.7 °C from $0.27 \pm 0.02 \text{ h}^{-1}$ to $0.33 \pm 0.03 \text{ h}^{-1}$.

Two different studies applied rbALE to increase tolerance for inhibitors present in corn stover hydrolysate^[23] and for methanol, which is an impurity found in certain glycerol waste streams.^[24] A fixed amount of inhibitor was added, and cells were selected for growth. The corn stover evolved strain showed a higher degradation rate of the inhibitors furfural, HMF, vanillin, syringaldehyde, 4-hydroxybenzaldehyde, and acetic acid.^[23] When grown on corn stover hydrolysate, the evolved strain also showed a 68% higher glutamic acid titer and 35% higher yield than the parental strain. The increased glucose consumption rate probably resulted from a higher *ptsI* expression, which codes for a component of the phosphotransferase system (PTS). In the methanol study, tolerance was increased by using media with 50 mM methanol.^[24] The evolved strain showed a higher growth rate under methanol concentrations between 500 mM and 2000 mM. Sequencing identified 29 mutations and combined engineering of two of them, *metY* (A165T) and *cat*(Q342²), increased the methanol tolerance of

the parental strain to that of the evolved strain. Interestingly, both the reverse engineered strain and the evolved strain lost the ability to grow on ethanol. This showed that, in contrast to an observed beneficial cross-tolerance for isobutanol after ALE for increased temperatures,^[27] a single-target ALE approach is typically associated with significant negative trade-off effects.

3.2. Increasing Feedstock Flexibility

The utilization of alternative, non-native carbon sources is key to the implementation of non-glucose based second or third generation processes in modern biotechnology, which provides a strong incentive to engineer industrial strains that utilize most of the components present in these substrate feeds. Attractive targets to realize a flexible feedstock concept are, for example, carbon sources derived from complex polymers (e.g., starch, cellulose, or xylan), di- and monosaccharides (e.g., arabinose or xylose), and C1 compounds, such as methanol.^[50,51] If a microbe has no native capacity to consume the particular substrate of interest, targeted engineering can provide an initial, nonoptimal strain, which can subsequently be improved by ALE. This combinatorial strategy was applied in three recent publications that describe improved growth on xylose, cellobiose, and methanol (Table 1).^[22,29,31]

Two different rbALE approaches were described for the co-utilization of xylose and methanol^[31] or xylose and cellobiose.^[29] In the xylose and methanol study, targeted engineering was first applied to obtain a strain that required both xylose and ribose for growth. After the first ALE, this strain showed an increased maximum specific growth rate, from 0.03 h^{-1} to 0.15 h^{-1} . Then, the evolved strain was rationally engineered for the co-utilization of xylose and methanol and submitted to another round of ALE. After 206 days, the evolved strain was able to grow, but the reported growth rate of 0.03 h^{-1} is still far away from industrial applications. Interestingly, the authors describe the *metY*(G1256A) mutation; another mutation in the same gene (*metY*(A165T)) was also found in the methanol tolerance study.^[24] An elegant approach to realize methanol-essential growth was recently reported for *E. coli*.^[52] The assimilation of methanol and gluconate was stoichiometrically coupled in a strain that had the ribulose monophosphate cycle. This study highlights the potential of metabolic rerouting for the successful implementation of synthetic methylotrophy in established industrial platform organisms.

In the xylose and cellobiose study, targeted engineering was first applied to obtain two different strains that could grow on cellobiose as the sole carbon source.^[29] ALE was performed until growth and cellobiose consumption did not improve anymore. Sequencing analysis revealed a high number of mutations (36 and 300), which could not be explained. After the ALE for cellobiose, a xylose consumption pathway was introduced and co-utilization of xylose and cellobiose was shown.

An improved growth rate on only D-xylose was shown by Radek et al.,^[22] using a rationally engineered strain that was subsequently evolved by high-throughput rbALE for 35 generations in only 13 days. A best performer showed more than a doubling of the maximum specific growth rate, compared to the

parental strain ($0.26 \pm 0.02 \text{ h}^{-1}$ and $0.10 \pm 0.02 \text{ h}^{-1}$, respectively). Sequencing analysis revealed putative loss-of-function mutations in the transcriptional regulator *IoIR*, involved in myo-inositol uptake and degradation, and *cg3388*, which encodes a putative transcriptional regulator. In a subsequent study, the functional loss of *IoIR* was shown to result in an upregulation of the gene *iolT1* (encoding the myo-inositol/proton symporter *IoIT1*), and additional reengineering could prove the importance of *IoIT1* for xylose uptake.^[50] Interestingly, the identified mutations are different from those identified by the xylose-methanol ALE study.^[51] Altogether, these examples once more highlight the importance of subsequent reengineering of key mutations to expand our knowledge of biological systems.

4. Metabolic Engineering to Guide Evolution

Combining evolutionary engineering with rationally engineered system perturbations is a promising approach for the identification of metabolic traits that can be beneficial for target molecule production.^[53] Gene knockouts are frequently used to not only investigate the metabolic and regulatory function of gene products,^[54,55] but also for predefining the cell's metabolic network with regard to precursor supply for biotechnological applications (Figure 2).^[56] Growth defects that result from

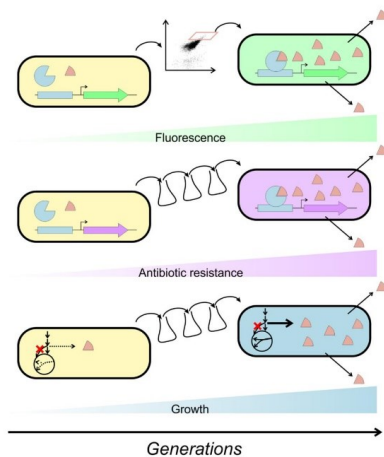


Figure 2. Overview of different synthetic evolution approaches to select for increased small molecule production. Synthetic regulatory circuits can be constructed to couple intracellular product concentration to a selectable output, for example to fluorescent protein synthesis,^[25] which can be selected for by FACS, or to synthesis of an antibiotic or auxotrophic marker.^[53] Metabolic engineering to guide evolution can be applied to increase small molecule production by selecting for growth rate.^[26]

system perturbations by introduction of gene knockouts provide a guided selection pressure toward fitness recovery. This can lead to alternative reoptimized metabolic states, for example by harnessing the underground metabolism by building on promiscuous enzyme activity of the particular species.^[54,57]

Schwentner et al.^[26] used the inactivation of anaplerotic reactions to evolve an alternative producer platform for the amino acid L-valine. This effort was motivated by the fact that metabolic engineering of *C. glutamicum* for L-valine production is typically based on complete inactivation or downregulation of the pyruvate dehydrogenase complex (PDHC).^[59,60] Schwentner et al. evolutionarily engineered an alternative route for increased pyruvate supply by inactivating the genes encoding the PEP and pyruvate carboxylase (*ppc* and *pyc*). The resulting strain was unable to grow on glucose as the sole carbon source since the replenishment of the oxaloacetate pool was impaired.^[61] Whole genome sequencing of the evolved strains revealed *icd*, encoding isocitrate dehydrogenase (ICD), as a consistent mutation target in all evolved strains. Re-engineering of the identified key mutations in *icd* revealed an upregulation of the glyoxylate shunt^[62] as an alternative route to replenish the oxaloacetate pool in the $\Delta ppc \Delta pyc$ background.^[26] However, further studies are necessary to identify the molecular mechanisms underlying the activation of the glyoxylate shunt as a secondary effect of reduced ICD activity. In accordance with assumptions of Baumgart et al.,^[63] the authors postulated that the reduced ICD activity and the resulting accumulation of isocitrate promote activation of the glyoxylate shunt.^[26] Based on a 1.9-fold increased intracellular L-valine level and a predicted increased flux through the PDHC, Schwentner et al. suggested that the re-engineered strains represent promising candidates for L-valine producers. Indeed, plasmid-based overexpression of L-valine biosynthesis genes (*pjC4ilvBNCE*) led to a fourfold-enhanced L-valine product yield in comparison to the wild type.^[26]

The study of Schwentner and co-workers demonstrated the potential of guided evolution strategies to improve glucose conversion to value-added compounds like L-valine. In contrast, the previously discussed guided evolution approaches of Lee et al.^[29] and Tuyishime et al.^[31] resulted in strains showing efficient co-utilization of the alternative substrates xylose and methanol or xylose and cellobiose, demonstrating the broad application spectrum of guided evolution strategies (Table 1).

McCloskey and colleagues intensively studied growth recovery of different *E. coli* knockout strains during ALE at the systems level. The combination of ALE and multiomics technologies provided deep insights into the versatile pathways allowing rebalancing of the metabolite and redox state.^[54,55] Furthermore, growth coupling approaches with *E. coli* used the strong evolutionary driving force towards redox homeostasis for increasing fermentative production of lactic acid,^[56] 1-butanol,^[64] and linear higher alcohols^[65] by inactivating competing NADH oxidizing enzymes. These examples highlight that not only the experimental conditions but also the genetic strain background need to be streamlined for the particular aim of the ALE.

5. The Benefit of Automation

A number of different ALE approaches are already established that can roughly be classified according to the cultivation conditions (i.e., chemostat vs repetitive batch culture), experimental throughput (i.e., lab-scale vs microscale bioreactor systems), and degree of automation (i.e., manual vs autonomous operation). See Dragosits et al.^[17] and Gresham et al.^[66] for a detailed discussion of the advantages and disadvantages of the chemostat and repetitive batch approach.

In recent years, the rbALE approach has become more popular due to its lower costs for operation, simpler experimental implementation, and easier expandability. In short, individual cultivation batches are sequentially inoculated until a termination point (e.g., no further improvement in fitness criterion) is reached. Adaptation and diversification of cells during rbALE depends on the applied selection pressure, which can greatly vary between conditions of feast (i.e., exponential growth) and famine (i.e., stationary phase).

In many cases, selection focuses on higher maximum growth rates, which are best supported by keeping the cells under balanced growth conditions throughout the whole rbALE experiment. The time window of exponential growth depends on the inoculation density, the lag-phase and the specific growth rate, and should become smaller with increasing number of beneficial mutations leading to faster cell population growth and earlier consumption of the usually fixed nutrient resources. Therefore, the application of conventional shaking flasks and offline OD measurements for monitoring biomass growth does not interface with appropriate standardization.

To overcome this limitation, several microbioreactor systems are available that are based on shaken microtiter plates (MTPs), e.g., "GrowthProfiler" (EnzyScreen), "Bioscreen C" (Oy Growth Curves) or "BioLector" (m2p-labs), which employ (quasi)-continuous biomass measurement via integrated image analysis, optical density, or backscatter (see Hemmerich et al.^[67] for an comprehensive overview). In addition, MTPs enable a higher throughput to perform replicate ALE experiments under identical conditions^[22,68] and thus provide access to important quantities for analyzing the evolutionary process, e.g., in terms of beneficial

mutation rates and the occurrence of fixed or converged mutations.^[69] It is noteworthy that the typical 1 mL working volume of standard MTPs is also sufficient to carry out further in-depth characterizations of evolved strains using available spectrophotometric assays^[70] and quantitative omics technologies.^[71]

However, when applied as stand-alone devices for rbALE, the above systems still require the manual passaging of cells from one MTP-well to another. Consequently, there is a high demand for automation to optimally balance available resources (i.e., personnel, material, and time) with the expected outcomes (i.e., strains with improved properties, new knowledge on cellular metabolism, and targets for rational strain design).

As a first step in this direction, a recently established a workflow was reported, which enables the performance of rbALE experiments in an automated manner.^[22] By building on an existing microbial phenotyping platform (i.e., Mini Pilot Plant integrating a BioLector system with liquid handling robotics^[72]), the workflow covers essential steps for autonomous rbALE, including preparation of different media compositions, their cool temporary storage and the repeated inoculation of individual MTP-cavities in the BioLector. The latter is supported by various online measurements (i.e., backscatter, pO₂, pH, and fluorescence) and ensures an instantaneous recording of metabolic adaptation events during ALE experimentation (Figure 3.2). Moreover, the setup not only enables dynamic adjustments of passage sizes for keeping genetic diversity, but (if required) also provides access to the full spectrum of applicable selection pressures.

6. Evolutionary Engineering of Small Molecule Production

Redirecting cellular metabolism toward the production of small molecules is challenging, because metabolic pathways have been evolved for tight control and our understanding of their regulation is incomplete.^[73] For ALE approaches, the difficulty lies in applying a pressure that selects for production of the compound of interest. While traditional approaches select for

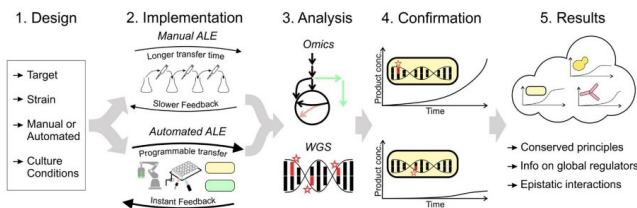


Figure 3. Outlook on future ALE strategies. Future ALE studies could implement multiple selection pressures, to optimize industrial performance of microbial strains. Automated approaches allow for simultaneous cultivation of multiple cultures, programmable transfer-time points and instant feedback, especially in combination with biosensors. ALE should be followed by whole genome sequencing analysis and confirmation of causal mutations via re-engineering approaches. Finally, online databases can be used to gather ALE results for different microbial organisms to ultimately increase our knowledge on biological systems.

suppressor mutants, the development of transcription factor-based biosensors enabled a direct coupling of product formation to an easily selectable output, which resulted in the first reports of “synthetic evolution.”^[16,74–76] For *C. glutamicum*, ALE for increased small molecule production has been shown for fatty acids, L-ornithine, putrescine, plasmid-based proteins, and L-valine (Table 1).

6.1. Selection for Suppressor Mutants

Early efforts on the improvement of small molecule production focused on the application of random mutagenesis (UV- or chemical mutagenesis) and on selection schemes based on the resistance to antimetabolites or product analogs inhibiting growth. Key enzymes of biosynthetic pathways (e.g., amino acid biosynthesis) are typically feedback-inhibited by the particular end-product of the pathway. Application of product analogous antimetabolites enabled the efficient isolation of mutations causing feedback-resistance of key enzymes.^[2]

Two groups have recently published work on improving small molecule production by selecting suppressor mutants. Takeno et al.^[32] used two different chemicals, Tween-40 and cerulenin, to select for mutants with a higher fatty acid production, using multiple selection steps on agar plates. Jiang et al.^[35] applied rbALE in media containing the desired product, L-ornithine, to select for mutants resistant to product inhibition. In both studies, the final product titer could be improved with the evolved strain, from $3.21 \pm 0.06 \text{ mg L}^{-1}$ to $279.95 \pm 8.50 \text{ mg L}^{-1}$ for fatty acids,^[32] and from $10.2 \pm 0.2 \text{ g L}^{-1}$ to $13.6 \pm 0.5 \text{ g L}^{-1}$ for L-ornithine.^[35] While Jiang et al. did not perform whole genome sequencing, Takeno et al. reported three mutations in the genes responsible for fatty acid synthesis. Takeno et al.^[33] repeated the selection step on cerulenin plates and identified one additional mutation. Re-engineering of this mutation in the previously evolved strain resulted in a 1.2-fold higher oleic acid titer. This shows how iterative selection of a parental strain can be used to find different mutations that have an additive effect on productivity. The L-ornithine evolved strain was further engineered for putrescine production.^[34] Random mutagenesis in combination with rbALE in media containing putrescine resulted in a strain showing a twofold higher final putrescine titer ($111.42 \pm 2.56 \text{ mM}$). Sequencing analysis revealed 78 SNPs; further analysis suggested that a decrease in the activity of the α -ketoglutarate decarboxylase OdhA and an increase in the major facilitator superfamily permease CgmA, a putative putrescine permease, contributed to a higher putrescine production.

6.2. Biosensor-Guided Evolution

Biosensors can greatly increase the throughput and selectivity for ALE, and thereby accelerate strain development (Figure 2). Initially, two studies reported on transcription factor-based biosensors that were designed to induce *eyfp* expression in response to higher intracellular amino acid concentrations in *C. glutamicum*. The pSenLys biosensor detects amino acids with a basic side chain^[13] and the Lrp biosensor detects L-methionine and branched chain amino acids.^[14] Both studies showed that

random mutagenesis coupled to FACS could be used to select for cells with higher productivity, illustrating the use of biosensors to screen for producers strains. However, applying random mutagenesis results in many mutations. Binder et al.^[13] reported 268 SNPs and a similar study reported 83 SNPs.^[77] This high number of mutations complicates the identification of causal ones.

In a recent study, Mahr et al.^[25] described a biosensor-guided ALE approach based on the native mutagenesis rate, by applying multiple rounds of selection using the Lrp biosensor (Table 1). The L-valine producer strain *C. glutamicum* Δ ace, which produces valine in the stationary phase, was used as the parental strain. Five iterative evolution steps were performed and, in each step, the top 10% fluorescent cells were transferred by FACS. After only five iterations, a single isolate showed a twofold increase in L-valine production compared to the parental strain. Only seven mutations were identified after whole genome sequencing. This is in large contrast to the higher number of mutations found when random mutagenesis is applied. The identified *ureD*(E188^S) mutation resulted in a truncated version of the urease accessory protein and was shown to increase the final L-valine titer (44.2 mM for evolved strain) by more than 100%.^[25] A low amount of mutations was also the result of a simple biosensor ALE experiment, in which iterative FACS selection was done with a strain containing a plasmid-based *egfp*. Only one mutation was identified, a loss of function of *parB*, which resulted in tenfold higher plasmid copy numbers.^[36] Further examples for biosensor-guided evolution approaches in *E. coli* are the coupling of mutation rate to small molecule production^[76] and the selection of improved producers via an antibiotic-based positive and negative selection strategy.^[38]

The mutations identified by ALE can be further investigated by targeted mutagenesis followed by biosensor mediated screening, which was shown for the *argB* gene using pSenLys^[78] and for the RBS sequence in *tktA* using the ShiR-based shikimate sensor.^[79] An interesting method was described by Binder et al.,^[80] who designed a rapid recombineering and screening approach, based on the RecT recombinase from prophage Rac and the pSenLys sensor, which was applied to screen for mutations in *murE* with an impact on L-lysine production.^[80] Furthermore, studies in *E. coli* have demonstrated the use of multiplexed genome engineering in combination with barcoding to increase mutation rates and enable tracking of mutations.^[81,82] Altogether, these approaches demonstrate the potential of transcription factor-based biosensors for the design of synthetic evolution schemes to enable the improvement of microbial small molecule production.

7. Conclusions

In engineering disciplines, the possibility of process improvement via evolution is a feature unique to biotechnology. But what do we learn from the comparison of different ALE endeavors? Evolution of living organisms is shaped by the particular environmental conditions (like media composition, gas supply, etc.), the genetic background, and by the mode of cultivation (batch, chemostat, solid media), including passage time and culture volume. For example, LaCroix et al.^[41]

achieved a significant increase of growth rates in a shorter time compared to the Lenski experiment, because batch cultures were propagated during the exponential phase rather than inoculated from stationary phase cultures. These examples demonstrate that an ALE setup must be efficiently tailored to the particular scientific aim. However, most studies only focus on one aspect of industrial performance (e.g., productivity or stress tolerance). A more integrative approach could combine several improvement targets into one experiment. Especially, the development of biosensors could aid in this field, as they allow for coupling of producers to a selectable output under industrial conditions. This could even be extended with high-throughput technologies enabling the design of novel ALE strategies (Figure 3).

Several recent examples highlight the fact that ALE or untargeted selection strategies should be complemented by sequencing, and possibly by transcriptome analyses and re-engineering to obtain novel information on enzyme properties or pathway regulation. Especially in the absence of a random mutagenesis step, the amount of mutations is most of the times low enough to allow for reverse engineering and an in-depth characterization of specific mutations. This is in contrast to classical genome breeding approaches where a high number of induced mutations (e.g., via UV radiation) may result in the accumulation on non-beneficial side mutations having a negative impact on genome stability. Often, different mutations found in different studies can result in an additive effect, but it can be hard to keep track of all described mutations. Recent database efforts such as the AleDB could aid in improving our understanding of metabolic regulations.^[69] A more systemic analysis of ALE experiments combined with sophisticated data management will accelerate the identification of causative key mutations and epistatic interactions resulting in improved strain properties.

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Conflict of Interest

The authors declare no conflict of interest.

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4.2. Lichtblicke in der mikrobiellen Stammentwicklung

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Contributor Role	Contributor
Conceptualization	JW 30 %, JF 70 %
Formal Analysis	-
Investigation/Experiments	-
Methodology	-
Project Administration	JW 20 %, JF 80 %
Software	-
Supervision	JF 100 %
Visualization	JW 100 %
Writing – Original Draft Preparation	JW 70 %, JF 30 %
Writing – Reviewing & Editing	JW 40 %, JW 60 %

Overall contribution JW: 50 %

Figures were prepared by JW. The writing of the original draft was mainly done by JW. JW took part in the revision and editing process.

Lichtblicke in der mikrobiellen Stammentwicklung

Johanna Wiechert, Julia Frunzke

Biosensoren der Zelle nutzen die natürliche Sensorik eines Organismus, um etwa eine Metabolitenkonzentration in ein optisches Signal umzuwandeln. Als vielseitig verwendbares Werkzeug helfen sie, bakterielle Stämme für die biotechnische Produktion zu entwickeln und zu optimieren.

◆ Seit Jahrtausenden nutzt der Mensch das enorme metabolische Potenzial von Mikroorganismen, um Brot, Käse oder Wein herzustellen. Seit dem 20. Jahrhundert werden mit Metabolic Engineering gezielt mikrobielle Stämme erzeugt, mit denen sich unter anderem Proteine, Aminosäuren, organische Säuren und Vorstufen von Polymeren produzieren lassen.¹⁾ Einfache, nachwachsende Substrate in mikrobiellen Prozessen sind eine kostengünstige Alternative zu erdölbasierten und energieintensiven chemischen Prozessschritten. Über fünf Millionen Tonnen Aminosäuren produziert die Industrie jährlich mit dem Bodenbakterium *Corynebacterium glutamicum* (*C. glutamicum*).²⁾

Mikroorganismen sind typischerweise nicht von Natur aus dafür ausgestattet, bestimmte Substanzen in großen Mengen herzustellen. Produktionsstämme zu entwickeln und die biotechnischen Prozesse zu optimieren, ist oft das zentrale Problem. Viele der heute verwendeten Produktionsstämme stammen aus klassischer Zufallsmutagenese und anschließenden chromatographischen und massenspektrometrischen Selektionsverfahren.³⁾ Die Mutationsrate etwa durch UV-Licht zu steigern ermöglicht eine hoch diverse Stammbibliothek. Jedoch ist eine

effiziente Hochdurchsatzanalyse von Stammbibliotheken nur begrenzt möglich, da sie dauert und teuer ist.³⁾ Eine Lösung für dieses Problem sind genetisch kodierte Biosensoren, die eine intrazelluläre Metabolitenkonzentration in ein optisches, leicht messbares Signal übersetzen.

Biosensoren als biotechnisches Werkzeug

◆ Mikroorganismen nutzen ein umfangreiches Repertoire an Mechanismen, das auf Proteinen oder kleinen RNA basiert, um intra- und extrazelluläre Signale wahrzunehmen und darauf zu reagieren.

Abhängig von physiologischen Parametern wie der intrazellulären Konzentration bestimmter Stoffwechselprodukte kontrollieren die Transkriptionsfaktoren die Genexpression über die Bindung an den entsprechenden Promotor des Zielgens. Vereint man diese Zielpromotoren mit einem Aktuatoren, lässt sich dieses effektorabhängig exprimieren (Abbildung 1A, S. 590).

Ist der Aktuator ein Fluoreszenzgen, wird die intrazelluläre Metabolitenkonzentration in ein Fluoreszenzsignal übersetzt. Mit solchen Biosensoren lassen sich biotechnisch relevante Metaboliten mit optischen Methoden auf Einzelzellebene sichtbar machen. Bei-

spiele für Biosensoren, die in der Vergangenheit in *C. glutamicum* etabliert wurden, sind der Lrp-⁴⁾ und der LysG-Biosensor.⁵⁾

Der Lrp-Biosensor basiert auf dem Transkriptionsfaktor Lrp (leucine-responsive protein) und macht die intrazellulären L-Methionin-, L-Valin-, L-Leucin- und L-Isoleucin-Konzentrationen sichtbar (Abbildung 1B, S. 590).⁴⁾ Eine erhöhte intrazelluläre Konzentration dieser Aminosäuren führt dazu, dass Lrp an seinen Zielpromotor (*brnFE*) bindet. Außerdem wird dadurch die Expression eines spezifischen Exportsystems aktiviert.⁶⁾ Durch die Fusion des Zielpromoters mit dem Reporter gen *eyfp* (enhanced yellow fluorescent protein) wird die intrazelluläre Konzentration der Effektoramino säuren in ein Fluoreszenzsignal übersetzt.⁴⁾ →

QUERGELESEN

- » Biosensoren weisen Aminosäuren, Dicarbonsäuren oder Alkohole nach.
- » Genetisch kodierte Biosensoren übersetzen intrazelluläre Metabolitenkonzentrationen in ein optisch messbares Signal, etwa Fluoreszenz. Das macht Metabolite in Hochdurchsatzscreenings erfassbar.
- » Mit solchen Sensoren lassen sich Mikroorganismen so manipulieren, dass sie große Mengen einer bestimmten Substanz produzieren.

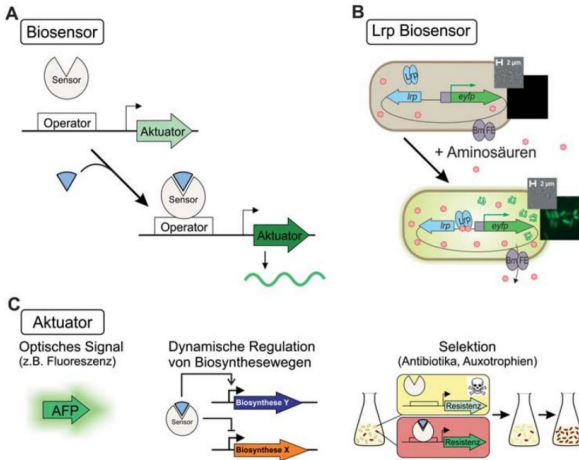


Abb. 1. A) Aufbau eines Transkriptionsfaktor(TF)-basierten Biosensors. Abhängig vom Effektor (blaues Dreieck) bindet der TF an den Zielpromotor und beeinflusst so die Transkription des Aktuatorgens. B) Aufbau und Funktion des Lrp-Biosensors.⁴⁾ C) Es sind verschiedene Ausgangssignale des Biosensors wählbar.^{1,4)}

Der Aufbau des LysG-Biosensors basiert auf einem ähnlichen Prinzip, ebenso wie etliche andere Beispiele aus den letzten Jahren.⁵⁾

Mit Biosensoren lassen sich nicht nur Aminosäuren detektieren. So gibt es Biosensoren, die Dicarbonsäuren (Succinat) und Alkohole (Butanol) nachweisen.⁷⁾

Die Art des Signals eines Biosensors lässt sich je nach Anwendung wählen. Sie produzieren nicht nur effektorabhängig Autofluoreszenzproteine (AFP), sondern die Biosensoren lassen sich auch in zellinterne oder -externe regulatorische Schaltkreise integrieren, um einzelne Biosynthesewege dynamisch zu regulieren (Abbildung 1C). Die Expression der Biosynthesegene ist dabei beispielsweise von der Verfügbarkeit einer bestimmten metabolischen Vorstufe abhängig. Alternativ lässt sich durch die effektorabhängige Expression von Selektionsmarkern wie Antibiotikaresistenzen⁷⁾ das Überleben der Zellen an ihre Produktivität koppeln: Nur Zellen mit den gewünschten Eigenschaften vermehren sich (Abbildung 1C).

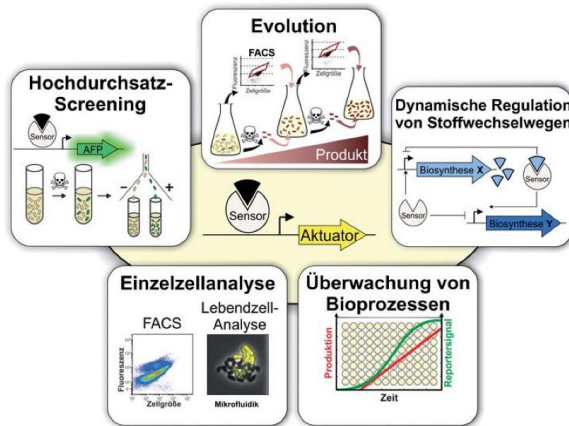


Abb. 2. Anwendungen genetisch kodierter Biosensoren in der Biotechnologie. Biosensoren, die die intrazelluläre Konzentration eines spezifischen Metaboliten in ein optisches Signal wie Fluoreszenz übersetzen, dienen der nichtinvasiven Überwachung von Bioprosessen und für die zeitlich aufgelöste Visualisierung der Produktion auf Einzelzellebene. In Hochdurchsatzscreenings oder bei adaptiver Evolution ermöglichen Biosensoren, Klone mit erhöhter Produktivität aus großen Stammbibliotheken oder Populationen auszuwählen und anzureichern.²⁾

Anwendung von Biosensoren

◆ Biosensoren lassen sich vielseitig anwenden (Abbildung 2).¹⁾ Sie ermöglichen etwa, Bioprosesse im Bioreaktor mit hoher Zeitauflösung zu überwachen. Mit dem Lrp-Biosensor ließ sich für einen *C. glutamicum*-l-Valin-Produzenten ($\Delta aceE$) eine zeitliche Entkopplung von Wachstum und l-Valin-Produktion nachweisen.⁴⁾ Dass Mikroorganismen gleichen Genotyps variable Phänotypen bilden können, ist eine universelle Überlebensstrategie, kann jedoch die Ausbeute in biotechnischen Produktionen reduzieren.⁸⁾ Wie die Analyse des l-Valin-Produzenten *C. glutamicum* $\Delta aceE$ im mikrofluidischen Kultivierungssystem kombiniert mit dem Lrp-Biosensor zeigt, entstehen während der l-Valin-Produktion unproduktive Subpopulationen.⁹⁾

Neben Mikroskopie eignet sich Durchflusszytometrie, um Fluoreszenz auf Einzelzellebene zu messen. Die intrazelluläre Produktkonzentration sichtbar zu machen, ist die Voraussetzung, um Zellen unterschiedlicher Produktivität fluoreszenzaktiviert zu sortieren (fluoreszenzaktivierte Sortierung, FACS).

Biosensorgestützte FACS-Hochdurchsatzscreenings von Mutantenbibliotheken, die durch Zufallsmutagenese erstellt wurden, sind Methode der Wahl, um Klone mit gesteigerter Produktivität zu isolieren.^{4,5} Auf Basis der Lrp- und LysG-Biosensoren ließen sich in ähnlichen Ansätzen mit FACS-Screening aus chemisch mutagenisierten *C. glutamicum*-Wildtypzellen Klone isolieren, die etwa zehnmal mehr Aminosäure produzieren als die Ursprungszelle.^{4,5}

Biosensorbasierte Evolution

Die vielen Mutationen, die durch Zufallsmutagenese entstehen, erschweren es, produktionsrelevante Mutationen zu identifizieren. Hier hilft der Blick auf natürliche Systeme und Organismen.

Auch natürliche Mutageneseraten von 10^{-10} bis 10^{-9} Mutationen pro Basenpaar und Replikation führen bereits zu genetischer Diversität (Abbildung 3, S. 592).¹⁰ Diese ist zusammen mit der Selektion vorteilhafter Mutationen die Triebfeder der Evolution. Im natürlichen Lebensraum des Mikroorganismus etablieren sich vor allem fitnessrelevante Mutationen.

Dass sich evolutive Ansätze für die Stammoptimierung anwenden lassen, hat sich bereits an einer verbesserten Anpassung an Stressbedingungen gezeigt. Die Toleranz von *C. glutamicum* gegenüber Isobutanol und Hitze steigerte sich durch wiederholtes Einwirken des Stressstimulus.¹¹ Dabei besteht ein direkter Zusammenhang zwischen der Fitness des Organismus und der zu optimierenden Stammeigenschaft, sodass während der experimentellen Evolution vorteilhafte

Mutationen entstanden. Typischerweise treten bei solch evolutionsbasierten Ansätzen nur wenige Mutationen auf – ein Vorteil verglichen mit der Zufallsmutagenese.

Bei biotechnisch relevanten Metaboliten funktioniert dieses evolutionsbasierte Vorgehen jedoch nur eingeschränkt. Denn oft sinkt mit gesteigerter Produktion die Fitness der Zellen. Das lässt sich durch Biosensoren ändern und liefert damit die Basis, um mit biosensorgestützten Evolutionsansätzen mikrobielle Produktionsstämme zu verbessern. In einer aktuellen Studie geschah dies durch die Kombination von FACS-Hochdurchsatz-Screenings und dem optischen Signal des Lrp-Biosensors. Durch wiederholte FACS-Screenings ließ sich ein künstlicher Selektionsdruck auf die Produktivität des L-Valin-Produzenten *C. glutamicum* $\Delta accE$ ausüben.¹² Innerhalb weniger Tage steigerten abwechselnde Sortierung und Rekulтивierung sowohl das Wachstum als auch den Produktivität um bis zu 100 Prozent (Abbildung 3A, S. 592). Die anschließende Genomanalyse isolierter Klone zeigt nur sieben Mutationen. Die Analyse dieser Mutationen zeigt eine gesteigerte Produkt- (L-Valin) und eine reduzierte Nebenproduktbildung (L-Alanin) sowie eine positive Wirkung auf das Zellwachstum (Abbildungen 3B und C).¹² →

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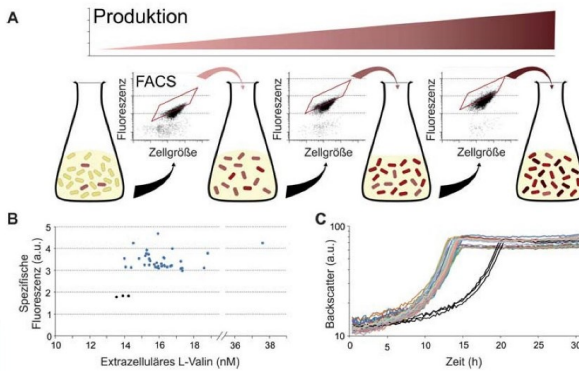


Abb. 3. Biosensorbasierte Evolution des L-Valin-Produzenten *C. glutamicum* Δ aceE. A) Evolutionsansatz: Natürliche Mutagenese führt zu genetischer Variabilität. Das Signal des Lrp-Biosensors dient als künstlicher Selektionsdruck. Durch wiederholte Sortierungen und Reklutierungen reichern sich Zellen mit hohem Sensorsignal an. B) Klone des evolvierten (blau) Δ aceE-Sensorstamms haben ein stärkeres Lrp-Sensorsignal und produzieren mehr L-Valin als nicht evolvierte Klone (schwarz). Zudem bilden sie schneller mehr Biomasse (C).¹²⁾

Neue Biosensoren entwickeln

◆ Biotechnische Produktionsstämme basieren in den meisten Fällen auf gut untersuchten Transkriptionsfaktoren. Damit entsteht gewissermaßen ein neuer Flaschenhals bei der zeit- und kosteneffizienten Entwicklung neuer Schaltkreise und Sensoren.

Promotorbibliotheken, Transkriptom- und Proteomdaten können das Repertoire nützlicher Transkriptionsfaktoren zwar erweitern,

jedoch sind Sensitivität, dynamischer Bereich oder Effektorspezifität natürlicher Systeme nicht immer optimal für die jeweilige Anwendung. Hier bietet die synthetische Biologie durch Kombinieren orthogonaler Bausteine Lösungsansätze, um Schaltkreise oder Sensoren mit den gewünschten Eigenschaften zu entwerfen. Dennoch wissen wir nach wie vor zu wenig über biologische Systeme, um uns auf rationales Design zu beschränken. Sowohl um neue Biosensoren zu entwickeln als

auch um neue produktionsrelevante Mutationen zu identifizieren, sehen wir Potenzial in der Kombination von synthetischer Biologie und mikrobieller Evolution.

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Genetisch codierte Biosensoren für die Entwicklung mikrobieller Produktionsstämme im Hochdurchsatz nutzt das Unternehmen SenseUp in Jülich: www.senseup.de



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- 4.3. Supplemental material to “Deciphering the rules underlying xenogeneic silencing and counter-silencing of Lsr2-like proteins using CgpS of *Corynebacterium glutamicum* as a model”

1

Supplemental Material to:

Deciphering the rules underlying xenogeneic silencing and counter-silencing of Lsr2-like proteins using CgpS of *Corynebacterium glutamicum* as a model

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Content:**Supplemental Text**

Text S1: Supplementary information on methods used in this study.

Supplemental Tables

Table S1: TSS analysis of relevant phage and non-phage-related CgpS target promoters after prophage induction with mitomycin C.

Table S2: Strains, plasmids, oligonucleotides, and DNA sequences.

Supplemental Figures

Figure S1: Counter-silencing of CgpS target promoters.

Figure S2: Effects of inserted sequences independent of GntR binding.

Figure S3: Effects of directionality of inserted GntR binding site on counter-silencing efficiency.

Figure S4: It is all about context: impact of GntR binding on the output of the P_{lys} promoter in the presence or absence (Δ phage) of CgpS.

Figure S5: Impact of GntR binding site (BS) position on inducibility of P_{cg1999} -based promoter constructs.

Figure S6: Definition of the minimal region required for silencing.

1 **SUPPLEMENTAL MATERIAL**

2

3 **Text S1: Supplementary information on methods used in this study**

4 **Growth conditions, monitoring of cell growth and fluorescence during microtiter**
5 **cultivation and cultivation in the microfluidic chip device**

6 **Growth conditions**

7 For all cultivations of *C. glutamicum* strains, brain heart infusion (BHI, Difco Laboratories,
8 Detroit, MI, USA) complex medium was inoculated with a single colony from a fresh agar
9 plate and incubated for 8 to 16 hours. All cultivation steps were performed at 30°C. For
10 reporter-based assays, BHI pre-cultures were used to inoculate a second overnight pre-
11 culture in CGXII minimal medium (1) supplemented with 25 µg/ml kanamycin and 100/111
12 mM glucose or 100 mM gluconate. Subsequently, the pre-culture was used to inoculate
13 the main culture at a start OD₆₀₀ of 1. Main cultures were cultivated in CGXII medium with
14 25 µg/ml kanamycin and 100/111 mM glucose, 100 mM gluconate or 100 mM fructose. *E.*
15 *coli* DH5α and BL21 (DE3) were used for plasmid amplification and *cgpS* overexpression,
16 respectively. Strains were cultivated in Lysogeny Broth (LB) media or on LB agar plates
17 at 37°C. If needed, 50 µg/ml kanamycin was added. *Vibrio natriegens* VmaxTM (Synthetic
18 Genomics, San Diego, CA, USA) was used for GntR (Cg2783) protein production and
19 cells were cultivated in BHIN complex medium (BHI + 15 g/l NaCl) supplemented with 50
20 µg/ml carbenicillin at 30°C.

21 **Microtiter cultivation to monitor cell growth and fluorescence**

1

22 Reporter-based analysis in microliter scale was performed in the BioLector®
23 microcultivation system (m2p-labs, Aachen, Germany) (2). Therefore, 750 µl of the main
24 culture (see Growth conditions) were cultivated in 48-well FlowerPlates (m2p-labs,
25 Aachen, Germany) at 30°C and 1200 rpm. Biomass production was measured as
26 backscattered light intensity of sent light with a wavelength of 620 nm (signal gain factor
27 20). Venus fluorescence was measured with an excitation wavelength of 508 nm and
28 emission wavelength of 532 nm (signal gain factor 60). Samples were measured at 15
29 min intervals. Arbitrary units (a.u.) of specific fluorescence were calculated by dividing the
30 Venus signal by the backscatter signal per time point (2). Obtained specific fluorescence
31 values were background corrected by subtracting values of strains harboring the control
32 plasmid pJC1-*venus-term* (no promoter in front of *venus*) (3) which were cultivated under
33 comparable conditions. Shown fold changes were calculated based on the ratio of induced
34 versus non-induced reporter outputs and correspond to reporter inducibility.

35

36 **Cultivation in microfluidic chip device**

37 The temporal dynamics of the genetic toggle switch were analyzed on single-cell level by
38 cultivating *C. glutamicum* wild type cells harboring the plasmid-based construct in an in-
39 house developed microfluidic platform (4, 5). The chip design and the experimental setup
40 was performed as described before (6). Phase contrast as well as Venus and E2-Crimson
41 fluorescence were imaged at 20 min intervals by fully motorized inverted Nikon Eclipse Ti
42 microscope (Nikon GmbH, Düsseldorf, Germany) as described previously (4, 5, 7). The
43 exposure times for phase contrast was 100 ms, for Venus 200 ms and for E2-crimson 300
44 ms. Cells were cultivated in the microfluidic chip system in CGXII medium supplemented

45 with 25 µg/ml kanamycin and either 100 mM gluconate or 111 mM glucose. Continuous
46 medium supply with a flow rate of 200 nl/min and waste removal was achieved by a high-
47 precision syringe pump system (neMESYS, Cetoni GmbH, Korbussen, Germany) using
48 disposable syringes (Omnifix-F Tuberculin, 1 ml; B. Braun Melsungen AG, Melsungen,
49 Germany). After 17 hours of cultivation, the carbon source supply was switched from
50 gluconate to glucose or vice versa by changing the syringes and the connecting tubing to
51 ensure an immediate medium change. The temperature was set to 30°C during the
52 complete cultivation using an incubator system (PeCon GmbH, Erbach, Germany). Data
53 analysis was performed using the image-processing package Fiji (8) which is based on
54 ImageJ (9) on colony level. Obtained fluorescence data were background normalized and
55 plotted with GraphPad prism 7.00 (GraphPad Software, La Jolla, CA, USA).

56

57 **Construction of strain Δ phage::P_{cgpS}-cgpS and design of disruptive counter-**
58 **silencing constructs**

59 **Construction of strain Δ phage::P_{cgpS}-cgpS**

60 Re-integration of *cgpS* in the prophage-free strain Δ phage (MB001) (3) with its native
61 promoter was performed with the pK19mobsacB-1199_1201-P_{cgpS}-*cgpS* integration
62 plasmid. This plasmid contains the *cgpS* gene fused to its native promoter, which was
63 flanked by 500 bp upstream and downstream regions of the integration site (intergenic
64 region of cg1199-cg1201). Two step homologous recombination and selection was
65 performed as described previously (10). Successful integration was verified by
66 sequencing using the oligonucleotides cg1199_1201_seq_fw and cg1199_1201_seq_rv
67 (Table S2G).

68

69 Design of disruptive counter-silencing constructs

70 Reporter studies were performed based on plasmid pJC1 (approximately 30 copies per
71 cell) (11, 12). All counter-silencing constructs were based on the same design scheme.
72 First, the native promoter region was amplified from *C. glutamicum* genomic DNA, fused
73 to the gene *venus* via a consistent linker, which contains a ribosomal binding site, and
74 inserted into the pJC1 plasmid using Gibson assembly (13). To ensure that all regulatory
75 promoter elements are present, forward primers were designed so that the 5'-sequence
76 end coincided with the upstream end of the CgpS binding peak (14). Reverse primers
77 were chosen so that the promoter constructs contain the first 30 bp of the coding
78 sequence. The resulting plasmids serve as template for the counter-silencer constructs.
79 Overlap PCR was performed for the insertion of the GntR binding site (BS:
80 TATGATAGTACCAAT) (15) at different positions. All constructed plasmids are listed in
81 Table S2C and oligonucleotides are listed in Table S2D.

82

83 Determination of transcriptional start sites (TSS)

84 For the determination of the TSS, *C. glutamicum* wild type cells were cultivated in CGXII
85 supplemented with 111 mM glucose starting at an OD₆₀₀ of 1. After one-hour cultivation
86 at 30°C, the SOS response was induced by adding 600 nM MMC leading to prophage
87 induction (16). 50 ml cultures were harvested on ice after one, three and six hours of
88 cultivation at 30°C, respectively. Pellets after centrifugation (5300 g, 4°C, 15 min) were
89 snap-frozen in liquid nitrogen and stored at -80°C until use. Total RNA was prepared using
90 the RNeasy Mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer

91 protocol. Subsequently, all three RNA samples were pooled in approximately equal
92 amounts. The determination of the TSS and data analysis was performed by Vertis
93 Biotechnology AG (Vertis Biotechnology AG, Freising, Germany) using the Cappable-seq
94 method developed by Ettwiller and Schildkraut (17). Obtained reads were mapped against
95 the reference *C. glutamicum* genome BX927147 (18). Relative read score at a certain
96 position and in a certain orientation (+ or - strand) ($RRS_{io} = (\text{number of reads at position } i \text{ in orientation } o / \text{total number of mapped reads}) * 1000000$) were calculated by the
97 company for a non-enriched control library (cutoff: 0) and for the enriched Cappable-seq
98 library (cutoff: 5). The enrichment score (enrichment score = $\log_2 (RRS_{io} \text{ TSS} / RRS_{io} \text{ control})$) was calculated based on the RRS_{io} of both libraries (cutoff: 3). Five bp of
100 upstream and downstream region were used for clustering of TSS. Relevant TSS located
101 in the promoter region (500 bp upstream of the start codon) and directed in gene
102 orientation were assigned to relevant phage genes. Multiple TSS mapped to the same
103 promoter were ranked depending on their enrichment scores (Table S1).

104
105

106 **Analyses of AT-rich stretches in CgpS binding regions**

107 Scanning of the *C. glutamicum* genome (BX927147 (18)) for AT-rich stretches was
108 performed using a custom python script (submitted to GitHub:
109 https://github.com/afilipch/afp/blob/master/genomic/get_at_stretches.py). AT-rich
110 stretches were determined as the longest possible sequences with at least 70%
111 adenosine/thymidine (AT)-content and a number of guanosines (G) and cytidines (C)
112 below a particular threshold. The scan was performed multiple times with incrementing
113 the limit for maximal allowed G/C interruptions inside AT-rich sequences. The results of

114 these multiple scans were then pulled together without further collapsing, meaning that
115 the discovered AT-rich sequences with different G/C numbers may overlap each other.
116 AT-rich regions were then grouped based on their lengths and G/C numbers (or number
117 of AT steps). The grouping was done in a way to fulfil two requirements: the groups should
118 be roughly equal and cover the whole dynamic range of the selected parameters.
119 Subsequently, the fraction of AT-rich stretches overlapping (full overlap) with CgpS
120 binding peaks were identified for each group (sequences were previously deposited in the
121 GEO database (ncbi.nlm.nih.gov/geo) under accession number GSE80674).

122

123 **Protein purification, surface plasmon resonance (SPR) spectroscopy and**
124 **electrophoretic mobility shift assay (EMSA)**

125 **Protein purification**

126 C-terminal Strep-tagged CgpS (Cg1966) was heterologously overproduced in *E. coli* BL21
127 (DE3) (19) harboring the plasmid pAN6-*cgpS*-Strep (14). Cell cultivation and protein
128 purification was performed as described before by Pfeifer and colleagues (14) except for
129 an increased amount of 15 mM d-desthiobiotin in the elution buffer. Purified Strep-tagged
130 CgpS was used for surface plasmon resonance measurements.

131 GntR (Cg2783) with a 21 amino acids long N-terminal decahistidine tag
132 (MGHHHHHHHHSSGHIEGRH) was heterologously overproduced in *Vibrio natriegens*
133 Vmax™ (Synthetic Genomics, San Diego, CA, USA) harboring the plasmid pET16b-*gntR1*
134 (15). Cells were grown in BHIN complex medium (BHI + 15 g/l NaCl) supplemented with
135 50 µg/ml carbenicillin and incubated at 30°C. Protein production was induced by the
136 addition of 1 mM Isopropyl-β-D-1-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.3. After
6

137 four hours of subsequent cultivation, cells were harvested by centrifugation (5300 g, 4°C,
138 15 min). The pellet was resuspended in TNI20 buffer (20 mM Tris/HCl pH 7.9, 300 mM
139 NaCl and 20 mM imidazole) and cells were disrupted by three passages through a French
140 pressure cell (SLM Aminco, Spectronic Instruments, Rochester, NY, USA) at 172 mPa.
141 To remove cell debris, cell lysate was centrifuged at 5300 g, 4°C for 15 min and
142 subsequently ultracentrifuged for one hour (229000 g, 4°C). His-tagged GntR protein from
143 the supernatant was purified with gravity-flow chromatography using Ni-NTA agarose
144 columns (QIAGEN, Venlo, Netherlands). Columns were washed with TNI20 buffer
145 followed by protein elution with TNI200 buffer (20 mM Tris/HCl pH 7.9, 300 mM NaCl and
146 200 mM imidazole). Protein fractions were pooled and buffer was exchanged against TG
147 buffer (30 mM Tris/HCl pH 7.5, 10% (v/v) glycerol) using a PD-10 Desalting Column (GE
148 Healthcare, Chicago, IL, USA). Subsequently, GntR was snap-frozen in liquid nitrogen
149 and stored at -80°C before it was used for EMSAs.

150

151 **Surface plasmon resonance (SPR) spectroscopy**

152 Binding of Strep-tagged CgpS to native or synthetic target promoters was analyzed by
153 SPR analysis in a Biacore T200 and a Biacore 3000 device (GE Healthcare, Freiburg,
154 Germany) using carboxy-methyl dextran sensor chips pre-coated with streptavidin
155 (XanTec SAD500L (XanTec Bioanalytics GmbH, Düsseldorf, Germany) and Sensor Chip
156 SA (GE Healthcare, Freiburg, Germany). As first step, DNA-fragments covering the
157 respective promoters or the promoter region of the gene cg3336 (negative control) were
158 amplified by using biotinylated primer *via* two-step PCR as described in Table S2E. All
159 experiments were carried out at a constant temperature of 25°C in HBS-EP+ buffer (10

160 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.05% (v/v) detergent P20). Before
161 immobilization of the DNA fragments, the chips were equilibrated by three injections of 1
162 M NaCl/50 mM NaOH using a flow rate of 10 μ l/min. Then, 10 nM of the respective double-
163 stranded biotinylated DNA fragment was injected at a flow rate of 10 μ l/min for a total
164 contact time of 420 s. The chips were then washed by injecting 1 M NaCl/50 mM
165 NaOH/50% (v/v) isopropanol. Approximately 300-550 RU (response units) of the relevant
166 DNA fragment was bound per flow cell. Binding analysis of CgpS with the five DNA
167 fragments P_{cg3336} , P_{cg1999} , $P_{cg1999_A-T/G-C}$, the native phage promoter P_{lys} or the
168 corresponding counter-silencer construct $P_{lys_CS_0}$ were performed at a flow rate of 30
169 μ l/min in HBS-EP+ buffer at 25°C. Various concentrations of CgpS (1 nM–5000 nM)
170 dissolved in HBS-EP+ buffer, were passed over the flow cells for 180 s, and the complexes
171 formed were allowed to dissociate for 420 s before the next cycle started. After each cycle,
172 the surface was regenerated by injection of 2.5 M NaCl for 30 s, followed by 0.5% (w/v)
173 SDS for 60 s, at a flow rate of 30 μ l/min. All experiments were performed at 25°C.
174 Sensorgrams were recorded using Biacore T200 Control Software 2.0 or Biacore 3000
175 Control Software 4.1.2, respectively, and analyzed with Biacore T200 Evaluation Software
176 2.0, BIAevaluation software 4.1.1 or TraceDrawer software 1.5 (Ridgeview Instruments,
177 Uppsala, Schweden). The surface of flow cell 1 was not coated and used to obtain blank
178 sensorgrams for subtraction of the bulk refractive index background. The referenced
179 sensorgrams were normalized to a baseline of 0. Peaks in the sensorgrams at the
180 beginning and the end of the injection are due to the run-time difference between the flow
181 cells for each chip.

182

183 Electrophoretic mobility shift assay (EMSA)

184 Binding of GntR to the native P_{lys} promoter and the corresponding counter-silencer
185 construct $P_{lys_CS_0}$ was analyzed by electrophoretic mobility shift assays (EMSAs).
186 Primers and templates used for the PCR amplification of DNA fragments are listed in
187 Table S2F. The PCR products were purified from an agarose gel with the PCR clean-up
188 and gel extraction kit of Macherey Nagel (Düren, Germany). 14 nM DNA fragments
189 covering the promoter regions (P_{lys} : 518 bp; $P_{lys_CS_0}$: 533 bp) were incubated for 15 min
190 at room temperature with varying amounts (0, 28, 70, 140, 180 or 210 nM) of N-terminal
191 tagged GntR (decahistidine tag) (15) in binding buffer (200 mM Tris/HCl pH 7.5, 200 mM
192 KCl, 25 mM MgCl₂, 25% (v/v) glycerol, 0.5 mM EDTA). If indicated, 100 mM gluconate or
193 glucose was added to the binding buffer. All samples were loaded onto a native 10%
194 polyacrylamide gel (loading dye: 0.01% (w/v) xylene cyanol dye, 0.01% (w/v)
195 bromophenol blue dye, 20% (v/v) glycerol, 1xTBE (89 mM Tris base, 89 mM boric acid, 2
196 mM Na₂-EDTA)) and electrophoresis was performed at 170 V and room temperature with
197 1x TBE buffer. Gels were stained with SybrGreen I following the manufacturer instructions
198 (Sigma-Aldrich, St. Louis, MO, USA).

199

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Table S1: TSS analysis of relevant phage and non-phage-related CgPS target promoters after prophage induction with mitomycin C.

Table S1: TSS analysis of relevant phage CgPS target promoters after prophage induction with mitomycin C. TSS positions of phage-related CgPS targets located maximal 500 bp upstream of the start codon and orientated in the appropriate direction are listed. TSS identified in previous studies of Pfeifer-Sancar and colleagues (K. Pfeifer-Sancar, A. Mentz, C. Rückert, and J. Kallinowski. BMC Genomics 14:888, 2013. doi: 10.1186/1471-2164-14-888) (accession number of the sequencing run: ERR358919 (https://trace.ncbi.nlm.nih.gov/Traces/trace/?un=ERR358919)) are given in column TSS ERR358919. TSS identified in this study after mitomycin C induction were identified by using the method developed by Etwiller and Schickel (E. Etwiller and M. Schickel. BMC Genomics 13:102, 2012. doi: 10.1186/1471-2164-13-102) (accession number of the sequencing run: ERR358919) and are given in column TSS CgPS. TSS identified in this study after mitomycin C induction were identified by using the method developed by Pfeifer, M. Hünnefeld, O. Popa, T. Pönn, D. Kohlmeier, M. Baumgart, and J. Frenzke. Nucleic Acids Res 44:10117-10131, 2016. doi: 10.1093/nar/gkv692; GEO database (ncbi.nlm.nih.gov/gso), accession number GSE41132) are listed. Promoters were grouped into three different classes based on the CgPS coverage peak width (Class 0, < 500 bp (grey); class 1, 500 bp - 850 bp (orange); class 2, > 850 bp (blue)). Distance TSS-Position maximal CgPS coverage peak*, TSS located upstream of maximal CgPS binding peak, negative value; downstream, positive values.

Locus	Class	Gene start	Gene end	Strand	CgPS peak start	CgPS peak end	Peak width (bp)	Position maximal CgPS peak	TSS ERR35819	TSS CgPS	Distance TSS-Position maximal CgPS coverage peak*	Distance TSS-Position maximal CgPS coverage peak*	Enrichment CAPPABEL E_SEQ	Enrichment CAPPABEL E_SEQ	Relative read score (RRS)	Distance TSS-Position maximal CgPS coverage peak*
cg1891	Class 2	1781586	1781023	-	1781272	1782247	975	1781685	/	1781664	516	4.84	21	78		
cg1895	Class 2	1786945	1784363	-	1786615	1787489	874	1787044	/	1787045	559	4.54	-1	100		
										1787064	86	4.1	-20	119		
cg1896	Class 0	1786316	1787201	-	1788157	1788501	344	1788341	/	/	/	/	/	/		
cg1897	Class 1	1790301	1789376	-	1789987	1790769	782	1790389	1790360	1790326	139	3.31	63	25		
										1790360	1188	3.15	29	59		
										1790779	82	3.19	-390	478		
cg1905	Class 0	1795186	1796295	+	1794804	1795079	275	1794937	1794978	1794881	155	4.11	-56	305		
										1795037	317	4.06	100	149		
										1795152	67	6.06	215	34		
cg1910	Class 0	1799441	1799319	-	1799243	1799650	407	1799454	1799699	1799486	211	4.4	-32	45		
										1799564	264	3.58	-110	123		
										1799659	7460	3.58	-245	258		
cg1911	Class 1	1800229	1799492	-	1799994	1800619	625	1800361	1800279	1800361	625	3.58	-245	258		

cg1917	Class 2	1804049	1803624	-	1803784	1804670	876	1804202	1804078	1800279	4586	4.08	82	50
		1800374	368							1800374	368	5.68	-13	145
		1800383	242							1800383	242	4.6	-22	154
		1800405	83							1800405	83	4.05	-44	176
		1800583	125							1800583	125	6.96	-222	354
1804049	246								1804049	246	6.36	153	0	
1804061	217								1804061	217	4.95	141	12	
1804078	10441								1804078	10441	4.31	124	29	
1804110	279								1804110	279	3.08	92	61	
1804153	136								1804153	136	4.28	49	104	
cg1936	Class 2	1816589	1817726	+	1816583	1816747	1064	1816143	/	1816311	1126	7.33	168	48
cg1938	Class 1	1817923	1818111	+	1817286	1818040	754	1817726	/	/	/	/	/	/
cg1940	Class 2	1818904	1819278	+	1818041	1818913	872	1818495	/	1818523	1047	5.78	28	81
										1818536	1066	6.74	41	68
										1818543	232	4.4	46	61
cg1955	Class 1	1832237	1833538	+	1831721	1832564	843	1832185	/	1832083	71	3.15	-102	154
cg1957	Class 1	1836400	1835618	-	1836032	1836584	552	1836450	1836678	1836417	80	4.73	33	17
										1836440	61	3.61	10	40
										1836678	280005	3.95	-228	278
										1836890	136	5.09	-240	290
										1836701	56	5.8	-251	301
										1836716	121	4.11	-266	316
										1836755	71	6.14	-305	355
										1836807	542	5.18	-357	407
cg1959 (prf)	Class 1	1838024	1839799	+	1837413	1838254	841	1837816	/	1837928	82	3.77	12	196

cg2008	Class 1	1898703	1899208	-	1898376	1899221	845	1898780	1899761	/	1894632	93	6.53	-89	123	
		1894705										1894705	60	5.9	-162	196
		1894864										1894864	202	6.66	-321	355
cg2011	Class 1	1902388	1901273	-	1902030	1902751	721	1902443	/		1902413	61	3.93	30	25	
											1902437	242	5.92	6	49	
cg2014	Class 2	1904355	1903474	-	1904017	1904600	883	1904446	1904428		1904355	110	6.77	91	0	
											1904428	28397	7.2	18	73	
cg2018	Class 1	1910132	1909434	-	1909773	1910579	806	1910175	/		1910180	5139	5.51	-5	48	
											1910430	83	6.37	-255	298	
											1910587	67	6.06	-422	465	
cg2020	Class 1	1911980	1910970	-	1911615	1912370	765	1912012	/							
cg2022	Class 1	1915265	1913802	-	1914947	1915574	627	1915392	/		1915290	223	4.48	2	25	
											1915305	68	3.28	-13	40	
											1915315	2282	4.25	-23	50	
cg2023	Class 1	1915801	1915442	-	1915575	1916307	732	1915888	/		1915810	94	4.97	78	9	
											1915863	313	3.77	36	52	
											1916048	117	5.28	-160	247	
cg2032	Class 1	1927482	1926016	-	1927172	1927972	800	1927582	1927569		1927569	4881	4.99	13	87	
											1927931	126	3.17	-249	349	
											1927946	248	6.37	-364	464	
cg2043	Class 0	1937384	1936440	-	1937475	1937714	239	1937547	1937565		1937387	846	4.87	150	13	
											1937460	143	6.16	87	76	
											1937547	647	5.53	0	163	
										1937565	6447	4.78	-18	181		

Table S1. TSS analysis of non-phase-related CpGs target promoters after induction with mitomycin C. TSS positions, located maximal 500 bp upstream of the start codon and orientated in the appropriate direction are listed. TSS identified in previous studies of Pfeifer-Sancar and colleagues (K. Pfeifer-Sancar, A. Menz, C. Ruckert, and J. Kallrosek. BMC Genomics 14:888, 2013; doi: 10.1186/1471-2164-14-888) (accession number of the sequencing run: ERR356819 (https://trace.ncbi.nlm.nih.gov/Traces/sra/?run=ERR356819)) are given in column TSS. ERR356819. TSS determination after mitomycin C induction was performed by Veris Biotechnology AG (Freising, Germany) using the Cappable seed method developed by Eitwiler and Schidkrait (L. Eitwiler, J. Buswell, Hummelfeld, O. Pops, T. Polen, D. Kontheyer, M. Baumgart, and J. Frunzke. Nucleic Acids Res 44:10177-10131, 2016; doi: 10.1093/nar/gkw652). Additionally, positions of maximal CpGs coverage and ranges of CpGs binding peaks (E. Pfeifer, M. Hummelfeld, O. Pops, T. Polen, D. Kontheyer, M. Baumgart, and J. Frunzke. Nucleic Acids Res 44:10177-10131, 2016; doi: 10.1093/nar/gkw652) are listed. Distance TSS-Position maximal CpGs coverage peak. TSS located upstream of maximal CpGs binding peak. Relative value of CpGs coverage peak. CpG peak end. CpG peak start. Strand. Gene start. Gene end. Locus. CpG peak width (bp). Peak width. Position maximal CpGs peak. TSS. ERR356819. Cappable _SEQ. Relative read score. Enrichment score. Cappable _SEQ. Distance TSS-ATG. TSS-ATG. Position maximal CpGs coverage peak.

Locus	Gene start	Gene end	Strand	CpG peak start	CpG peak end	Peak width (bp)	Position maximal CpGs peak	TSS. ERR356819. Cappable _SEQ	Relative read score	Enrichment score	Cappable _SEQ	Distance TSS-ATG	
cg0002	1920	1594	-	1863	2163	301	2007	/	/	/	/	106	-23
cg0160	136566	136120	-	136573	136691	119	136649	136543	/	/	/		
cg0432	380843	379848	-	380625	381100	476	380893	380902	380902	600	3.84	-9	59
								380975	380975	476	3.99	-82	132
								381009	381009	157	4.97	-116	166
								381209	381209	85	5.41	-316	366
cg0435	381949	383112	+	381669	381862	194	381726	381491	381491			-235	458
								381726	381726			0	223
								381861	381861			135	88
								381491	381491	1816	3.93	-235	458
								381726	381726	55	4.19	0	223
								381861	381861	103	4.69	135	88
cg0442	389249	390172	+	389104	389315	212	389207	389249	389249			42	0
								389249	389249	1534	5.45	42	0
cg0723	640893	639775	-	640718	641333	616	641032	641007	641007			25	114
								641007	641007	107	6.73	25	114
cg0726	642340	641690	-	642039	642708	670	642376	/	/	/	/		
								642358	642358	90	4.9	18	18
								642386	642386	1285	6.63	-10	46

cg1028	960467	969310	-	960435	960630	396	960616	/	960571	343	4.61	45	104
									960580	262	3.79	36	113
									960615	282	7.14		148
cg1347	1258070	1256202	-	1258077	1258437	361	1258267	/	/				
cg1444	1346402	1346890	+	1346292	1346511	220	1346400	1346402	1346230	485	5.6	-170	172
cg1517	1409328	1409023	-	1409317	1409598	282	1409451	1409389	1409389	59	4.88	62	61
cg1609	1499791	1498259	-	1499717	1499669	253	1499819	/	1499936	1102	4.86	-117	145
cg2462	2349440	2349552	+	2348310	2349518	209	2346453	2346571	2349160	62	4.37	-293	280
									2349208	240	4.21	-245	232
cg2722	2594635	2595282	+	2594491	2594680	170	2594601	2594635	2594285	3274	4.22	-316	350
									2594474	318	4.85	-127	161
cg2783	2647165	2647917	+	2646731	2647420	690	2647059	2647114	2647114	2028	3.42	55	51
cg2896	2757790	2755646	-	2757694	2757991	298	2757962	2757823	/			39	33
cg2903	2763214	2762783	-	2763310	2763542	233	2763408	2763250	2763250			158	36
									2763255			153	41
									2763263			145	49
									2763371			37	157
cg3057	2919709	2918842	-	2919541	2920009	469	2919730	2919739	2763371	194	5.6	37	157
									2919739	411	3.88	-9	30
									2919828	94	5.56	-198	219
cg3342	3190514	3190191	-	3190595	3190816	224	3190716	/	3190624	944	5.13	92	110

1 **SUPPLEMENTAL MATERIAL**2 **SUPPLEMENTARY INFORMATION: TABLE S2**

3

4 **TABLE S2: Strains, plasmids, oligonucleotides and DNA sequences**5 **Table S2A: Strains used in this study.**

Strain	Relevant characteristics	Reference
<i>E. coli</i>		
DH5 α	F ⁻ Φ 80/ <i>lacZ</i> Δ M15 Δ (<i>lacZ</i> YA- <i>argF</i>) U169 <i>recA1 endA1 hsdR17</i> (<i>r_K⁻, m_K⁺</i>) <i>phoA supE44 thi-1 gyrA96 relA1</i> λ ⁻ , strain used for cloning procedures	Invitrogen
BL21(DE3)	F- <i>ompT hsdS_B</i> (<i>r_B-m_B-</i>) <i>gal dcm</i> BL21(DE3), strain used for protein production	(1)
<i>Vibrio natrigens</i>		
<i>Vibrio natrigens</i> Vmax TM		Synthetic Genomics, San Diego, CA, USA
<i>C. glutamicum</i>		
ATCC 13032	Biotin-auxotrophic wild type	(2)
Δ <i>gntR1/2</i>	Derivate of ATCC 13032 with in-frame deletions of genes <i>gntR1</i> (cg2783) and <i>gntR2</i> (cg1935)	(3)
Δ phage (MB001)	Derivate of ATCC 13032 with deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752), and CGP3 (cg1890-cg2071)	(4)
Δ phage::P _{<i>cgpS</i>} - <i>cgpS</i>	Derivate of Δ phage (MB001) with deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752), and CGP3 (cg1890-cg2071) and re-integrated <i>cgpS</i> together with its native promoter in the	This work

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intergenic region of cg1199-
cg1201

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9 **Table S2B: Plasmids from other studies used in this study.**

Plasmid	Relevant characteristics	Source or reference
pJC1	<i>Kan^R</i> , <i>Amp^R</i> ; <i>oriV_{C.g.}</i> , <i>oriV_{E.c.}</i> (<i>C. glutamicum</i> / <i>E. coli</i> shuttle vector)	(5)
pJC1- <i>venus-term</i>	<i>Kan^R</i> , pJC1 derivative carrying the <i>venus</i> coding sequence followed by a terminator sequence of <i>Bacillus subtilis</i>	(4)
pJC1- <i>P_{lysin}-e2-crimson</i>	<i>Kan^R</i> ; pJC1 derivative containing a 250 bp promoter region of the <i>lys</i> gene (cg1974) and the first 30 bp of the coding sequence fused to <i>e2-crimson</i> gene via a linker containing a stop codon and a RBS	(6)
pK19- <i>mobsacB</i>	<i>Kan^R</i> ; plasmid for allelic exchange in <i>C. glutamicum</i> ; (<i>oriT</i> , <i>oriV_{E.c.}</i> , <i>sacB</i> , <i>lacZα</i>)	(7)
pAN6	<i>Kan^R</i> ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for gene expression under control of the <i>P_{tac}</i> promoter; (<i>P_{tac}</i> , <i>lacIq</i> , pBL1 <i>oriV_{C.g.}</i> , pUC18 <i>oriV_{E.c.}</i>)	(3)
pAN6- <i>cgpS-Strep</i>	<i>Kan^R</i> ; pAN6 derivative containing the <i>cgpS</i> gene fused C-terminal without stop codon to a sequence encoding a Strep-tag	(8)
pET16b	<i>Amp^R</i> ; vector for overexpression of genes in <i>E.</i> <i>coli</i> , adding a C-terminal hexahistidine affinity tag to the	Novagen

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	synthesized protein (pBR322 <i>oriVE.c., P_{TT}, lacI</i>)
pET16b- <i>gntR1</i>	<i>Amp^R</i> ; pET16b derivative for (3) overproduction of GntR1 with an N-terminal decahistidine tag.

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12 **Table S2C: Plasmids constructed in this work.** Oligonucleotide pairs, which were used
13 for PCR, are given as numbers (Table S2D). DNA templates are indicated in brackets
14 behind the oligonucleotides. The used backbones including the restriction enzymes used
15 for linearization are listed behind (*). Information about region of CgpS bound area and
16 positions of maximal CgpS binding coverage were based on previous studies from Pfeifer
17 and colleagues (8).

Plasmid	Construction	Relevant characteristics
Phage promoter-based constructs		
pJC1-P _{cg1897} - <i>venus</i>	Gibson assembly: 459/460 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of <i>cg1897</i> (468 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1897} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 459/394 (pJC1-P _{cg1897} - <i>venus</i>) and 393/116 (pJC1-P _{cg1897} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1-P _{cg1897} - <i>venus</i> derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg1936} - <i>venus</i>	Gibson assembly: 449/450 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of <i>cg1936</i> (676

3

	pJC1- <i>venus-term</i> *BamHI *BcuI	bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1936} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 449/452 (pJC1-P _{cg1936-<i>venus</i>}) and 451/116 (pJC1-P _{cg1936-<i>venus</i>}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1936-<i>venus</i>} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg1940-<i>venus</i>}	Gibson assembly: 444/445 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of cg1940 (563 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1940} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 444/447 (pJC1-P _{cg1940-<i>venus</i>}) and 446/116 (pJC1-P _{cg1940-<i>venus</i>}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1940-<i>venus</i>} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg1955-<i>venus</i>}	Gibson assembly: 387/388 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of cg1955 (516 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1955} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 387/390 (pJC1-P _{cg1955-<i>venus</i>}) and 389/116 (pJC1-P _{cg1955-<i>venus</i>}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1955-<i>venus</i>} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{prfP-<i>venus</i>}	Gibson assembly: 200/201 (<i>C. glutamicum</i> genome) and 115/116	<i>Kan</i> ^R ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the

	(pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	promoter of P_{priP} (611 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1- P_{priP} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 200/191 (pJC1- P_{priP} - <i>venus</i>) and 190/116 (pJC1- P_{priP} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- P_{priP} - <i>venus</i> derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1- P_{lys} - <i>venus</i>	Gibson assembly: 117/114 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1- P_{lys} ::GntR_BS_pos-100- <i>venus</i>	Gibson assembly: 117/119 (pJC1- P_{lys} - <i>venus</i>) and 118/116 (pJC1- P_{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- P_{lys} - <i>venus</i> derivative with an inserted GntR BS 100 bp upstream of the position of maximal CgpS binding.
pJC1- P_{lys} ::GntR_BS_pos-50- <i>venus</i>	Gibson assembly: 117/114 (pEK-A2- P_{lys} ::GntR_BS-50 bp upstream) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- P_{lys} - <i>venus</i> derivative with an inserted GntR BS 50 bp upstream of the position of maximal CgpS binding.
pJC1- P_{lys} ::GntR_BS_pos-25- <i>venus</i>	Gibson assembly: 117/178 (pJC1- P_{lys} - <i>venus</i>) and 177/116 (pJC1- P_{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- P_{lys} - <i>venus</i> derivative with an inserted GntR BS 25 bp upstream of the position of maximal CgpS binding.
pJC1- P_{lys} ::GntR_BS_pos-20- <i>venus</i>	Gibson assembly: 117/168 (pJC1- P_{lys} - <i>venus</i>) and 167/116 (pJC1- P_{lys} - <i>venus</i>) into	Kan^R ; pJC1- P_{lys} - <i>venus</i> derivative with an inserted GntR BS 20 bp upstream of the position

	pJC1- <i>venus-term</i> *BamHI *BcuI	of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-15- <i>venus</i>	Gibson assembly: 117/176 (pJC1-P _{lys} - <i>venus</i>) and 175/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 15 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-10- <i>venus</i>	Gibson assembly: 117/174 (pJC1-P _{lys} - <i>venus</i>) and 173/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 10 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-5- <i>venus</i>	Gibson assembly: 117/172 (pJC1-P _{lys} - <i>venus</i>) and 171/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 5 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-4- <i>venus</i>	Gibson assembly: 117/244 (pJC1-P _{lys} - <i>venus</i>) and 243/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 4 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-3- <i>venus</i>	Gibson assembly: 117/246 (pJC1-P _{lys} - <i>venus</i>) and 245/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 3 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-2- <i>venus</i>	Gibson assembly: 117/248 (pJC1-P _{lys} - <i>venus</i>) and 247/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 2 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} ::GntR_BS_pos-1- <i>venus</i>	Gibson assembly: 117/250 (pJC1-P _{lys} - <i>venus</i>) and 249/116 (pJC1-P _{lys} - <i>venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS 1 bp upstream of the position of maximal CgpS binding.
pJC1-P _{lys} _CS_0- <i>venus</i>	Gibson assembly: 117/125 (pJC1-P _{lys} - <i>venus</i>) and 124/116	<i>Kan</i> ^R ; pJC1-P _{lys} - <i>venus</i> derivative with an inserted GntR BS

	(pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	directly upstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+1-venus</i>	Gibson assembly: 117/212 (pJC1- <i>P_{lys}-venus</i>) and 211/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 1 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+2-venus</i>	Gibson assembly: 117/252 (pJC1- <i>P_{lys}-venus</i>) and 251/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 2 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+3-venus</i>	Gibson assembly: 117/254 (pJC1- <i>P_{lys}-venus</i>) and 253/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 3 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+4-venus</i>	Gibson assembly: 117/256 (pJC1- <i>P_{lys}-venus</i>) and 255/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 4 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+5-venus</i>	Gibson assembly: 117/180 (pJC1- <i>P_{lys}-venus</i>) and 179/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 5 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+10-venus</i>	Gibson assembly: 117/182 (pJC1- <i>P_{lys}-venus</i>) and 181/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 10 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+15-venus</i>	Gibson assembly: 117/184 (pJC1- <i>P_{lys}-venus</i>) and 183/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 15 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+20-venus</i>	Gibson assembly: 117/186 (pJC1- <i>P_{lys}-</i>	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an

	<i>venus</i>) and (pJC1- <i>P_{lys}-venus</i>) pJC1- <i>venus</i> -term *BamHI *BcuI	185/116	inserted into pJC1- <i>venus</i> -term *BamHI *BcuI	GntR BS 20 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+25-venus</i>	Gibson assembly: 117/121 (pJC1- <i>P_{lys}-venus</i>) and (pJC1- <i>P_{lys}-venus</i>) pJC1- <i>venus</i> -term *BamHI *BcuI	120/116	inserted into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 25 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::GntR_BS_pos+50-venus</i>	Gibson assembly: 117/188 (pJC1- <i>P_{lys}-venus</i>) and (pJC1- <i>P_{lys}-venus</i>) pJC1- <i>venus</i> -term *BamHI *BcuI	187/116	inserted into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted GntR BS 50 bp downstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::control_sequence_1_pos0-venus</i>	Gibson assembly: 117/380 (pJC1- <i>P_{lys}-venus</i>) and (pJC1- <i>P_{lys}-venus</i>) pJC1- <i>venus</i> -term *BamHI *BcuI	379/116	inserted into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted control sequence 1 (CATTAAATGATAATGC) directly upstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}::control_sequence_2_pos0-venus</i>	Gibson assembly: 117/382 (pJC1- <i>P_{lys}-venus</i>) and (pJC1- <i>P_{lys}-venus</i>) pJC1- <i>venus</i> -term *BamHI *BcuI	381/116	inserted into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with an inserted control sequence 2 (TATGATAGTAGCAAT) directly upstream of the position of maximal CgpS binding.
pJC1- <i>P_{lys}(5'Δ300bp)-venus</i>	Gibson assembly: 385/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI			<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with a 300 bp truncation of the 5'-promoter region.
pJC1- <i>P_{lys}(5'Δ350bp)-venus</i>	Gibson assembly: 386/116 (pJC1- <i>P_{lys}-venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI			<i>Kan^R</i> ; pJC1- <i>P_{lys}-venus</i> derivative with a 350 bp truncation of the 5'-promoter region.
pJC1- <i>P_{lys}(5'Δ300bp)_CS_0-venus</i>	Gibson assembly: 385/116 (pJC1- <i>P_{lys}_CS_0-venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI			<i>Kan^R</i> ; pJC1- <i>P_{lys}_CS_0-venus</i> derivative with a 300 bp truncation of the 5'-promoter region.
pJC1- <i>P_{lys}(5'Δ350bp)_CS_0-venus</i>	Gibson assembly: 386/116 (pJC1- <i>P_{lys}_CS_0-venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI			<i>Kan^R</i> ; pJC1- <i>P_{lys}_CS_0-venus</i> derivative with a

	<i>P_{lys_CS_0-venus}</i> into pJC1- <i>venus-term</i> *BamHI *BcuI	350 bp truncation of the 5'-promoter region.
pJC1-P _{cg1977} - <i>venus</i>	Gibson assembly: 435/436 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of cg1977 (653 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1977} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 435/438 (pJC1-P _{cg1977-venus}) and 437/116 (pJC1-P _{cg1977-venus}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1-P _{cg1977-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} - <i>venus</i>	Gibson assembly: 395/396 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of cg1999 (448 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg1999} ::GntR_BS_pos-30- <i>venus</i>	Gibson assembly: 395/575 (pJC1-P _{cg1999-venus}) and 574/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 30 bp upstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} ::GntR_BS_pos-20- <i>venus</i>	Gibson assembly: 395/577 (pJC1-P _{cg1999-venus}) and 576/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 20 bp upstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} ::GntR_BS_pos-10- <i>venus</i>	Gibson assembly: 395/579 (pJC1-P _{cg1999-venus}) and 578/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus-term</i> *BamHI *BcuI	<i>Kan^R</i> ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 10 bp upstream of the position of maximal CgpS binding.

pJC1-P _{cg1999} ::GntR_BS_pos-5- <i>venus</i>	Gibson assembly: 395/573 (pJC1-P _{cg1999-venus}) and 572/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 5 bp upstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 395/398 (pJC1-P _{cg1999-venus}) and 397/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} ::GntR_BS_pos+5- <i>venus</i>	Gibson assembly: 395/571 (pJC1-P _{cg1999-venus}) and 570/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 5 bp downstream of the position of maximal CgpS binding.
pJC1-P _{cg1999} ::GntR_BS_pos+10- <i>venus</i>	Gibson assembly: 395/581 (pJC1-P _{cg1999-venus}) and 580/116 (pJC1-P _{cg1999-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg1999-venus} derivative with an inserted GntR BS 10 bp downstream of the position of maximal CgpS binding.
pJC1-P _{cg2014-venus}	Gibson assembly: 439/440 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of cg2014 (545 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg2014} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 439/442 (pJC1-P _{cg2014-venus}) and 441/116 (pJC1-P _{cg2014-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg2014-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg2020-venus}	Gibson assembly: 408/409 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of cg2020 (390 bp) and the first 30 bp of the coding sequence fused to the reporter

		gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg2020} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 408/411 (pJC1-P _{cg2020-venus}) and 410/116 (pJC1-P _{cg2020-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg2020-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg2022-venus}	Gibson assembly: 416/417 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of cg2022 (309 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg2022} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 416/419 (pJC1-P _{cg2022-venus}) and 418/116 (pJC1-P _{cg2022-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg2022-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
pJC1-P _{cg2032-venus}	Gibson assembly: 412/413 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of cg2032 (490 bp) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1-P _{cg2032} ::GntR_BS_pos0- <i>venus</i>	Gibson assembly: 412/415 (pJC1-P _{cg2032-venus}) and 414/116 (pJC1-P _{cg2032-venus}) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{cg2032-venus} derivative with an inserted GntR BS directly upstream of the position of maximal CgpS binding.
Synthetic P_{cg1999} promoter variants		
pJC1-P _{cg1999_A-T/G-C-venus}	Gibson assembly: 501/505 (pJC1-P _{cg1999_A-T/G-C}) and 115/116 (pJC1- <i>venus</i> -term) into	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying a synthetic promoter based on a 50 bp region of P _{cg1999} containing

	<p>pJC1-<i>venus</i>-term *BamHI *BcuI</p>	<p>core promoter elements (TSS, -10 and -35 box), the identified binding motif and the nucleotide associated with maximal CgpS coverage. GC-profile of P_{cg1999} was maintain in the adjacent flanks but A/T and G/C were swapped. The promoter was fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS. The distance between TSS, start of coding region and artificial 30 bp coding region as well as the linker containing the RBS were consistent with the template sequence of P_{cg1999}.</p>
<p>pJC1-P_{cg1999}_rand-<i>venus</i></p>	<p>Gibson assembly: 497/498 (pEX-K168-Synthetic-sequence1) and 115/116 (pJC1-<i>venus</i>-term) into pJC1-<i>venus</i>-term *BamHI *BcuI</p>	<p><i>Kan</i>^R; pJC1-<i>venus</i>-term derivative carrying a synthetic promoter based on a 50 bp region of P_{cg1999} containing core promoter elements (TSS, -10 and -35 box), the identified binding motif and the nucleotide associated with maximal CgpS coverage. The adjacent sequences were randomized and fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS. The distance between TSS, start of coding region and artificial 30 bp coding region as well as the linker containing the RBS were consistent</p>

		with the template sequence of P _{cg1999} .
Synthetic P_{lys} promoter variants		
pJC1-P _{lys} _A-T/G-C_70bp- <i>venus</i>	Gibson assembly: 560/601 (gene strand P _{lys} _A-T/G-C) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying a synthetic promoter based on a 70 bp region of P _{lys} containing core promoter elements (TSS, -10 and -35 box). GC-profile of P _{lys} was maintain in the adjacent flanks but A/T and G/C were swapped. The promoter was fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS. The distance between TSS, start of coding region and artificial 30 bp coding region as well as the linker containing the RBS were consistent with the template sequence of P _{lys} .
pJC1-P _{lys} _A-T/G-C_80bp- <i>venus</i>	Gibson assembly: 560/618 (pJC1-P _{lys} _A-T/G-C_70bp- <i>venus</i>) and 617/116 (pJC1-P _{lys} _A-T/G-C_70bp- <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying a synthetic promoter based on a 80 bp region of P _{lys} containing core promoter elements (TSS, -10 and -35 box) and the identified binding motif. GC-profile of P _{lys} was maintain in the adjacent flanks but A/T and G/C were swapped. The promoter was fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS. The distance between TSS, start of coding region

		and artificial 30 bp coding region as well as the linker containing the RBS were consistent with the template sequence of P_{lys} .
pJC1- $P_{lys_A-T/G-C_70bp-venus}$	Gibson assembly: 560/601 (gene strand $P_{lys_A-T/G-C}$) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- <i>venus-term</i> derivative carrying a synthetic promoter based on a 100 bp region of P_{lys} containing core promoter elements (TSS, -10 and -35 box), the identified binding motif and the nucleotide associated with maximal CgpS coverage (8). GC-profile of P_{lys} was maintain in the adjacent flanks but A/T and G/C were swapped. The promoter was fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS. The distance between TSS, start of coding region and artificial 30 bp coding region as well as the linker containing the RBS were consistent with the template sequence of P_{lys} .
Further reporter constructs		
pJC1- $P_{gntK-venus}$	Gibson assembly: 203/204 (<i>C. glutamicum</i> genome) and 115/116 (pJC1- <i>venus-term</i>) into pJC1- <i>venus-term</i> *BamHI *BcuI	Kan^R ; pJC1- <i>venus-term</i> derivative carrying the P_{gntK} promoter (307 bp) (P_{cg2732}) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.
pJC1- $P_{gntK-e2-crimson}$	Gibson assembly: 203/204 (<i>C. glutamicum</i>	Kan^R ; pJC1- <i>venus-term</i> derivative carrying the

	genome) and 259/260 (pJC1- <i>P_{lys}</i> - <i>e2-crimson</i>) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>P_{gntK}</i> promoter (307 bp) (<i>P_{cg2732}</i>) and the first 30 bp of the coding sequence fused to the reporter gene <i>e2-crimson</i> via a linker containing a stop codon and an artificial RBS.
GntR-dependent toggle		
pJC1- <i>P_{lys_CS_0-venus-T-P_{gntK-e2-crimson}}</i>	Gibson assembly: 117/263 (<i>P_{lys_CS_0-venus}</i>) and 261/262 (<i>P_{gntK-e2-crimson}</i>) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the construct <i>P_{lys_CS_0-venus}</i> and the oppositely oriented <i>P_{gntK-e2-crimson} construct.</i>
Templates used for preparation of DNA fragments for surface plasmon resonance analysis		
pJC1- <i>P_{cg3336}</i>	Gibson assembly: 516/517 (<i>C. glutamicum</i> genome) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying a 1238 bp region covering the promoter region of <i>cg3336</i>
pJC1- <i>P_{cg1999}</i>	Gibson assembly: 520/521 (<i>C. glutamicum</i> genome) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying a 1363 bp region covering the promoter region of <i>c1999</i>
pJC1- <i>P_{cg1999_A-T/G-C}</i>	Gibson assembly: 501/503 (pEX-K168-Cg1999-AT-GCSwitch) and 502/504 (pEX-K168-Cg1999-AT-GCSwitch) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the sequence <i>P_{cg1999_A-T/G-C} encoded in pEX-K168-Cg1999-AT-GCSwitch (Table S2H) with nucleotide exchanges to ATG at positions 331-333</i>
pJC1- <i>P_{lys_CS_0}</i>	Gibson assembly: 117/125 (<i>C. glutamicum</i> genome) and 124/404 (<i>C. glutamicum</i> genome) into pJC1- <i>venus-term</i> *BamHI *BclI	<i>Kan^R</i> ; pJC1- <i>venus-term</i> derivative carrying the CgpS bound area of the promoter of <i>lys</i> (<i>cg1974</i>) (444 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding and the first 179 bp of the <i>lys</i> gene.

cgpS integration plasmid	
pK19mobsacB-1199_1201- P _{cgpS} -cgpS	Gibson assembly: <i>Kan</i> ^R ; pK19- <i>mobsacB</i> E227/E228 (C. glutamicum genome), E245/E115 (C. glutamicum genome) and E229/E230 (C. glutamicum genome) into pK19mobsacB *HindIII *EcoRI
	assembly: <i>Kan</i> ^R ; pK19- <i>mobsacB</i> derivative for the re-integration of <i>cgpS</i> and its native promoter (500+351bp) into the intergenic region of cg1199-cg1201 (500 bp upstream and downstream flanking region).

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19

20 **Table S2D: Oligonucleotides used in this study for plasmid constructions.**

Oligonucleotide number	Sequence (5'→3')
114	TGATATCTCCTTCTTAAAGTTCAATTTTTCGGCATTGCG CCTTAAATCGC
115	TGAACTTTAAGAAGGAGATATCATATGGTGAGCAAGGG CGAGGAG
116	AAAACGACGGCCAGTACTAGTTACTTGTACAGCTCGTC CATGCC
117	AGCGACGCCGAGGGGGATCCGCTCAAGGAAGAGTT CTTCATTGGTC
118	GCCTTTATGATAGTACCAATTCGAGAACTGGGTGTAGT GATTCTG
119	GTTCTCGAATTGGTACTATCATAAAGGCTTTTTCTCTCA TGACCTACCC
120	GGGAACATTATGATAGTACCAATTAACGGGTAAAGGT AAAGGACAAACG
121	CCCGTTAATTGGTACTATCATAATGTTCCCTGGTACT ACAAAATTAAC
124	GATACTAGAGTTATGATAGTACCAATTTAATTTGTAGTA TCCAGGGAAC
125	AAATTAATTGGTACTATCATAACTCTAGTATCTTATTAA TTTCTGTTAC
167	CAGATATGATAGTACCAATAATTAATAAGATACTAGAGT TTAATTTGTAG
168	CTTATTAATTATTGGTACTATCATATCTGTTACTATTAAC ATATAGGTTT
171	GATACTTATGATAGTACCAATAGAGTTAATTTGTAGTA TCCAGGGAAC

16

172	CTCTATTGGTACTATCATAAGTATCTTATTAATTTCTGTT ACTATTAAC
173	GTATGATAGTACCAATATACTAGAGTTTAAATTTGTAGTA TCCAGGG
174	GTATATTGGTACTATCATACTTATTAATTTCTGTTACTAT TAACATATAG
175	ATTATATGATAGTACCAATATAAGATACTAGAGTTTAAAT TTGTAGTATCC
176	CTTATATTGGTACTATCATATAATTTCTGTTACTATTAAC ATATAGG
177	TATATGATAGTACCAATACAGAAATTAATAAGATACTAG AGTTTAAATTTG
178	ATTTCTGTATTGGTACTATCATATACTATTAACATATAG GTTTTTAAAG
179	GTTTAATTATGATAGTACCAATTTGTAGTATCCAGGGAA CATTAACGG
180	AAATTGGTACTATCATAATTAAGCTCTAGTATCTTATTA TTTCTGTTAC
181	GTATATGATAGTACCAATGTATCCAGGGAACATTAAC GGGTAAAG
182	TACATTGGTACTATCATATACAAATTAAGCTCTAGTATC TTATTAATTTT
183	GTATCTATGATAGTACCAATCAGGGAACATTAACGGG TAAAGGTAAAG
184	CCCTGATTGGTACTATCATAGATACTACAAATTAAGCT TAGTATC
185	CCAGGGTATGATAGTACCAATAACATTAACGGGTAA GGTAAAGGAC
186	GTTATTGGTACTATCATACCCTGGATACTACAAATTA CTCTAGTATC
187	GGTAAAGGACAAATATGATAGTACCAATCGAACATGGC GATTAAGGCGC
188	GTTTCGATTGGTACTATCATATTTGTCCTTTACCTTTACC CGTTTAAATG
190	CTTATGATAGTACCAATAAGCTTGTTTAAATTGAACTT CGTTATATTC
191	CTTATTGGTACTATCATAAGTAATAAAGAACTCAACGG TTTATTAAGAC
200	AGCGACGCCGCAGGGGGATCCTGATGTAACGCTTATA TTATTTTAAAG
201	TGATATCTCCTTCTTAAAGTTCATGGGTCGTGGCTGTC TGTGGTGTC
203	AGCGACGCCGCAGGGGGATCCGTATCAATGGAATCCG GGACGC
204	TGATATCTCCTTCTTAAAGTTCAGACAATATGTAAGCCT TCGGCTGC

211	GATACTAGAGTTTATGATAGTACCAATTAATTTGTAGTA TCCAGGGAAC
212	AATTAATTGGTACTATCATAAACTCTAGTATCTTATTAAT TTCTGTTAC
243	GATACTATATGATAGTACCAATGAGTTAATTTGTAGTA TCCAGGG
244	CTCATTGGTACTATCATATAGTATCTTATTAATTTCTGTT ACTATTAAC
245	GATACTAGTATGATAGTACCAATAGTTAATTTGTAGTA TCCAGGGAAC
246	CTATTGGTACTATCATACTAGTATCTTATTAATTTCTGTT AC
247	GATACTAGATATGATAGTACCAATGTTAATTTGTAGTA TCCAGGGAAC
248	CATTGGTACTATCATATCTAGTATCTTATTAATTTCTGTT AC
249	GTATGATAGTACCAATTTAATTTGTAGTATCCAGGGAA CATTAAAC
250	AATTAATAATTGGTACTATCATACTCTAGTATCTTATTAAT TTCTGTTAC
251	GAGTTTTATGATAGTACCAATAATTTGTAGTATCCAGGG AACATTAAC
252	AATTATTGGTACTATCATAAACTCTAGTATCTTATTAAT TTCTGTTAC
253	GAGTTTATATGATAGTACCAATATTTGTAGTATCCAGGG AACATTAAC
254	CTACAAATATTGGTACTATCATATAAACTCTAGTATCTT ATTAATTTCTG
255	GAGTTTAATATGATAGTACCAATTTGTAGTATCCAGGG AACATTAACG
256	CTACAAAATTGGTACTATCATATTAACCTCTAGTATCTT ATTAATTTCTG
259	TGAACTTTAAGAAGGAGATATCATATGGATAGCACTGA GAACGTCATC
260	AAAACGACGGCCAGTACTAGCTACTGGAACAGGTGGT GG
261	GAAAGGCTCAGTCGAAAGACTGGGCCTTTCGTTTTATC TACTGGAACAGGTGGTGG
262	AAAACGACGGCCAGTACTAGGTATCAATGGAATCCGG GACGC
263	GTCTTTCGACTGAGCCTTTCGTTTTATTTACTTGTACA GCTCGTCCATG
379	GATACTAGAGTCATTAATGATAATGCTTAATTTGTAGTA TCCAGGGAAC
380	AAATTAAGCATTATCATTAAATGACTCTAGTATCTTATTA TTCTGTTAC

381	GATACTAGAGTTATGATAGTAGCAATTTAATTTGTAGTA TCCAGGGAAC
382	AAATTAATTTGCTACTATCATAACTCTAGTATCTTATTAA TTTCTGTTAC
385	AGCGACGCCGCAGGGGGATCCGTGTAGTGATTCTGT TGCAGGTTTATG
386	AGCGACGCCGCAGGGGGATCCATATGTTAATAGTAAC AGAAATTAATAAG
387	AGCGACGCCGCAGGGGGATCCCACTCTCGCAACACTC GCTC
388	TGATATCTCCTTCTTAAAGTTCAGATCTTGTGCGGTCTA GATAATGCG
389	TATGATAGTACCAATAATAACTAGTATTTTTAATGACTTA C
390	CTAGTTATTATTGGTACTATCATATTTACTAAATATAG AAGTTAATTG
393	CACAATTATGATAGTACCAATTATTTACATACATGTGTTA TGTTAATTAC
394	ATTGGTACTATCATAATTGTGAATACTAAAGTTAATAGT TTC
395	AGCGACGCCGCAGGGGGATCCATTATGTGCGTATCGC TGCTGCTC
396	TGATATCTCCTTCTTAAAGTTCATCTGCGCTCGCCAGC AAGTG
397	GTTAGTTATGATAGTACCAATATATTAATTACTTGATTTA ATTGAAGGGG
398	ATTGGTACTATCATAACTAACTTAATTGAGTTTATTTTTA CGG
404	AAAACGACGGCCAGTACTAGGTCACGACCTGATTTTTT ACCTCG
408	AGCGACGCCGCAGGGGGATCCCGATGTATGTCAGCAA TGAGGTTG
409	TGATATCTCCTTCTTAAAGTTCAGTCATCCGACCCGCC ATTATTC
410	GAAACTTTATGATAGTACCAATCAATAACTTAGTAACTT TAAAGGGG
411	GATTGGTACTATCATAAAGTTTCTAGAATATAAGTTATT ACACAAAAACG
412	AGCGACGCCGCAGGGGGATCCATTTGGAATCTTGGA CTTTGGTGCC
413	TGATATCTCCTTCTTAAAGTTCAAGTTGCGGTTCTAAA ACTGTCG
414	CAATTTTAAATGTGTATGATAGTACCAATTTAAAGTTTCT ATATTCCATTCTAAAATAAC
415	AATTGGTACTATCATAACATTAATAATGAATATTAAT GTTGTTTAAAG

416	AGCGACGCCGCAGGGGGATCCGCAGATTCAGTGGTTC ACCAC
417	TGATATCTCCTTCTTAAAGTTCAGCTTTTCTCTGCACAG TATTTGTCAT
418	TAAGTTATGATAGTACCAATTTGTCATTAAGAAATGGG GTAGAGCTATG
419	CAAATTGGTACTATCATAACTTATTTAACTATTTAAAGA CTAATTAATTTAAC
435	AGCGACGCCGCAGGGGGATCCTTCTGCCTTGACAT GCGAGAAC
436	TGATATCTCCTTCTTAAAGTTCAGACTGTGGAGATGAT GCGGAATTTT
437	TAAAATATCGTATGATAGTACCAATGAGTTTATTTATGT GATTTGACCGG
438	CATTGGTACTATCATAACGATATTTAATCAGATAAATAA TACTAATAAC
439	AGCGACGCCGCAGGGGGATCCATCCCCTACAGGTATT TTGCGTAGTG
440	TGATATCTCCTTCTTAAAGTTCATCTTTATCCTTGCTT CGAAAATTAGC
441	CAAGTATGATAGTACCAATGTGATTATGCTTCACATTAA TACTTAATAAG
442	CATAATCACATTGGTACTATCATACTTGTCAAACAAATG CAACTTTTTT
444	AGCGACGCCGCAGGGGGATCCATATACCCAAGCACT TGGCGATC
445	TGATATCTCCTTCTTAAAGTTCAGTTGATGTGGCTGAC GTGGTG
446	CGCGTTTATGATAGTACCAATTCTCTATTTTAATTAATAT AATTAATGTAGTTTATTAAC
447	ATTAAAATAGAGAATTGGTACTATCATAAACGCGATAG GCGTGTATGTGG
449	AGCGACGCCGCAGGGGGATCCTGCTGCACGAATGCG TAACCTC
450	TGATATCTCCTTCTTAAAGTTCAGCCGAATATTCGGCG AGTTTTTAG
451	GAAAAGTCACTTATGATAGTACCAATTAAGCGCACGCT AAAAGCGAATTG
452	GCGCTTAATTGGTACTATCATAAGTGACTTTTCAATTGT GCTGTAATTG
459	AGCGACGCCGCAGGGGGATCCTGGAGTCGCGGGTGC TCAAC
460	TGATATCTCCTTCTTAAAGTTCATACCTCTCGACCCTGT TTATTAATAAG
497	AGCGACGCCGCAGGGGGATCCGCCTCTACGAGAC ATTGG

498	GATATCTCCTTCTTAAAGTTCAATAAGGCGAAATACATT TAATAGCAATC
501	AGCGACGCCGCAGGGGGATCCTATCAGGACGGCTCC TATATCGG
502	GATTTATAACTTCCCCAAATATCAATGTGCGTTTGTGAA CGACCGCTC
503	GCGGTCGTTCCAAAACGCACATTGATATTTGGGGAAGT TATAAATCAAG
504	AAAACGACGGCCAGTACTAGTGCACGGGCTAGACGAC CTAG
505	TGATATCTCCTTCTTAAAGTTCAAGACGCGAGCGGTCC TTCAC
516	AGCGACGCCGCAGGGGGATCCGCCTCCTCATCGATT CCGC
517	AAAACGACGGCCAGTACTAGCCGTAGCAGCGCTTGT TCG
520	AGCGACGCCGCAGGGGGATCCGACCACTACCTCAAG GGCGG
521	AAAACGACGGCCAGTACTAGCAGTTGCAGACGTGTAC GACAC
560	AGCGACGCCGCAGGGGGATC GTATATTTATGATAGTACCAATAATTACTTGATTTAATTG
570	AAGGGGTTTA TTATTGGTACTATCATAAATACTAATTAATTGAGTTT
571	ATTTTACGG AGTATGATAGTACCAATTTAGTATATTAATTACTTGATT AATTGAAGGG
572	TACTAAATTGGTACTATCATACTTAATTGAGTTTATTTT ACGGTTAAT
573	AAATTATATGATAGTACCAATAACCGTAAAAATAAACTC AATTAAGTTAG
574	TTTTACGGTTATTGGTACTATCATATAATTTACTCTAAG CGAAGACGCC
575	TATGATAGTACCAATATAAACTCAATTAAGTTAGTATAT TAATTACTTGATTTAATTG
576	TGAGTTTATATTGGTACTATCATATTTTACGGTTAATTT ACTCTAAGCG
577	AATATGATAGTACCAATTTAAGTTAGTATATTAATTACT GATTTAATTG
578	CTTAAATTGGTACTATCATATTGAGTTTATTTTACGGTT TAATTTACTC
579	TATTAATTATATGATAGTACCAATCTTGATTTAATTGAAG GGGTTTATAG
580	TTGGTACTATCATATAATTAATACTAATTAATTGAGT TTATTTTAC
581	ATGATATCTCCTTCTTAAAGTTCAAAAAAGCCGTAACG
601	

617	CAGAAATTAATAAGATACTTCTCAAATTAACATCATAG GTCCCTTG
618	TAATTTGAGAAGTATCTTATTAATTTCTGTTACTATTAAC ATATAGGTTT
619	ACAGAAATTAATAAGATACTAGAGTTAATTTGTAGTAT CGTCCCTTGTAAATTTGCCATTTC
620	AAAACGACGGCCAGTACTAGTTACTTGTACAGCTCGTC CATGCC
E115	TTATTCGAAAGGAATGCCTTCTTTTTTCG
E227	GACCATGATTACGCCAAGCTTGAAAATCGGGAGTGGG AAAGAG
E228	GTAGCTACACAGACGACCAGATTCGTGGGCGAAGTGG TTC
E229	AAGGCATTCTTTTCGAATAAGTGAACCTCAGAAATGCCA GGATTG
E230	AAAACGACGGCCAGTGAATTCGAAGACCGTCACCCAA CTAG
E245	CTGGTCGTCTGTGTAGCTAC

21

22 **Table S2E: Oligonucleotides used for the amplification of DNA probes for surface**

23 **plasmon resonance analysis.** Primers belonging to the same first PCR reaction are
 24 alternately shadowed in grey or white. The first PCR product was used as template for the
 25 second PCR reaction. The first primer was combined with the primer Biotin-primer (Biotin-
 26 GAGGAGTCGTCGATGTGGAGACC), which was 5'-fused to biotin.

Sample	Oligo-nucleotide number	Sequence (5'→3')	Template
P _{cg3336} (control)	M259	CGCCCTCACCGGTGG	pJC1-P _{cg3336}
	M260	GAGGAGTCGTCGATGTGGA GACCCCAAGATCTTTGGGG AGAGATTTTT	
P _{cg1999}	710	ACGTGCCCGATCTGCTGGA TC	pJC1-P _{cg1999}
	709	GAGGAGTCGTCGATGTGGA GACCATTTGTGGGCGCTTTA ATCGCTAAC	
P _{cg1999_A-T/G-C}	507	TGCACGGGCTAGACGACCT AG	pJC1-P _{cg1999_A-T/G-C}

22

	506	GAGGAGTCGTCGATGTGGA GACCTAAACACCCGCGAAAT TAGCGATTG	
<i>P_{lys}</i>	M263	CTCGACGACGGCCACTG	<i>C. glutamicum</i> genome
	M264	GAGGAGTCGTCGATGTGGA GACCAGTGCCTTCTTTGAGG CTTGA	
<i>P_{lys_CS_0}</i>	M263	CTCGACGACGGCCACTG	pJC1- <i>P_{lys_CS_0}</i>
	M264	GAGGAGTCGTCGATGTGGA GACCAGTGCCTTCTTTGAGG CTTGA	

27

28 **Table S2F: Oligonucleotides used for electrophoretic mobility shift assays.** Primers

29 and their corresponding templates are alternately shadowed in grey or white.

Sample	Oligo-nucleotide number	Sequence (5'→3')	Template
<i>P_{lys}</i>	117	AGCGACGCCGCAGGGGGATCCGC TCAAGGAAGAGTTCTTCATTGGTC	pJC1- <i>P_{lys-venus}</i>
	114	TGATATCTCCTTCTTAAAGTTCAAT TTTTCGGCATTGCGCCTTTAATCG C	
<i>P_{lys_CS_0}</i>	117	AGCGACGCCGCAGGGGGATCCGC TCAAGGAAGAGTTCTTCATTGGTC	pJC1- <i>P_{lys_CS_0-venus}</i>
	114	TGATATCTCCTTCTTAAAGTTCAAT TTTTCGGCATTGCGCCTTTAATCG C	

30

31

32 **Table S2G: Oligonucleotides used for sequencing.**

Oligonucleotide name	Sequence (5'→3')	Target
pJC1-MCS-fw	CAGGGACAAGCCACCCGCACA	All pJC1-based plasmids (Table S2C)
pJC1-MCS-rv	GGAAGCTAGAGTAAGTAGTTCGC	
R274-eYFPK5-seq-rv	GCAGGACCATGTGATCGCGC	pJC1- <i>P_{lys_CS_0-venus-T-P_{gntK-e2-crimson}}</i>
R216-venus-end-fw	CTCTCGGCATGGACGAGCTGTAC	
492-venus-seq-rv	CTCGAACTTCACCTCGGCGC	

23

M13-fw	CGCCAGGGTTTTCCAGTCAC	pK19 <i>mobsacB</i> - 1199_1201- <i>cgpS</i> Genomically re- integrated <i>P_{cgpS}</i> - sequence in the intergenic region of cg1199- cg1201
M13-rv	AGCGGATAACAATTTACACAGGA	
cg1199_1201_seq_fw	GTGGAAAAAATTGGGGTTTCCG	
cg1199_1201_seq_rv	GCTCATTGTCACAGATGGCG	

33

34

35 **Table S2H: Ordered DNA sequences.** All DNA sequences were synthesized by Eurofins

36 Genomics (Ebersberg, Germany) and provided as plasmids or DNA strands.

Ordered Sequences Plasmids	Sequence (5'→3')
pEX-K168-P _{cg1999_A} - T/G-C	TATCAGGACGGCTCCTATATCGGTGGGCAGGACAGC TCTCGCGTTGTTGTTACGAGAACGACCTAAACGACA ACCAATATAAACACCCGCGAAATTAGCGATTGTGAAT TTTGTGAAGAGTTGCCAAGATAGACAGTCGACACATC TACCAGCACCGCCTTAGTAACAGTCGTATCGATATAA AATGTTATACGAGTTAATCAAAGACGCAGACCTTTGC CACGAATACCACTAAGGCCCGCAGAAGCGAATCTCA TTTAAAAACCGTAAAAATAAACTCAATTAAGTTAGTAT ATTAATTACTTGATTATAACTTCCCCAAATATCATACT GCGTTTGTGAACGACCGCTCGCGTCTTAGCAGTGGT GCCGTCTCGAATTTGCTACGACCGTGCTCCCTATT CCACAAAGACGGACCACAGCGTCGCGCCCGCGGTC TCTAGGAGCTGTTGAAGCTAGGTCTGCTAGCCCGTG CA
pEX-K168- P _{cg1999_rand}	GCGCCTCTACGAGACATTGGATGACCTAGGCTAACT AGGCTTATAGAAGAGGGCACGCACGTACGGTATCGG AGCTTTCATTGCCATTGGTAGGATCAAACCCGTGAC GAGTTGCTCTCCATGCTACTATCAGAAGTACAAATT TGTAAGGCCCTGAACGCTTTCAGCCGATTACACAC AAGTGGTGGACTAATATTGGGGAGGCTGCTGCCAG ATCAATGAGCCTTAGGAGCAGATGATTAATACCGGC GCAATTAACCGTAAAAATAAACTCAATTAAGTTAGTA TATTAATTACTTGATTACCTTACCGGATCACGTTAAT GATTGCTATTAATGTATTTGCTTATATCTGCCTCG CTCAAGTTGCTTAAGCTGGCAACTGTTATTATATGG GTTGCCCGAGCACGGATGGCTCGCTTCAACTCCGCA

24

	TTGACAGTTTACACACCCGCCGAGACGGGGATTCCG ACT
pEK-A2- <i>P_{lys}</i> ::GntR_BS_pos-50	GCTCAAGGAAGAGTTCCTTCATTGGTCTTTTGTTCGG GTTGCCGTAGATGATTTTCAGGGTGGCTCAAAGTCAG GGAGGTGTAGTCGAATTCGATTGGGTGAAGCCGTGC GCGATTACGGTTTTTCGCGCTCGGTACCAGTAATAAAG CTTGCGATGCGGGAGTAATTAACCCGACCTTTGATAA ATACCTGAGTGCCTTCTTTGAGGCTTGATGCCTTGAT CTTTTCGGCGACTGGTGTGGCTGCACAGGTTGAGG GGTAACTGGGTAGGTCATGAGAGAAAAAGCCTTTCG AGAACTGGGTGTAGTGATTTCTGTTGCAGGTTTATGT AGACGAACTTATGATAGTACCAATTTAAAAACCTAT ATGTTAATAGTAACAGAAATTAATAAGATACTAGAGTT TAATTTGTAGTATCCAGGGAACATTAACCGGTAAG GTAAAGGACAAACGAACATGGCGATTAAGGCGCAA TGCCGAAAAAT
Ordered Sequences	
Gene strands	
<i>P_{lys}</i> _A-T/G-C	AGCGACGCCGACGGGGGATCCCGAGTTCCTTCTCAA GAAGTAACCAGAAAAACAACGCCCAACGGCATCTACT AAAGTCCCACCGAGTTTCAGTCCCTCCACATCAGCTT AAGCTAACCCACTTCGGACAGCGCTAATGCCAAAAG CGCGAGCCATGGTCAATTTTCGAACGCTACGCCCT CATTAAATTGGGCTGGAAACTATTTATGGACTCACGGA AGAAACTCCGAACTACGGAAGTAAAGCCGCTGA CCACGACCGACGTGTCCAACCTCCCAATTGACCCATC CAGTACTCTCTTTTTCGGAAAGCTCTTGACCCACAAG TGATTTCTGTTGCAGGTTTATGTAGACGAAACTTTAAA AAACCTATATGTTAATAGTAACAGAAATTAATTTCTAT GATCTCAAATTAACATCATAGGTCCCTTGTAATTTGC CCATTTCCATTTCCGTGTTTGCTTGATGCGCTAATTTCC GCGTTACGGCTTTTATGAACTTTAAGAAGGAGATAT CAT

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38 **Table S2I: Sequences of the native phage promoters *P_{cg1999}* and *P_{lys}* and the**
 39 **corresponding synthetic variants shown in Figure 2.** Highlighted are the core
 40 promoter region (blue), the ATG as translational start codon (bold red) and the stop codon
 41 introduced after 30 nucleotides (bold yellow). For the native *P_{lys}* promoter, the 100 bp core
 42 promoter region is highlighted.

Promoter	Sequence (5'→3')
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25

P_{cg1999}	
P _{cg1999}	ATAGTCCTGCCGAGGATATAGCCACCCGTCCTGTGC AGAGCGCAACAACAAGTGCTCTTGCTGGATTTGCTGT TGTTATATTTGTGGGCGCTTTAATCGCTAACACTTA AAACACTTCTCAACGCTTCTATCTGTCAGCTGTGTAG ATGGTCGTGGCGGAATCATTGTCAGCATAGCTATATT TTACAATATGCTCAATTAGTTTCTGCGTCTGGAAACG GTGCTTATGGTGATTCCGGGCGTCTTCGCTTAGAGTA AATTAAACCGTAAAAATAAACTCAATTAAGTTAGTATA TTAATTACTTGATTTAATTGAAGGGGTTTATAGTATGA CGCAAACACTTGCTGGCGAGCGCAGATGA
P _{cg1999_A-T/G-C}	TATCAGGACGGCTCCTATATCGGTGGGCAGGACAGC TCTCGCGTTGTTGTTACAGGAGAACGACCTAAACGACA ACCAATATAAACACCCGCGAAATTAGCGATTGTGAAT TTTGTGAAGAGTTGCGAAGATAGACAGTCGACACATC TACCAGCACCGCCTTAGTAACAGTCGTATCGATATAA AATGTTATACGAGTTAATCAAAGACGCAGACCTTTGC CACGAATACCACTAAGGCCCGCAGAAGCGAATCTCA TTTAAAAACCGTAAAAATAAACTCAATTAAGTTAGTAT ATTAATTACTTGATTTATAACTTCCCAAATATCAATG TGCGTTTGTGAACGACCGCTCGCGTCTGA
P _{cg1999_rand}	GCGCCTCTACGAGACATTGGATGACCTAGGCTAACT AGGCTTATAGAAGAGGGGCACGCACGTACGGTATCGG AGCTTTCATTGCCATTGGTAGGATCAAAAACCCGTGAC GAGTTGCTCTCCCATGCTACTATCAGAACTGACAATT TGTAGAGGCCCTGAACGCTTTCAGCCGATTACACAC AAGTGGTGGACTAATATTGGGGAGGCTGCTGCCAG ATCAATGAGCCTTAGGAGCAGATGATTAATACCGGC GCAATTAAACCGTAAAAATAAACTCAATTAAGTTAGTA TATTAATTACTTGATTTACCTTACCGGATCAGTTAAT GATTGCTATTAATGTATTTCCCTTATGA
P_{lys}	
P _{lys}	GCTCAAGGAAGAGTTCTTCATTGGTCTTTTGTTCGGG GTTGCCGTAGATGATTTCCAGGGTGGCTCAAAGTCAG GGAGGTGTAGTCGAATTCGATTGGGTGAAGCCTGTC GCGATTACGGTTTTTCGCGCTCGGTACCAGTAATAAAG CTTGCGATGCGGGAGTAATTAACCCGACCTTTGATAA ATACCTGAGTGCCCTTCTTTGAGGCTTGATGCCTTGAT CTTTTCGGCGACTGGTGCTGGCTGCACAGGTTGAGG GGTAACTGGGTAGGTCATGAGAGAAAAAGCCTTTCG AGAACTGGGTGTAGTGATTTCTGTTGCAGGTTTATGT AGACGAACTTTAAAAACCTATATGTTAATAGTAACA GAAATTAATAAGATACTAGAGTTAATTTGTAGTATCC AGGGAACATTAACGGGTAAGGTAAGGACAACG AACATGGCGATTAAGGCGCAATGCCGAAAAATGA
P _{lys_A-T/G-C_70 bp}	CGAGTTCCTTCTCAAGAAGTAACAGAAAAACAACGCC CAACGGCATCTACTAAAGTCCCACCGAGTTTCAGTCC

CTCCACATCAGCTTAAGCTAACCCACTTCGGACAGC
GCTAATGCCAAAAGCGCGAGCCATGGTCATTATTCCG
AACGCTACGCCCTCATTAAATGGGCTGGAAACTATTT
ATGGACTCACGGAAGAACTCCGAACTACGGAACCTA
GAAAAGCCGCTGACCACGACCGACGTGTCCAACCTCC
CCATTGACCCATCCAGTACTCTCTTTTTCGGAAAGCT
CTTGACCCACAAGTGATTTCTGTTGCAGGTTTATGTA
GACGAAACTTTAAAAAACCTATATGTTAATAGTAACAG
AAATTAATTCTATGATCTCAAATTAACATCATAGGT
CCCTTGTAATTTGCCATTTCCATTTCTGTTTGCTTG
ATGCGCTAATTTCCGCGTTACGGCTTTTTATGA

P_{lys}_A-T/G-C_80 bp CGAGTTCCTTCTCAAGAAGTAACCAGAAAAACAACGCC
CAACGGCATCTACTAAAGTCCCACCGAGTTTCAGTCC
CTCCACATCAGCTTAAGCTAACCCACTTCGGACAGC
GCTAATGCCAAAAGCGCGAGCCATGGTCATTATTCCG
AACGCTACGCCCTCATTAAATGGGCTGGAAACTATTT
ATGGACTCACGGAAGAACTCCGAACTACGGAACCTA
GAAAAGCCGCTGACCACGACCGACGTGTCCAACCTCC
CCATTGACCCATCCAGTACTCTCTTTTTCGGAAAGCT
CTTGACCCACAAGTGATTTCTGTTGCAGGTTTATGTA
GACGAAACTTTAAAAAACCTATATGTTAATAGTAACAG
AAATTAATAAGATACTTCTCAAATTAACATCATAGGT
CCCTTGTAATTTGCCATTTCCATTTCTGTTTGCTTG
ATGCGCTAATTTCCGCGTTACGGCTTTTTATGA

P_{lys}_A-T/G-C_100 bp CGAGTTCCTTCTCAAGAAGTAACCAGAAAAACAACGCC
CAACGGCATCTACTAAAGTCCCACCGAGTTTCAGTCC
CTCCACATCAGCTTAAGCTAACCCACTTCGGACAGC
GCTAATGCCAAAAGCGCGAGCCATGGTCATTATTTCG
AACGCTACGCCCTCATTAAATGGGCTGGAAACTATTT
ATGGACTCACGGAAGAACTCCGAACTACGGAACCTA
GAAAAGCCGCTGACCACGACCGACGTGTCCAACCTCC
CCATTGACCCATCCAGTACTCTCTTTTTCGGAAAGCT
CTTGACCCACAAGTGATTTCTGTTGCAGGTTTATGTA
GACGAAACTTTAAAAAACCTATATGTTAATAGTAACAG
AAATTAATAAGATACTAGAGTTTAAATTTGTAGTATCGT
CCCTTGTAATTTGCCATTTCCATTTCTGTTTGCTTG
ATGCGCTAATTTCCGCGTTACGGCTTTTTATGA

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Supplemental Figures

References are indicated as numbers. Numbers are given in the reference section of the publication.

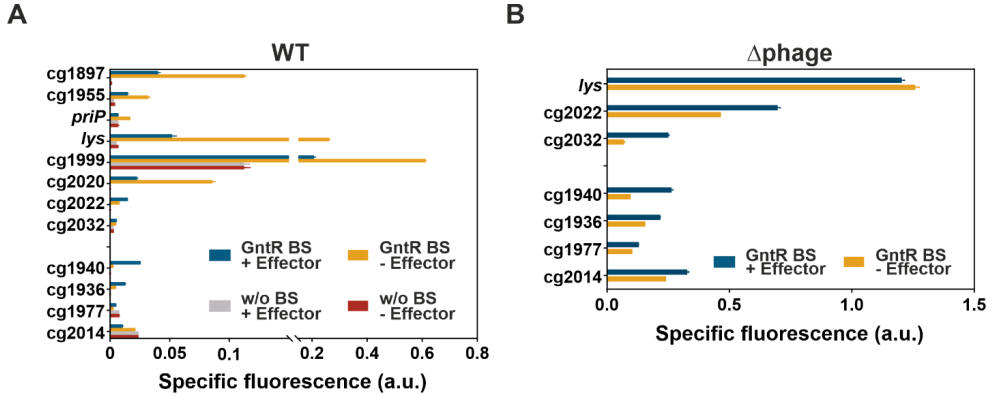


Figure S1: Counter-silencing of CgpS target promoters. A) Specific reporter outputs of native phage promoters and corresponding synthetic constructs which were used for the calculation of fold change ratios shown in Figure 3. **B)** To confirm functionality of synthetic promoter variants (GntR BS), which showed only low reporter outputs in the wild type, respective constructs were analyzed in the prophage-free strain Δ phage in the absence of the silencer CgpS. All constructs led to significant reporter outputs confirming synthetic promoter functionality ($n \geq 3$ biological triplicates).

A

native GntR BS P_{gntK}	T	A	T	G	A	T	A	G	T	A	C	A	A	T	GC: 27%	
control-seq 1	C	A	T	T	A	A	T	G	A	T	A	A	T	G	C	GC: 27%
control-seq 2	T	A	T	G	A	T	A	G	T	A	G	C	A	A	T	GC: 27%

Highly conserved nucleotides	T	A	T	G	A	T	A	G	T	A	C	A	A	T	
Weakly conserved nucleotides	C	A	T	T	A	A	T	G	A	T	A	A	T	G	C

B

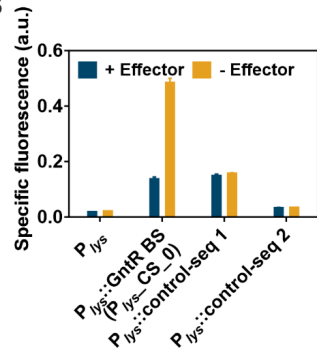


Figure S2: Effects of inserted sequences independent of GntR binding. **A)** The native GntR BS located within the P_{gntK} promoter contains four highly conserved and four weakly conserved nucleotides (43). As controls for sequence insertions, which do not allow for GntR binding, control sequences (control-seq) 1 and 2 were inserted at position 0 within the phage promoter P_{lys} . Control-seq 1 contained the same nucleotide composition as the GntR BS but the sequence was randomized. Control-seq 2 was similar to the GntR BS, but one conserved cytosine was replaced with guanine. Based on previous studies, this exchange was expected to abolish GntR binding (43). **B)** Specific reporter outputs of the promoter variants after five hours of cultivation. Promoter constructs (plasmid backbone pJC1) were fused to the reporter gene *venus* and analyzed in *C. glutamicum* wild type. Cells were cultivated in CGXII in the presence (100 mM gluconate) or absence (100 mM glucose) of the effector molecule gluconate in a microtiter cultivation system. The results shown in this graph demonstrate that mutation of the GntR operator site abolished GntR binding in vivo and that the observed counter-silencing effect strictly depends on GntR binding.

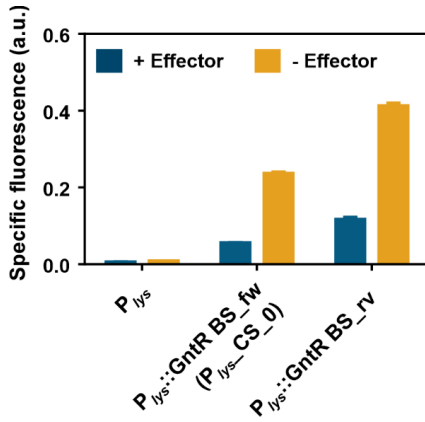


Figure S3: Effects of directionality of inserted GntR binding site on counter-silencing efficiency. The 15 bp long GntR BS was inserted either in a forward ($P_{lys}::GntR\ BS_fw$; $P_{lys_CS_0}$) or in a reverse orientation ($P_{lys}::GntR\ BS_rv$) in the P_{lys} promoter (position 0) to analyze if the directionality of the GntR BS influence counter-silencing. Promoter constructs (plasmid backbone pJC1) were fused to the reporter gene *venus* and analyzed in *C. glutamicum* wild type. Cells were cultivated in CGXII medium in the presence (100 mM gluconate) or absence (100 mM glucose) of the effector molecule gluconate in a microtiter cultivation system. Presented is the mean of the specific fluorescence after five hours of cultivation (n=3 biological triplicates). These results demonstrated that counter-silencing does not depend on the directionality of GntR operator sites.

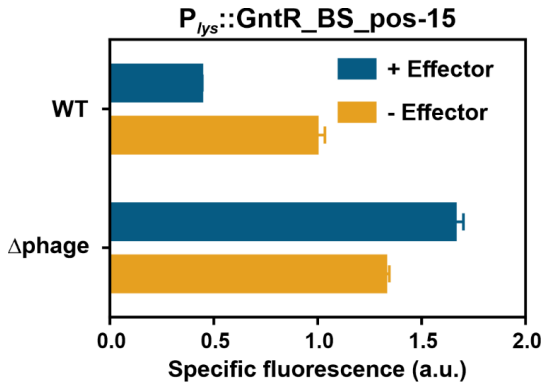


Figure S4: It's all about context – Impact of GntR binding on the output of the P_{lys} promoter in the presence or absence ($\Delta phage$) of CgpS. Reporter outputs (*venus* expression) driven by a P_{lys} promoter with an inserted GntR operator sequences 15 bp upstream of the maximal CgpS binding peak (11 bp downstream of TSS) in *C. glutamicum* wild type cells and in the absence of CgpS in the strain $\Delta phage$. Cells were cultivated in CGXII in the presence (100 mM gluconate) or absence (111 mM glucose) of the effector molecule gluconate in a microtiter cultivation system. Shown are mean values and standard deviation of reporter outputs of biological triplicates after five hours of cultivation.

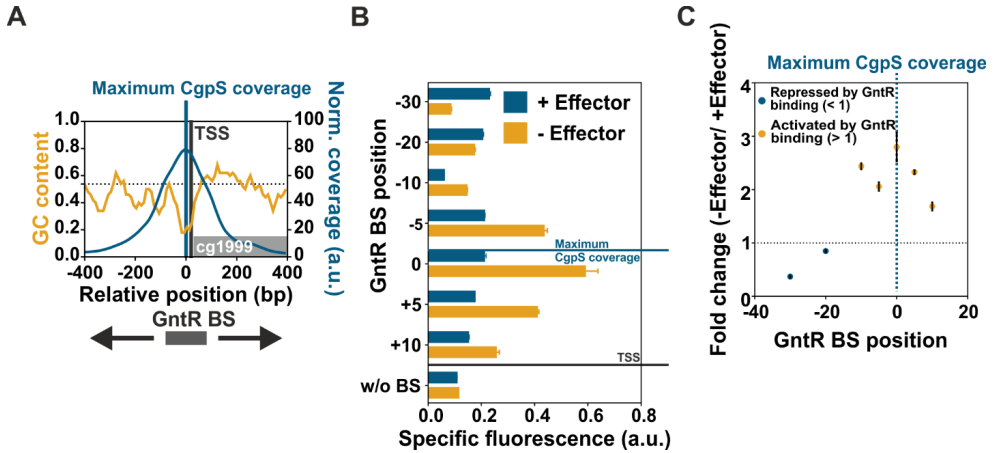


Figure S5: Impact of GntR binding site (BS) position on inducibility of P_{cg1999} -based promoter constructs. A) Inverse correlation of GC-profile and CgpS binding coverage of the phage promoter P_{cg1999} . The highest scored transcriptional start site (TSS) and the position of maximal CgpS binding affinity (4) are shown as vertical lines. BS positions refer to the sequence base associated with maximal CgpS binding peak. The position directly upstream of this nucleotide was defined as position 0. **B)** Impact of inserted GntR BS position on specific reporter outputs in the presence (gluconate) and absence (glucose) of the effector molecule gluconate. Positions of TSS and maximal CgpS coverage are marked by lines. **C)** Impact of GntR binding site position on counter-silencing efficiency of P_{cg1999} -based promoter constructs. Ratio of specific reporter outputs, shown in (B), were used for the calculation of their inducibility (fold change). Cells harboring the plasmid-based synthetic promoter constructs were grown in CGXII medium supplemented with either 100 mM glucose or 100 mM gluconate. Bars (B) and dots (C) represent the means and error bars the standard deviation of biological triplicates.

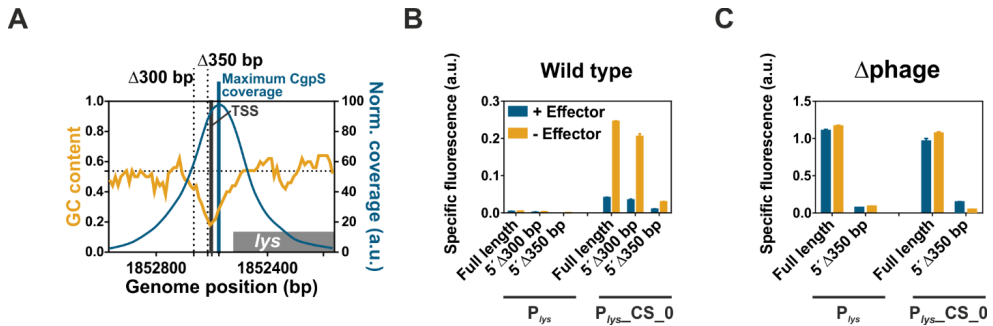


Figure S6: Definition of the minimal region required for silencing. **A**) Reporter outputs (*venus* expression) driven by 5'-truncated promoter versions ($\Delta 300$ and $\Delta 350$ bp truncations) of P_{lys} and $P_{lys_CS_0}$ were analyzed regarding silencing and counter-silencing efficiency. 5'-ends of full-length constructs coincided with the upstream end of the CgpS binding peak (P_{lys} : 389 bp, $P_{lys_CS_0}$: 404 bp upstream of the maximal CgpS binding peak). The distance between maximal CgpS binding peak and ATG was in all constructs 85 bp. Full length promoters were compared to variants ending 89 bp (5' $\Delta 300$) or 39 bp (5' $\Delta 350$) upstream of the maximal CgpS binding peak in P_{lys} . **B and C**) Specific reporter outputs of the promoter variants after five hours of cultivation. Promoter constructs were fused to the reporter gene *venus* (plasmid backbone pJC1) and analyzed in *C. glutamicum* wild type cells (**B**) and in the prophage-free strain Δ phage ($\Delta cgps$) (**C**). Cells were cultivated in CGXII in the presence (100 mM gluconate) or absence (100 mM glucose) of the effector molecule gluconate in a microtiter cultivation system. Shown are mean values and standard deviation of biological triplicates after five hours of cultivation. The results shown in this graph demonstrate that the region >89 bp upstream of the maximal CgpS binding peak of the P_{lys} promoter is neither involved in silencing nor in counter-silencing. Further 50 bp truncation strongly reduced promoter activity of both promoters also in the Δ phage strain.

- 4.4. Supplemental material to “Inducible expression systems based on xenogeneic silencing and counter-silencing and the design of a metabolic toggle switch”

Supporting Information

Inducible expression systems based on xenogeneic silencing and counter-silencing and the design of a metabolic toggle switch

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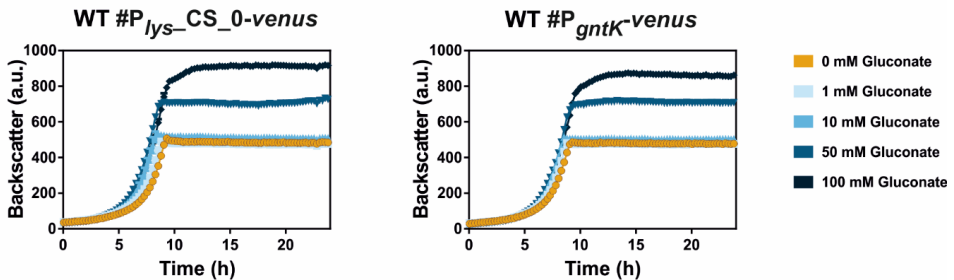
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Supplemental figures

A



B

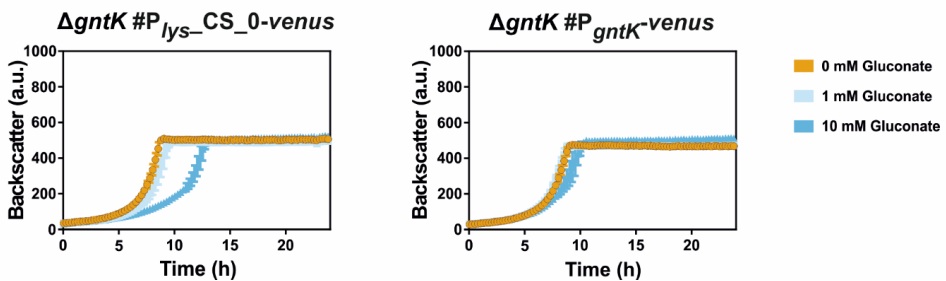


Figure S1: Growth curves corresponding to the analysis of tunability of the native P_{gntK} target promoter P_{gntK} and the synthetic counter-silencer promoter $P_{lys_CS_0}$ shown in Figure 4. Shown are backscatter values of *C. glutamicum* wild type cells (WT) (A) or *C. glutamicum* cells lacking the gene encoding the gluconate kinase ($\Delta gntK$) (B) harbouring the plasmid-based constructs pJC1- $P_{lys_CS_0-venus}$ or pJC1- $P_{gntK-venus}$ during cultivation in the presence of different gluconate concentrations. Graphs show the mean and error bars the standard deviation of biological triplicates over time. Cells were cultivated in a microtiter cultivation system in CGXII medium supplemented with constant amounts of glucose (100 mM in analysis of pJC1- $P_{gntK-venus}$ and 111 mM for characterization of pJC1- $P_{lys_CS_0-venus}$) and either no or varying amounts of gluconate as effector. Backscatter was measured at 15 min intervals. The presented data show that varying amounts of gluconate affect the final backscatter values but did not significantly influence growth rates of wild-type cells. In contrast, $\Delta gntK$ cells displayed reduced growth rates upon addition of gluconate.

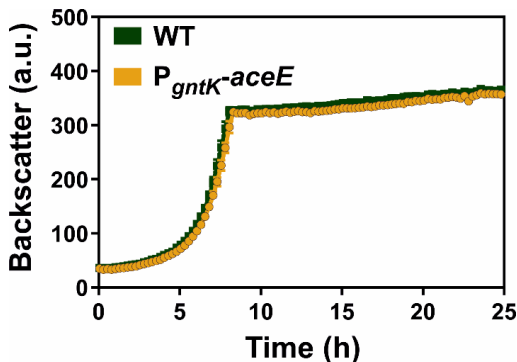


Figure S2: Growth of the strain with dynamically controlled $aceE$ expression ($P_{gntK-aceE}$) in comparison to *C. glutamicum* wild type cells, both harbouring the plasmid pJC1- $P_{lys_CS_0-venus}$. Shown are backscatter values of both *C. glutamicum* strains during cultivation in a microtiter cultivation system in CGXII minimal medium with 100 mM gluconate and 25 $\mu\text{g}/\text{ml}$ kanamycin. Strains had been pre-cultivated in CGXII containing 100 mM gluconate and 111 mM glucose. Graphs show the mean and error bars the standard deviation of biological triplicates over time. Backscatter was measured at 15 min intervals.

This experiment verified that growth of $P_{gntK}\text{-}aceE$ is not impaired in comparison to wild-type cells in the presence of gluconate (100 mM).

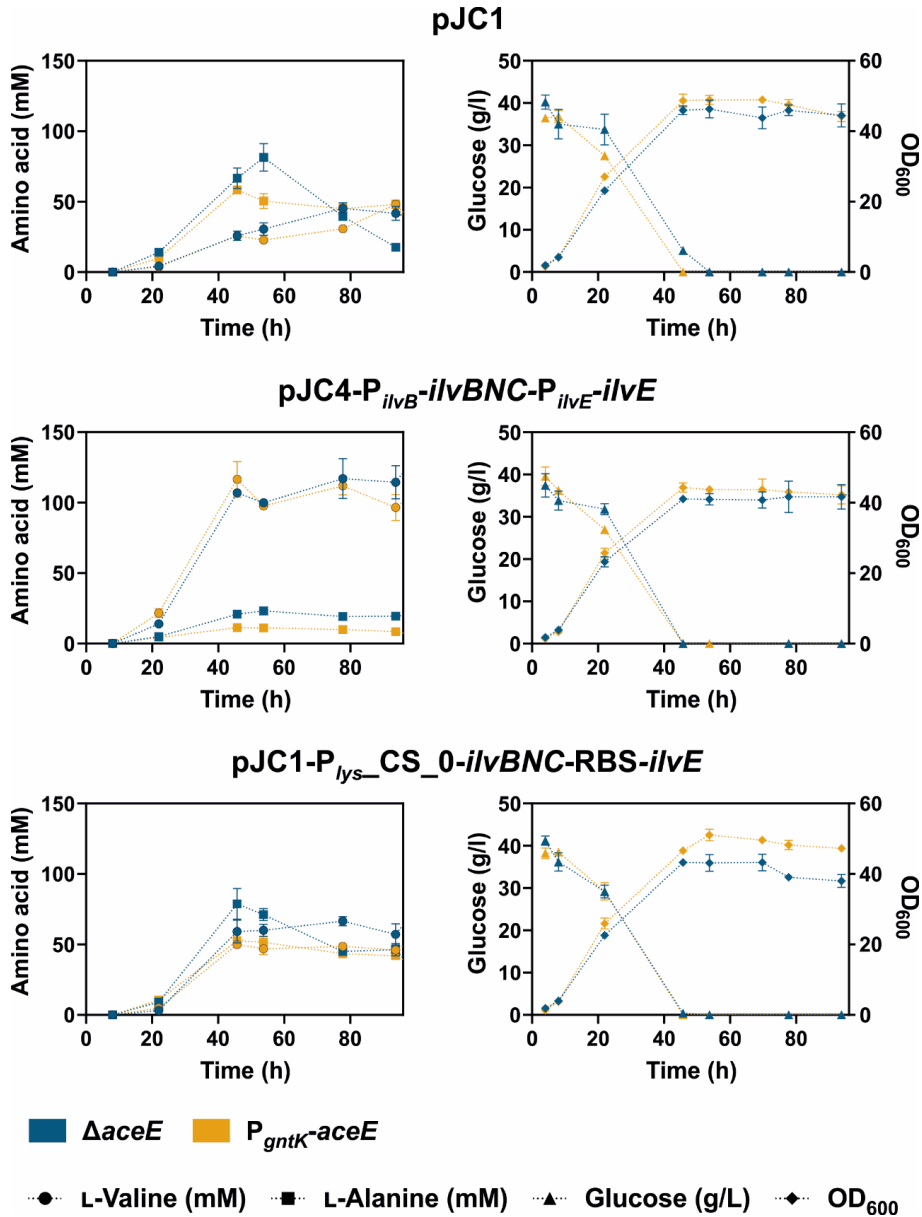


Figure S3: Growth, glucose consumption, product (L-valine) and by-product (L-alanine) formation during L-valine production. Cultivation of the strain with dynamically controlled $aceE$ expression ($P_{gntK}\text{-}aceE$) in comparison to the previously established $\Delta aceE$ strain (Blombach et al., 2007; Schreiner et al., 2005) harbouring either the empty control plasmid pJC1 (pJC1-*venus-term*), plasmid-based L-valine biosynthesis genes controlled by the counter-silencer promoter (pJC1- P_{lys} -CS_0- $illvBNC$ -RBS- $illvE$) or the natively regulated variant pJC4- P_{illvB} - $illvBNC$ - P_{illvE} - $illvE$ (pJC4- $illvBNC$ E) (Radmacher et al., 2002). Cultivation was performed in CGXII supplemented with 25 $\mu\text{g}/\text{ml}$ kanamycin, 222 mM glucose and 254 mM acetate. Graphs represent the values of biological triplicates and error bars the corresponding standard deviations after 8, 22, 46, 54, 78 and

94 h of cultivation. Measurements of glucose and L-valine in the supernatant over time revealed that glucose was almost completely consumed after 46 hours of cultivation and L-valine concentrations had mostly reached maximal values.

Supplemental tables

Table S1: Overview of prices for glucose, gluconate and acetate provided by Sigma-Aldrich (December 2019, <https://www.sigmaaldrich.com>). Considered were powders with minimal purity of 99% and an amount of 1 kg.

Carbon source	Purity	Amount	Price
D-Gluconic acid sodium salt (G9005)	≥99%	1 kg	49.80 €
D-(+)-Glucose (G8270)	≥99.5%	1 kg	48.50 €
Potassium acetate (P5708)	≥99%	1 kg	119 €

Table S2: Strains used in this study.

Strain	Relevant characteristics	Reference or source
<i>E. coli</i>		
DH5 α	F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (r _k ⁻ , m _k ⁺) <i>phoA supE44 thi-1 gyrA96 relA1 λ</i> , strain used for cloning procedures	Invitrogen
<i>C. glutamicum</i>		
ATCC 13032	Biotin-auxotrophic wild type	(Kinoshita et al., 1957)
Δ <i>aceE</i>	Derivate of ATCC 13032 with in-frame deletions of gene <i>aceE</i> (cg2466)	(Schreiner et al., 2005)
Δ <i>gntK</i>	Derivate of ATCC 13032 with in-frame deletions of gene <i>gntK</i> (cg2732)	This work
Δ P _{<i>aceE</i>} - <i>aceE</i>	Derivate of ATCC 13032 with in-frame deletions of gene <i>aceE</i> (cg2466) and its 300 bp upstream promoter region	This work
P _{<i>gntK</i>} - <i>aceE</i>	Derivate of Δ <i>aceE</i> with the re-integrated gene <i>aceE</i> (cg2466) under control of the promoter P _{<i>gntK</i>} . P _{<i>gntK</i>} (303 bp, P _{cg2732}). The first 30 bp of the coding sequence of <i>gntK</i> were fused to the <i>aceE</i> gene via a linker containing a stop codon and an artificial RBS.	This work

Table S3: Plasmids from other studies used in this work.

Plasmid	Relevant characteristics	Reference
pJC1	<i>Kan^R</i> , <i>Amp^R</i> ; <i>oriV_{C.g.}</i> , <i>oriV_{E.c.}</i> ; <i>C. glutamicum/E. coli</i> shuttle vector	(Cremer et al., 1990)
pJC1- <i>venus</i> -term	<i>Kan^R</i> ; pJC1 derivative carrying the <i>venus</i> coding sequence followed by a terminator sequence of <i>Bacillus subtilis</i>	(Baumgart et al., 2013)
pJC1-P _{cg1897} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1897 (468 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1936} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1936 (676 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1940} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1940 (563 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1955} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1955 (516 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1977} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1977 (653 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)

pJC1-P _{cg1999} ::GntR_BS_pos-30- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS 30 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1999} ::GntR_BS_pos-20- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS 20 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1999} ::GntR_BS_pos-10- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS 10 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1999} ::GntR_BS_pos-5- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS 5 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1999} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg1999} ::GntR_BS_pos+5- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene <i>cg1999</i> (448 bp) with an inserted GntR BS 5 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker	(Wiechert et al., 2020)

	containing a stop codon and an artificial RBS.	
pJC1-P _{cg1999} ::GntR_BS_pos+10- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg1999 (448 bp) with an inserted GntR BS 10 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg2014} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg2014 (545 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg2020} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg2020 (390 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{cg2022} ::GntR_BS_pos0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the gene cg2022 (309 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-100- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 100 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-25- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 25 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence	(Wiechert et al., 2020)

	fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	
pJC1-P _{lys} ::GntR_BS_pos-20- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 20 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-15- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 15 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-10- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 10 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-5- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 5 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-4- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 4 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-3- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 3 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016)	(Wiechert et al., 2020)

	and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	
pJC1-P _{lys} ::GntR_BS_pos-2- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 2 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos-1- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 1 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} _CS_0- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+1- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 1 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+2- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 2 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+3- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 3 bp downstream of the position of	(Wiechert et al., 2020)

	maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	
pJC1-P _{lys} ::GntR_BS_pos+4- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 4 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+5- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 5 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+10- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 10 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+15- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 15 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+20- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 20 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{lys} ::GntR_BS_pos+25- <i>venus</i>	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted	(Wiechert et al., 2020)

	GntR BS 25 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	
pJC1-P _{lys} ::GntR_BS_pos+50- <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of the <i>lys</i> gene (cg1974) (444 bp) with an inserted GntR BS 50 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{priP} _CS_0- <i>venus</i> (previously named pJC1-P _{priP} ::GntR_BS_pos0- <i>venus</i>)	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the CgpS bound area of the promoter of P _{priP} (611 bp) with an inserted GntR BS directly upstream of the position of maximal CgpS binding (Pfeifer et al., 2016) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pJC1-P _{gntK} - <i>venus</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the P _{gntK} promoter (307 bp) (P _{cg2732}) and the first 30 bp of the coding sequence fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	(Wiechert et al., 2020)
pEC-P _{tetR}	<i>Cm^R</i> , pGA1 <i>oriV_{Cg}</i> , <i>oriV_{Ec}</i> , <i>tetR</i> , P _{tet} ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector	(Huber et al., 2017)
pEKEx2	<i>Kan^R</i> , pBL1 <i>oriV_{Cg}</i> , pUC18 <i>oriV_{Ec}</i> , <i>lacIq</i> , P _{tac} ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector	(Eikmanns et al., 1994)
pK19- <i>mobsacB</i>	<i>Kan^R</i> , <i>oriT</i> , <i>oriV_{Ec}</i> , <i>sacB</i> , <i>lacZ</i> ; plasmid for allelic exchange in <i>C. glutamicum</i>	(Schäfer et al., 1994)
pJC1-P _{lys} _CS_0- <i>venus</i> -T-P _{gntK} -e2- <i>crimson</i>	<i>Kan^R</i> ; pJC1- <i>venus</i> -term derivative carrying the construct P _{lys} _CS_0- <i>venus</i> and the oppositely oriented P _{gntK} -e2- <i>crimson</i> construct.	(Wiechert et al., 2020)
pJC4-P _{ilvB} - <i>ilvBNC</i> -P _{ilvE} - <i>ilvE</i> (previously named pJC4- <i>ilvBNC</i>)	<i>Kan^R</i> , P _{ilvB} - <i>ilvBNC</i> , P _{ilvE} - <i>ilvE</i> (genes encoding the L-valine biosynthesis enzymes acetohydroxyacid synthase, isomeroreductase and transaminase B under control of their native promoters)	(Radmacher et al., 2002)

Table S4: Plasmids constructed in this work. Oligonucleotide pairs used for PCR are given as numbers (Table S5) with DNA templates indicated in brackets behind. The used backbones including the restriction enzymes used for linearization are listed behind (*). Used primers for sequencing are given as numbers (Table S6).

Plasmid	Construction	Relevant characteristics	Primer used for sequencing
pJC1-P _{priP} ::GntR_BS_pos-100- <i>venus</i>	Gibson assembly: 200/280 (pJC1-P _{priP} -	<i>Kan^R</i> ; pJC1-P _{priP} - <i>venus</i> (Wiechert et al., 2020)	R12, R13

	<i>venus</i>) and 279/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	derivative with an inserted GntR BS 100 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	
pJC1-P _{prip} ::GntR_BS_pos-50- <i>venus</i>	Gibson assembly: 200/278 (pJC1-P _{prip} - <i>venus</i>) and 277/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 50 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	R12, R13
pJC1-P _{prip} ::GntR_BS_pos-10- <i>venus</i>	Gibson assembly: 200/276 (pJC1-P _{prip} - <i>venus</i>) and 275/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 10 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	R12, R13
pJC1-P _{prip} ::GntR_BS_pos-5- <i>venus</i>	Gibson assembly: 200/288 (pJC1-P _{prip} - <i>venus</i>) and 287/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 5 bp upstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	R12, R13
pJC1-P _{prip} ::GntR_BS_pos+5- <i>venus</i>	Gibson assembly: 200/290 (pJC1-P _{prip} - <i>venus</i>) and 289/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 5 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	R12, R13
pJC1-P _{prip} ::GntR_BS_pos+10- <i>venus</i>	Gibson assembly: 200/282 (pJC1-P _{prip} - <i>venus</i>) and 281/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 10 bp downstream of the position of maximal CgpS binding (Pfeifer et al., 2016).	R12, R13
pJC1-P _{prip} ::GntR_BS_pos+50- <i>venus</i>	Gibson assembly: 200/284 (pJC1-P _{prip} - <i>venus</i>) and 283/116 (pJC1-P _{prip} - <i>venus</i>) into pJC1- <i>venus</i> -term *BamHI *BcuI	<i>Kan</i> ^R ; pJC1-P _{prip} - <i>venus</i> (Wiechert et al., 2020) derivative with an inserted GntR BS 50 bp downstream of the position of maximal	R12, R13

pJC1- <i>tetR</i> -P _{tet} - <i>venus</i>	547/548 (pEC-P _{tetR}) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BclI	CgpS binding (Pfeifer et al., 2016). <i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the <i>tetR</i> gene and the corresponding P _{tet} promoter fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	R12, R13, 492
pJC1- <i>lacI</i> -P _{tac} - <i>venus</i>	Gibson assembly: 545/546 (pEKEx2) and 115/116 (pJC1- <i>venus</i> -term) into pJC1- <i>venus</i> -term *BamHI *BclI	<i>Kan</i> ^R ; pJC1- <i>venus</i> -term derivative carrying the <i>lacI</i> gene and the corresponding P _{tac} promoter fused to the reporter gene <i>venus</i> via a linker containing a stop codon and an artificial RBS.	R12, R13, 293, 399, 492, 546
pJC1-P _{lys_CS_0} - <i>ilvBNC</i> -RBS- <i>ilvE</i>	Gibson assembly: 114/117 (pJC1-P _{lys_CS_0} - <i>venus</i>), 349/350 (pJC4- <i>ilvBNCE</i>) and 351/352 (pJC4- <i>ilvBNCE</i>) into pJC1- <i>venus</i> -term *BamHI *BclI	<i>Kan</i> ^R , pJC1- <i>venus</i> -term derivative carrying the operon <i>ilvBNC</i> and the gene <i>ilvE</i> (L-valine biosynthesis genes) which were combined in a synthetic operon controlled by the counter-silencer promoter P _{CS_0} .	R12, R182, R183, R215, 487, 488, 489, 490
pK19- <i>mobsacB</i> -Δ <i>gntK</i>	Gibson assembly: 207/208 (C. <i>glutamicum</i> genome) and 209/210 (C. <i>glutamicum</i> genome) into pK19- <i>mobsacB</i> *BamHI *EcoRI	<i>Kan</i> ^R , pK19- <i>mobsacB</i> derivative for the chromosomal deletion of the <i>gntK</i> gene (cg2732) (530 bp upstream and 524 bp downstream flanking regions).	M19, M20
pK19- <i>mobsacB</i> -ΔP _{aceE} - <i>aceE</i>	Gibson assembly: 62/112 (C. <i>glutamicum</i> genome) and 113/65 (C. <i>glutamicum</i> genome) into pK19- <i>mobsacB</i> *BamHI *EcoRI	<i>Kan</i> ^R , pK19- <i>mobsacB</i> derivative for the chromosomal deletion of the <i>aceE</i> gene (cg2466) and its 300 bp upstream promoter region (501 bp upstream and 500 bp downstream flanking regions).	M19, M20
pK19- <i>mobsacB</i> -P _{gntK} - <i>aceE</i>	Gibson assembly: 62/63 (C. <i>glutamicum</i> genome), 97/92 (C.	<i>Kan</i> ^R , pK19- <i>mobsacB</i> derivative for the chromosomal integration of the <i>aceE</i>	M19, M20, 93, 94, 95, 101, 102

glutamicum gene (cg2466) und genome), 70/71 (*C. glutamicum* genome) and 64/65 (*C. glutamicum* genome) into pK19-*mobsacB* *BamHI *EcoRI control of the P_{gntK} promoter (333 bp, P_{cg2732}) (501 bp upstream and 500 bp downstream flanking regions).

Table S5: Oligonucleotides used for plasmid constructions.

Oligonucleotide number	Sequence (5'→3')
279	CGCTCGATATGATAGTACCAATTCACGTGCAGCAGCACTCCC
280	GAATTGGTACTATCATATCGAGCGTTACGAACCATAACTG
277	GTGCACGTGTATGATAGTACCAATCAACTGTGCGCTAAATGCGTC
278	CACAGTTGATTGGTACTATCATACACGTGCACACATATGCGCG
275	GTTTTATGATAGTACCAATCTTTACTAAGCTTGTTAAATTGAAAC
276	GTAATAAAGATTGGTACTATCATAAACTCAACGGTTTAAAGACGC
287	CTTTATATGATAGTACCAATTTACTAAGCTTGTTAAATTGAAAC
288	GTAATTGGTACTATCATATAAAGAACTCAACGGTTTAAAG
289	CTAAGCTTATGATAGTACCAATTTGTTAAATTGAACTTCGTTATTC
290	CAATTGGTACTATCATAAGCTTAGTAATAAAGAACTCAACGG
281	GCTTGTATGATAGTACCAATAAATTGAACTTCGTTATTTCTG
282	GTTTCAATTTATTGGTACTATCATAAAACAAGCTTAGTAATAAAGAAAC
283	GAAAGTAAGTTATGATAGTACCAATAATTAAGTACTTCGGCTCCAGC
284	CTTAATTATTGGTACTATCATAACTTACTTTCTTAAATCAGAATATAAC
285	CTTTACTATATGATAGTACCAATAGCTTGTTAAATTGAACTTCG
286	CAAGCTATTGGTACTATCATATAGTAATAAAGAACTCAACGG
547	AGCGACGCCGAGGGGATCCTTAAGACCCACTTTCACATTTAAGTTGTTTTTC
548	ATGATATCTCCTTCTAAAGTTCAGTGTATCAACAAGCTGGGGATCTTAAGC
115	TGAACCTTAAGAAGGAGATATCATATGGTGAGCAAGGGCGAGGAG
116	AAAACGACGGCCAGTACTAGTACTTGTACAGCTCGTCCATGCC
545	AGCGACGCCGAGGGGATCCTCAAGCCTTCGTCAGTGGTCCC
546	ATGATATCTCCTTCTAAAGTTCAGGATCCTCTAGAGTCGACCTGC
114	TGATATCTCCTTCTAAAGTTCATTTTTTCGGCATTCGCGCTTAAATCGC
117	AGCGACGCCGAGGGGATCCGCTCAAGGAAGAGTTCCTCATTGGTC
349	TGAACCTTAAGAAGGAGATATCATGTGAATGTGGCAGCTTCTCAAC
350	ATGTATATCTCCTTCTAAAGTAAACAAAATTTTCTAGTTAAAGCGTTTCTGCGCG AGC
351	GTTTAACTTAAAGAAGGAGATATACATGTGTATCTGTCAGGTAGCAGG
352	AAAACGACGGCCAGTACTAGTTAGCCAACCAAGTGGGTAAAGC
207	CAGTGCAGCTTAGAGGATCATGGTGGCGTCATGCTCGGC
208	GTCTGTAACCGAGCATCTCTCCTAGACAATATGTAAGCCTTCGGCTG
209	GAGAGATGCTCGGTTACAGACGCAGAGTGGGTTCCGAACAAATAA
210	GTTGTAACGACGGCCAGTGAATTGCAGGTCGAGTCTTCCCACAG
62	CAGTGCAGCTTAGAGGATCTCGATGGACTCGTGATCAGC
112	GTCTGTAACCGAGCATCTCTCAAAGAATTATCGGGTAGTTTCCCGC
113	GAGAGATGCTCGGTTACAGACATCACCTCAAGGGACAGATAAATCC
65	GTTGTAACGACGGCCAGTGAATTCGTGAGCAATTCAGCAGGAAC
63	GGACGAGCTGTACAAGTAAACTAGTAAAGAATTATCGGGTAGTTTCCCGC

97	CTAGTTTACTTGTACAGCTCGTCCGAGGATCGTCTCCGCGAAGAG
92	CATTCCACACCTCCTGTTGGGTGCAAAATATGTAAGCCTTCGGCTGC
70	GACCCAACAGGAGGTGTGAAATGGCCGATCAAGCAAACTTGGTG
71	GGATTTATCTGTCCCTTGAGGTGATTTATTCCTCAGGAGCGTTTGGATC
64	GATCCAACGCTCCTGAGGAATAAATCACCTCAAGGGACAGATAAATCC

Table S6: Oligonucleotides used for plasmid sequencing.

Oligonucleotide number	Sequence (5'→3')
M19	CGCCAGGGTTTTCCAGTCAC
M20	AGCGGATAACAATTTACACAGGA
R12	CAGGGACAAGCCACCCGACA
R13	GGAAGCTAGAGTAAGTAGTTCGC
R182	CGATTCTATGGACCCTGCCACC
R183	GGTGGCAGGCTCCATAGGAATCG
R215	CTGCGTTCTGATTAATCTGTATCAGG
93	CTCTGGCAGGTAGCCACCG
94	CTGCCAGAGCGTCGTGAGAAC
95	CACACCACGGGGACTGTGG
101	CTATGGAACCTGAATTCACAGGC
102	CTTCAGGTGCCTCACGGTAGG
293	CGCCGCTTCCACTTTTTCCCG
399	CACCAAACGTTTCGGCGAGAAGC
487	GGTCAACGATGAGCTTGAGCTC
488	CTGCTGGAAACCACCAAGGC
489	CACTCGGATTGCGCCATTC
490	GCCACATTTGGTGCTGGCC
492	CTCGAACTCACCTCGGGCC
546	ATGATATCTCCTTCTTAAAGTTCAGGATCCTCTAGAGTCGACCTGC

Table S7: Oligonucleotides used for sequencing of chromosomal modifications.

Strain	Oligonucleotide number	Sequence (5'→3')
<i>ΔgntK</i>	313	GCCCACTGCTCAGCGATTTC
	314	CGGGGTCGAGTTCCTTGATCC
ΔP_{aceE} - <i>aceE</i>	104	CCAGGGCTCCTTCTTTACCAATG
	105	CGTTCTCCCCGGCACTGTG
P_{gntK} - <i>aceE</i>	93	CTCTGGCAGGTAGCCACCG
	94	CTGCCAGAGCGTCGTGAGAAC
	95	CACACCACGGGGACTGTGG
	101	CTATGGAACCTGAATTCACAGGC
	104	CCAGGGCTCCTTCTTTACCAATG
	105	CGTTCTCCCCGGCACTGTG

Table S8: Oligonucleotides used for quantitative Real-time PCR (qRT-PCR).

Target gene	Oligo-nucleotide number	Sequence (5'→3')
venus	554	GCGCACCATCTTCTTCAAGG
	555	CGGCGGTGATATAGACGTTGTG
ddh	558	AGCAGGTATGGAGCAACTTCG
	559	TGATTACCACCGGCGACAC

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4.5. Supplemental material to “Impact of CO₂/HCO₃⁻ availability on anaplerotic flux in pyruvate dehydrogenase complex-deficient *Corynebacterium glutamicum* strains”

1 **Supplemental Material to:**
 2 The impact of CO₂/HCO₃⁻ availability on anaplerotic flux in PDHC-deficient
 3 *Corynebacterium glutamicum* strains

4
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 13
 14 **Content:**

15
 16 **Table S1:** Peak areas for compounds determined via GC-ToF analysis for PDHC-deficient
 17 strains.

18 **Table S2:** Analysis of differential gene expression (0.7- and 1.7-fold change) of *C.*
 19 *glutamicum* $\Delta aceE \Delta pyc$ and $\Delta aceE$ growing on CGXII containing 154 mM acetate and
 20 222 mM glucose.

21 **Table S3:** Overview on plasmids constructed in this study.

22 **Table S4:** Oligonucleotides used in this work.

23
 24 **Figure S1:** Effect of different PTS-sugars on growth of *C. glutamicum* $\Delta aceE$ and $\Delta aceE$
 25 Δpyc .

26 **Figure S2:** Growth and glucose consumption of different *C. glutamicum* PDHC-deficient
 27 strains.

28 **Figure S3:** Effect of PTS-sugar fructose on growth of *ptsG*-deficient *C. glutamicum* $\Delta aceE$
 29 and $\Delta aceE \Delta pyc$.

30 **Figure S4:** Effect of gluconate and ribose on growth of *ptsG*-deficient *C. glutamicum*
 31 $\Delta aceE \Delta pyc$.

32 **Figure S5:** Addition of HCO₃⁻ restored growth defects of *pyc*-deficient *C. glutamicum*
 33 strains

34 **Figure S6:** Growth of the PEPCx-deficient strain $\Delta aceE \Delta ppc$.

35 **Figure S7:** Effect of culture volume on the growth of *C. glutamicum* $\Delta aceE$ and $\Delta aceE$
 36 Δpyc .

37 **Figure S8:** Deletion of *pgi* in PDHC-deficient strains to reroute flux through the pentose
 38 phosphate pathway.

39 **Figure S9:** Growth phase dependent sampling of *C. glutamicum* $\Delta aceE$ and $\Delta aceE \Delta pyc$
 40 for DNA microarrays and GC-ToF analysis.

41 **Figure S10:** Adaptive laboratory evolution of *C. glutamicum* $\Delta aceE \Delta pyc$ on acetate.

42

43 **Table S1: Peak areas for compounds determined via GC-ToF analysis for PDHC-**
 44 **deficient strains.**

	Area					
	WT	$\Delta aceE$ E1*	$\Delta aceE$ Δpyc E1*	$\Delta aceE$ Δpyc E2*	$\Delta aceE$ HCO ₃ **	$\Delta aceE$ Δpyc HCO ₃ **
Amino acids						
Alanine	2503	1303	845	8321	3277	5299
Aspartate	998	402	<1	383	296	460
Glutamine	6603	2554	1393	4430	3060	4166
Glutamate	30122	9836	3249	21157	17374	21093
N-acetyl-Glutamate	305	178	116	140	144	216
Glycine	197	110	-	168	164	192
Lysine	217	124	112	216	174	257
Proline	9611	4918	432	8127	8609	4835
Proline+CO ₂	917	625	73	620	737	407
Serine	90	52	<1	-	68	80
Valine	177	436	753	1125	410	285
Glycolysis						
Glucose	3819	10761	7152	5901	3702	3270
Glycerol + Phosphate	1591	804	801	929	1248	1262
Glycerol-3-phosphate	1229	751	299	666	928	961
3-phospho-Glycerate	49	78	35	51	117	111
Pyruvate	51	85	-	278	156	492
TCA cycle						
Malate	218	259	-	90	-	214
Succinate	1689	2135	597	2848	2093	5719
Further						
Lactate	2436	934	784	915	1686	401
Trehalose	31837	11813	29109	7409	32045	8603
Urea	233	193	168	144	247	69

45 Strains were cultivated in 50 ml CGXII containing 154 mM acetate and 222 mM glucose. Compounds were identified
 46 based on retention time using in-house database JuPoD.* = E1 corresponds to the first sample extraction where $\Delta aceE$
 47 reached in the exponential phase, while $\Delta aceE \Delta pyc$ was still in lag phase. E2 corresponds to the second sample
 48 extraction where $\Delta aceE \Delta pyc$ reached the exponential phase. ** = Medium was supplemented with 100 mM HCO₃.

49

50 **Table S2: Analysis of differential gene expression (0.7- and 1.7-fold change) of *C.***
 51 ***glutamicum* $\Delta aceE \Delta pyc$ and $\Delta aceE$ growing on CGXII containing 154 mM acetate**
 52 **and 222 mM glucose. mRNA ratio ≤ 0.70 = downregulation in $\Delta aceE \Delta pyc$ (shaded in**
 53 **red), mRNA ratio ≥ 1.7 = upregulation in $\Delta aceE \Delta pyc$ (shaded in green), p-values < 0.05 .**

Gene	Annotation	mRNA ratio	p-value
cg0791	<i>pyc</i> , pyruvate carboxylase	0.01	0.01
cg1377	<i>ssuC</i> , aliphatic sulfonates transmembrane ABC transporterprotein	0.04	0.02
cg1376	<i>ssuD1</i> , alkanesulfonate monooxygenase	0.06	0.01
cg1379	<i>ssuB</i> , aliphatic sulfonates ATP-binding ABC transporterprotein	0.08	0.00
cg0120	esterase/lipase/thioesterase family protein	0.12	0.00
cg0111	hypothetical protein cg0111	0.17	0.00
cg3219	<i>ldhA</i> , NAD-dependent L-lactate dehydrogenase	0.18	0.02
cg1147	<i>ssuI</i> , FMN-binding protein required for sulfonate and sulfonate ester utilization	0.19	0.00
cg1156	<i>ssuD2</i> , monooxygenase for sulfonate utilization	0.20	0.00
cg2962	uncharacterized enzyme involved in biosynthesis of extracellular polysaccharides	0.23	0.00
cg1905	hypothetical protein cg1905	0.25	0.00
cg2320	predicted transcriptional regulator	0.26	0.00
cg3226	putative L-lactate permease	0.27	0.01
cg1484	putative secreted protein	0.28	0.01
cg3315	bacterial regulatory protein, MarR family	0.28	0.00
cg0957	<i>fas-IB</i> , fatty acid synthase	0.29	0.02
cg3118	<i>cysI</i> , sulfite reductase (hemoprotein)	0.31	0.02
cg1907	putative phosphopantothenoylecysteine synthetase/decarboxylase	0.31	0.01
cg0812	<i>dtsR1</i> , acetyl/propionyl-CoA carboxylase beta chain	0.31	0.01
cg1906	hypothetical protein cg1906	0.32	0.00
cg1454	putative aliphatic sulfonates uptake ABC transporter secreted solute-binding protein	0.32	0.01
cg2958	<i>butA</i> , L-2,3-butanediol dehydrogenase/acetoin reductase	0.33	0.02
cg3179	<i>fadD2</i> , acyl-CoA synthase	0.35	0.00

cg3117	hypothetical protein cg3117	0.36	0.01
cg0683	permease	0.36	0.00
cg3316	universal stress protein UspA or related nucleotide-binding protein	0.37	0.00
cg2685	short chain dehydrogenase	0.37	0.03
cg1380	<i>ssuA</i> , aliphatic sulfonate binding protein	0.38	0.02
cg0277	<i>dccT</i> , dicarboxylate uptake system (succinate, fumarate or L-malate)	0.40	0.04
cg3119	<i>cysJ</i> , probable sulfite reductase (flavoprotein)	0.41	0.00
cg0175	secreted protein, signal peptide	0.41	0.02
cg3116	<i>cysH</i> , phosphoadenosine-phosphosulfate reductase	0.42	0.01
cg1003	5-formyltetrahydrofolate cyclo-ligase	0.42	0.03
cg1268	<i>glgA</i> , glycogen synthase	0.42	0.02
cg3227	<i>lldD</i> , quinone-dependent L-lactate dehydrogenase LldD	0.43	0.01
cg1066	<i>urtE</i> , ABC-type urea uptake system, ATP binding protein	0.43	0.01
cg0241	hypothetical protein cg0241	0.44	0.00
cg1399	permease of the major facilitator superfamily	0.44	0.01
cg0973	<i>pgi</i> , glucose-6-phosphate isomerase	0.44	0.03
cg0240	membrane protein	0.45	0.00
cg2645	<i>clpP1</i> , ATP-dependent Clp protease proteolytic subunit	0.45	0.01
cg2699	hypothetical protein cg2699	0.45	0.03
cg0284	drug exporter of the RND superfamily	0.45	0.04
cg2891	<i>pqq</i> , pyruvate chinon oxidoreductase	0.47	0.02
cg1744	<i>pacL</i> , cation-transporting ATPase	0.47	0.03
cg2707	hypothetical protein cg2707	0.47	0.01
cg2953	<i>xyIC</i> , benzaldehyde dehydrogenase	0.48	0.01
cg2456	Zn-ribbon protein, possibly nucleic acid-binding	0.48	0.02
cg2715	hypothetical protein cg2715	0.48	0.01
cg1740	putative nucleoside-diphosphate-sugar epimerase	0.48	0.00
cg0697	hypothetical protein cg0697	0.49	0.02
cg2342	dehydrogenase	0.49	0.02
cg0514	hypothetical membrane protein	0.49	0.00
cg3112	predicted permease	0.49	0.03

cg3092	hypothetical protein cg3092	0.50	0.03
cg0316	secreted protein	0.50	0.00
cg3223	NADPH-dependent FMN reductase	0.50	0.01
cg1741	hypothetical protein cg1741	0.50	0.01
cg0359	hypothetical protein cg0359	0.51	0.03
cg1345	<i>narK</i> , putative nitrate/nitrite transporter	0.51	0.04
cg3218	pyruvate kinase	0.52	0.01
cg0129	<i>putA</i> , proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase	0.52	0.00
cg2910	transcriptional regulator, LacI family	0.52	0.00
cg2800	<i>pgm</i> , phosphoglucomutase	0.52	0.02
cg1080	putative multicopper oxidase	0.52	0.00
cg3224	<i>LidR</i> , Repressor of the cg3226- <i>l</i> ldD lactate operon	0.52	0.01
cg1081	ABC-type multidrug transport system, ATPase component	0.52	0.00
cg1742	hypothetical protein cg1742	0.52	0.01
cg1619	putative DNA gyrase inhibitor	0.53	0.01
cg2890	putative amino acid processing enzyme	0.53	0.01
cg3279	putative dehydrogenase-fragment	0.53	0.02
cg1045	hypothetical protein cg1045	0.53	0.04
cg1246	hypothetical protein cg1246	0.53	0.01
cg0892	hypothetical protein cg0892	0.53	0.04
cg0387	<i>adhE</i> , putative zinc-type alcohol dehydrogenase transmembrane	0.53	0.03
cg2500	bacterial regulatory proteins, ArsR family	0.53	0.03
cg2118	transcriptional regulator of sugar metabolism, DeoR family	0.53	0.01
cg2810	Na ⁺ /H ⁺ -dicarboxylate symporter family	0.54	0.02
cg1647	ABC-type multidrug transport system, permease component	0.54	0.00
cg1002	putative secreted protein	0.54	0.01
cg1452	hypothetical protein cg1452	0.55	0.00
cg3402	copper chaperone	0.55	0.01
cg3004	<i>gabD2</i> , succinic semialdehyde dehydrogenase	0.55	0.00
cg2305	<i>hisD</i> , histidinol dehydrogenase	0.55	0.05
cg3304	<i>dnaB</i> , replicative DNA helicase	0.55	0.00

cg1068	probable oxidoreductase	0.55	0.01
cg1374	hypothetical protein cg1374	0.56	0.01
cg3280	putative secreted protein	0.56	0.02
cg1238	hypothetical protein cg1238	0.56	0.02
cg0039	putative transcriptional regulator	0.56	0.01
cg1111	<i>eno</i> , phosphopyruvate hydratase	0.57	0.03
cg1618	hypothetical protein cg1618	0.57	0.01
cg3340	<i>dadA</i> , putative D-amino acid dehydrogenase (deaminating)	0.57	0.02
cg2318	putative secreted vitamin B12-binding lipoprotein	0.57	0.00
cg0807	hypothetical protein cg0807	0.57	0.03
cg2598	hypothetical protein cg2598	0.57	0.00
cg3225	putative serine/threonine-specific protein phosphatase	0.57	0.03
cg2340	ABC-type amino acid transport system, secreted component	0.57	0.00
cg0513	hypothetical membrane protein	0.58	0.00
cg0777	siderophore ABC transporter, ATP-binding protein	0.58	0.02
cg1545	permease of the major facilitator superfamily	0.58	0.04
cg0755	<i>metY</i> , O-acetylhomoserine sulfhydrylase	0.58	0.01
cg2537	<i>brnQ</i> , branched-chain amino acid uptake carrier	0.58	0.03
cg1525	<i>polA</i> , DNA polymerase I	0.58	0.01
cg0685	homolog of metal-dependent protease, putative molecular chaperone	0.58	0.01
cg2291	<i>pyk</i> , pyruvate kinase	0.58	0.01
cg2770	hypothetical protein cg2770	0.58	0.03
cg3128	ABC-type transport system, ATPase component	0.59	0.01
cg0780	membrane protein ribonuclease BN-like family	0.59	0.01
cg0611	secreted protein	0.59	0.00
cg0176	permease	0.59	0.02
cg0358	hydrolase or acyltransferase	0.59	0.01
cg0122	putative glycerol 3-phosphate dehydrogenase	0.59	0.05
cg2304	<i>hisC</i> , histidinol-phosphate aminotransferase	0.59	0.01
cg1537	<i>ptsG</i> , glucose-specific enzyme II BC component of PTS	0.59	0.02
cg1791	<i>gap</i> , glyceraldehyde-3-phosphate dehydrogenase	0.59	0.02

cg0976	<i>pcrA</i> , ATP-dependent helicase PCRA	0.59	0.01
cg0260	<i>moaC</i> , molybdenum cofactor biosynthesis protein C	0.60	0.03
cg0464	<i>ctpA</i> , copper-transporting ATPase	0.60	0.02
cg0662	FAD/FMN-containing dehydrogenase	0.60	0.03
cg2247	hypothetical protein cg2247	0.60	0.02
cg1017	<i>metS</i> , methionyl-tRNA synthetase	0.60	0.02
cg1330	similar to GTP pyrophosphokinase	0.60	0.00
cg2459	<i>ptpA</i> , protein-tyrosine-phosphatase	0.60	0.01
cg0766	<i>icd</i> , isocitrate dehydrogenase	0.60	0.01
cg2877	<i>avtA</i> , aminotransferase, uses alanine, keto-isovalerate and ketobutyrate	0.61	0.01
cg2743	<i>fas-IA</i> , fatty acid synthase	0.61	0.04
cg2457	hypothetical protein cg2457	0.61	0.02
cg1195	sulfate permease or related transporter (MFS superfamily)	0.61	0.01
cg2556	uncharacterized iron-regulated membrane protein	0.61	0.00
cg1027	<i>ldd</i> , D-lactate dehydrogenase	0.61	0.02
cg0404	nitroreductase family	0.61	0.00
cg2835	predicted acetyltransferase	0.61	0.01
cg3399	permease of the major facilitator superfamily	0.62	0.05
cg2162	<i>thyX</i> , alternative thymidylate synthase	0.62	0.02
cg0896	hypothetical protein cg0896	0.62	0.02
cg2104	<i>galE</i> , UDP-glucose 4-epimerase	0.62	0.02
cg0131	putative oxidoreductase	0.62	0.00
cg3057	putative secreted protein	0.62	0.00
cg1790	<i>pgk</i> , phosphoglycerate kinase	0.62	0.02
cg2558	related to aldose 1-epimerase	0.62	0.00
cg2554	<i>rbsK2</i> , probable ribokinase protein	0.62	0.02
cg2536	<i>metC</i> , cystathionine beta-lyase	0.62	0.00
cg0239	hypothetical protein cg0239	0.63	0.02
cg2178	<i>nusA</i> , transcription elongation factor NusA	0.63	0.04
cg1908	hypothetical protein cg1908	0.63	0.05
cg1603	hypothetical protein cg1603	0.63	0.02

cg1247	putative secreted protein	0.63	0.00
cg3228	hypothetical protein cg3228	0.63	0.04
cg1106	hypothetical protein cg1106	0.63	0.03
cg3158	<i>nagA2</i> , beta-N-acetylglucosaminidase precursor	0.63	0.02
cg1691	<i>arc</i> , AAA+ ATPase ARC, pupylation machinery	0.63	0.00
cg0781	membrane protein	0.63	0.04
cg0934	hypothetical protein cg0934	0.63	0.02
cg2867	<i>gpx</i> , glutathione peroxidase	0.64	0.02
cg1683	superfamily II DNA and RNA helicase	0.64	0.01
cg2605	predicted acetyltransferase	0.64	0.02
cg1883	putative secreted protein	0.64	0.05
cg3331	<i>ogt</i> , methylated-DNA--protein-cysteine methyltransferase	0.64	0.00
cg1375	putative thioredoxin	0.64	0.01
cg2529	<i>treS</i> , trehalose synthase (maltose alpha-D-glucosyltransferase)	0.64	0.00
cg1400	DNA polymerase III subunit epsilon	0.64	0.00
cg2778	hypothetical protein cg2778	0.64	0.01
cg2248	hypothetical protein cg2248	0.64	0.01
cg0684	<i>papA</i> , prolyl aminopeptidase A	0.64	0.02
cg2577	predicted multitransmembrane, metal-binding protein	0.64	0.04
cg2431	putative transcriptional regulator	0.64	0.01
cg1831	bacterial regulatory protein, ArsR family	0.65	0.05
cg3332	putative quinone oxidoreductase	0.65	0.01
cg0712	secreted protein	0.65	0.00
cg1599	hypothetical protein cg1599	0.65	0.00
cg0297	hypothetical protein cg0297	0.65	0.03
cg2455	hypothetical protein cg2455	0.65	0.01
cg2572	hypothetical protein cg2572	0.65	0.01
cg0512	<i>hemB</i> , delta-aminolevulinic acid dehydratase	0.65	0.00
cg0171	secreted protein	0.65	0.00
cg2521	<i>fadD15</i> , long-chain fatty acid CoA ligase	0.65	0.01
cg0174	putative transport protein	0.65	0.04
cg1680	hypothetical protein cg1680	0.65	0.01

cg0376	<i>dnaX</i> , putative DNA polymerase III, delta subunit	0.65	0.03
cg2033	putative secreted protein	0.66	0.02
cg0680	hypothetical protein cg0680	0.66	0.04
cg2924	<i>cysS</i> , cysteinyl-tRNA synthetase	0.66	0.01
cg1206	PEP phosphonmutase or related enzyme	0.66	0.01
cg3236	<i>msrA</i> , peptide methionine sulfoxide reductase	0.66	0.00
cg0481	<i>mshA</i> , glycosyltransferase	0.66	0.01
cg0686	acetyltransferase, GNAT family	0.66	0.02
cg1035	TatD related DNase	0.66	0.03
cg3067	hypothetical protein cg3067	0.66	0.01
cg0388	Zn-dependent hydrolase	0.66	0.04
cg2193	putative lysophospholipase	0.66	0.01
cg1018	probable ATP-dependent DNA helicase protein	0.66	0.03
cg1423	putative oxidoreductase (related to aryl-alcohol dehydrogenase)	0.66	0.01
cg1284	<i>lipT</i> , type B carboxylesterase	0.67	0.03
cg2597	<i>me</i> , probable ribonuclease E (RNase E) protein	0.67	0.05
cg0796	<i>prpD1</i> , 2-methylcitrate dehydratase	0.67	0.01
cg2117	<i>ptsI</i> , phosphoenolpyruvate:sugar phosphotransferase system enzymei	0.67	0.01
cg0081	putative tautomerase	0.67	0.01
cg1630	<i>odhI</i> , essential for glutamine utilization, regulates ODH activity	0.67	0.03
cg2795	NADPH quinone reductase or related Zn-dependent oxidoreductase	0.67	0.02
cg3253	<i>mcbR</i> , TetR-type transcriptional regulator of sulfur metabolism	0.67	0.03
cg1600	predicted rRNA methylase	0.68	0.01
cg3069	hypothetical protein cg3069	0.68	0.02
cg3068	<i>fda</i> , fructose-bisphosphate aldolase	0.68	0.01
cg0067	<i>gabD3</i> , succinate-semialdehyde dehydrogenase (NADP+)	0.68	0.00
cg0450	hypothetical protein cg0450	0.68	0.04
cg2687	<i>metB</i> , cystathionine gamma-synthase	0.68	0.00
cg2323	<i>treY</i> , maltooligosyl trehalose synthase	0.68	0.01
cg3185	hypothetical protein cg3185	0.68	0.01
cg1384	putative NUDIX hydrolase	0.68	0.00

cg3350	<i>nagK</i> , fumarylpyruvate hydrolase	0.68	0.04
cg0232	hypothetical secreted protein	0.69	0.00
cg2432	MUTT/NUDIX family protein	0.69	0.03
cg3034	<i>def</i> , peptide deformylase	0.69	0.02
cg2421	<i>sucB</i> , dihydrolipoamide acetyltransferase	0.69	0.00
cg1401	<i>ligA</i> , DNA ligase	0.69	0.03
cg3423	<i>trxC</i> , thioredoxin	0.69	0.01
cg0128	secreted protein, signal peptide	0.69	0.03
cg0496	thiol-disulfide isomerase or thioredoxin	0.69	0.05
cg1048	haloacid dehalogenase/epoxide hydrolase family	0.69	0.03
cg0015	<i>gyrA</i> , DNA gyrase subunit A	0.69	0.03
cg0854	<i>pmmA</i> , phosphomannomutase	0.69	0.01
cg1318	DNA repair exonuclease	0.69	0.03
cg0873	<i>aroA</i> , 3-phosphoshikimate 1-carboxyvinyltransferase	0.69	0.01
cg0154	haloacid dehalogenase-like hydrolase	0.70	0.04
cg2444	hypothetical protein cg2444	0.70	0.03
cg0745	NAD-dependent deacetylase	0.70	0.01
cg2716	<i>hyi</i> , hydroxypyruvate isomerase	0.70	0.02
cg3154	<i>udgA2</i> , UDP-glucose 6-dehydrogenase	0.70	0.01
cg0910	inositol monophosphatase	0.70	0.03
cg0489	hypothetical membrane protein	0.70	0.00
cg3168	SAM-dependent methyltransferase	0.70	0.01
cg3015	hypothetical protein cg3015	1.70	0.01
cg1414	uncharacterized component of ribose/xylose transport systems	1.70	0.00
cg1355	<i>prfA</i> , peptide chain release factor 1	1.71	0.02
cg2138	<i>gluC</i> , glutamate permease	1.71	0.03
cg1366	<i>atpA</i> , ATP synthase subunit A	1.71	0.05
cg3008	<i>porA</i> , main cell wall channel protein	1.72	0.03
cg2374	<i>murE</i> , UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	1.72	0.03
cg1367	<i>atpG</i> , ATP synthase subunit C	1.72	0.02
cg2272	mutM1, formamidopyrimidine-DNA glycosylase	1.73	0.00

cg1498	RecG-like helicase	1.73	0.01
cg0719	<i>crtYe</i> , C50 carotenoid epsilon cyclase	1.74	0.01
cg3266	<i>tnp5c</i> (ISCg5c), transposase	1.74	0.00
cg1706	<i>arsC1</i> , arsenate reductase	1.74	0.02
cg2263	hypothetical protein cg2263	1.75	0.00
cg1314	<i>putP</i> , proline transport system	1.75	0.00
cg1349	membrane protein containing CBS domain	1.75	0.03
cg1122	putative secreted protein	1.75	0.05
cg1412	ribose/xylose/arabinose/galactoside ABC-type transport system, permease component	1.75	0.02
cg1123	<i>greA</i> , transcription elongation factor GreA	1.76	0.03
cg0577	<i>rpoC</i> , DNA-directed RNA polymerase beta subunit	1.78	0.03
cg1617	GTP-binding protein EngA	1.78	0.01
cg2990	<i>speE</i> , spermidine synthase	1.78	0.00
cg2624	<i>pcaR</i> , transcriptional regulator of 4-hydroxybenzoate, protocatechuate, p-cresol pathway	1.78	0.02
cg0690	<i>groES</i> , chaperonin 10 Kd subunit	1.78	0.01
cg2807	<i>tnp11a</i> (ISCg11a), transposase-fragment	1.79	0.00
cg1041	<i>pdxK</i> , pyridoxal/pyridoxine/pyridoxamine kinase	1.79	0.00
cg2184	ATPase component of peptide ABC-type transport system, contains duplicated ATPase domains	1.79	0.00
cg3027	<i>mrpE</i> , hypothetical protein cg3027	1.80	0.02
cg2600	<i>tnp1d</i> (ISCg1d), transposase	1.80	0.00
cg0203	<i>iolE</i> , 2-Keto-myo-inositol dehydratase	1.80	0.00
cg0621	substrate-specific component SCO2325 of predicted cobalamin ECF transporter	1.81	0.00
cg1218	<i>ndnR</i> , transcriptional repressor of NAD de novo biosynthesis genes	1.81	0.00
cg1586	<i>argG</i> , argininosuccinate synthase	1.82	0.01
cg2269	predicted permease	1.82	0.00
cg1413	secreted sugar-binding protein	1.82	0.01
cg0717	<i>crtEb</i> , hypothetical protein cg0717	1.82	0.00
cg0057	<i>pknB</i> , eukaryotic-type serine/threonine kinase	1.83	0.00
cg1602	<i>recN</i> , DNA repair protein RecN	1.86	0.00
cg2467	ABC transporter ATP-binding protein	1.86	0.00

cg2196	putative secreted or membrane protein	1.86	0.05
cg3385	<i>catA3</i> , catechol 1,2-dioxygenase	1.86	0.00
cg1149	hypothetical protein cg1149	1.87	0.04
cg1364	<i>atpF</i> , ATP synthase subunit B	1.88	0.02
cg2559	<i>aceB</i> , malate synthase	1.88	0.02
cg0693	<i>groEL</i> , 60 KDA chaperonin (protein CPN60) (groel protein) C-terminal fragment	1.88	0.04
cg1363	<i>atpE</i> , ATP synthase subunit C	1.88	0.02
cg0691	<i>groEL</i> , 60 KDA chaperonin (protein CPN60) (HSP60)-N-terminal fragment	1.89	0.02
cg1040	ATPase component of ABC transporters with duplicated ATPase domains	1.90	0.00
cg1902	putative secreted protein	1.91	0.02
cg0692	<i>tnp1c</i> (ISCg1c), transposase	1.92	0.00
cg1418	secreted siderophore-binding lipoprotein	1.92	0.00
cg1213	<i>tnp1a</i> (ISCg1a), transposase	1.93	0.04
cg2052	putative secreted protein	1.94	0.02
cg2676	ABC-type dipeptide/oligopeptide/nickel transport systems, permease component	1.94	0.01
cg1780	<i>devB</i> , 6-phosphogluconolactonase	1.94	0.01
cg1362	<i>atpB</i> , ATP synthase subunit A	1.95	0.03
cg2265	<i>smc</i> , chromosome segregation ATPase	1.96	0.00
cg0204	<i>iolG</i> , putative oxidoreductase myo-inositol 2-dehydrogenase	1.96	0.04
cg3386	<i>tcbF</i> , maleylacetate reductase	1.96	0.01
cg0223	<i>iolT1</i> , myo-Inositol transporter	1.97	0.04
cg1488	<i>leuD</i> , isopropylmalate isomerase small subunit	1.97	0.00
cg1354	<i>rho</i> , transcription termination factor Rho	1.98	0.01
cg1792	putative transcriptional regulator-WhiA homolog	1.98	0.01
cg2470	secreted ABC transporter substrate-binding protein	1.98	0.00
cg3047	<i>ackA</i> , acetate/propionate kinase	1.99	0.01
cg1417	acetyltransferase	2.00	0.00
cg1348	membrane protein containing CBS domain	2.01	0.03
cg1725	<i>mutA</i> , methylmalonyl-CoA mutase, subunit	2.01	0.01
cg0062	<i>ppp</i> , protein phosphatase	2.02	0.02

cg1779	<i>opcA</i> , putative subunit of glucose-6-P dehydrogenase	2.04	0.01
cg0323	conserved hypothetical secreted protein	2.05	0.01
cg3195	flavin-containing monooxygenase (FMO)	2.06	0.04
cg1812	<i>pyrF</i> , orotidine 5-phosphate decarboxylase	2.07	0.02
cg1344	<i>narG</i> , nitrate reductase 2, alpha subunit	2.07	0.01
cg0417	<i>capD</i> , probable dTDP-glucose 4,6-dehydratase transmembrane protein	2.08	0.00
cg1813	<i>carB</i> , carbamoyl-phosphate synthase large subunit	2.12	0.01
cg3363	<i>trpB</i> , tryptophan synthase subunit beta	2.14	0.00
cg0720	<i>crtI2</i> , phytoene dehydrogenase (desaturase)	2.15	0.01
cg0321	Na ⁺ /H ⁺ antiporter subunit	2.16	0.01
cg0419	glycosyltransferase	2.16	0.01
cg1365	<i>atpH</i> , ATP synthase subunit D	2.16	0.01
cg0418	putative aminotransferase	2.17	0.01
cg0325	hypothetical protein cg0325	2.22	0.02
cg1215	<i>nadC</i> , quinolinate phosphoribosyltransferase	2.23	0.01
cg3016	hypothetical protein cg3016	2.24	0.01
cg3096	<i>ald</i> , alcohol dehydrogenase	2.24	0.04
cg2939	ABC-type dipeptide/oligopeptide/nickel transport system, fused permease and ATPase components	2.29	0.02
cg3028	<i>mrpF</i> , hypothetical protein cg3028	2.32	0.00
cg3029	<i>mrpG</i> , multisubunit Na ⁺ /H ⁺ antiporter, g subunit	2.34	0.01
cg3025	<i>mrpC</i> , hypothetical protein cg3025	2.34	0.01
cg2675	ATPase component of ABC-type transport system, contains duplicated ATPase domains ATPase component of ABC-type transport system, contains duplicated ATPase domains	2.36	0.01
cg1582	<i>argB</i> , acetylglutamate kinase	2.37	0.04
cg2240	<i>thiF</i> , molybdopterin biosynthesis protein MoeB	2.38	0.04
cg3391	<i>oxiD</i> , myo-Inositol dehydrogenase	2.40	0.00
cg0324	<i>mnhD</i> , NADH dehydrogenase subunit N	2.41	0.01
cg2610	ABC-type dipeptide/oligopeptide/nickel transport system, secreted component	2.46	0.00
cg3017	hypothetical protein cg3017	2.54	0.00
cg0837	hypothetical protein cg0837	2.66	0.03

cg0322	predicted conserved membrane protein	2.69	0.00
cg3026	<i>mrpD</i> , NADH-ubiquinone oxidoreductase/multisubunit Na ⁺ /H ⁺ antiporter, D subunit	2.86	0.01
cg3390	<i>myo</i> -Inositol catabolism, sugar phosphate isomerase/epimerase	2.89	0.01
cg3360	<i>trpG</i> , anthranilate synthase component II	2.97	0.01
cg0059	<i>pknA</i> , serine/threonine protein kinase	3.01	0.00
cg0838	helicase	3.06	0.01
cg3359	<i>trpE</i> , anthranilate synthase component I	3.08	0.00
cg0165	ABC-2 type transporter	3.12	0.01
cg0061	<i>rodA</i> , putative FTSW/RODA/SPOVE family cell cycle protein	3.13	0.00
cg1724	<i>meaB</i> , accessory protein of methylmalonylCoA mutase	3.18	0.01
cg0018	hypothetical membrane protein	3.19	0.00
cg0163	N-acetylglucosaminyltransferase	3.21	0.00
cg1583	<i>argD</i> , acetylornithine aminotransferase	3.26	0.01
cg0060	<i>pbpA</i> , D-alanyl-D-alanine carboxypeptidase	3.31	0.00
cg3362	<i>trpCF</i> , bifunctional indole-3-glycerol phosphate synthase/phosphoribosylanthranilate isomerase	3.31	0.01
cg0162	membrane spanning protein	3.33	0.00
cg1214	<i>nadS</i> , cysteine desulfurase-like protein involved in Fe-S cluster assembly	3.44	0.00
cg2636	<i>catA1</i> , catechol 1,2-dioxygenase	3.50	0.01
cg1584	<i>argF</i> , ornithine carbamoyltransferase	3.81	0.01
cg3361	<i>trpD</i> , anthranilate phosphoribosyltransferase	3.83	0.01
cg1585	<i>argR</i> , arginine repressor	3.96	0.00
cg0844	type II restriction enzyme, methylase subunit	6.36	0.00
cg2430	hypothetical protein cg2430	13.96	0.00

55 **Table S3: Overview on plasmids constructed in this study.** In Table 2 in the main text
 56 provides a list of all plasmids used in this study.

Plasmid	Backbone	Restriction-enzymes	Oligonucleotides for insert	Template	Method
pK19 <i>mobsacB</i> - Δ <i>ptsG</i> -P3	pK19 <i>mobsacB</i> (1)	EcoRI, BamHI	A7-ptsG-leftflank-fw A8-ptsG-leftflank-rv A9-ptsG-rightflank-fw A10-ptsG-rightflank-rv	Genomic DNA (<i>C. glutamicum</i>)	Gibson assembly
pK19 <i>mobsacB</i> - Δ <i>pgi</i>	pK19 <i>mobsacB</i> (1)	EcoRI, BamHI	A21- <i>pgi</i> -leftflank-fw A22- <i>pgi</i> -leftflank-rv A23- <i>pgi</i> -rightflank-fw A24- <i>pgi</i> -rightflank-rv	Genomic DNA (<i>C. glutamicum</i>)	Gibson assembly
pJC1- <i>pyc</i>	pJC1- <i>venus</i> - term-BS (2)	BamHI, BcuI	A31- <i>pyc</i> -pJC1-fw A32- <i>pyc</i> -pJC1-rv	Genomic DNA (<i>C. glutamicum</i>)	Gibson assembly

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59 **Table S4: Oligonucleotides used in this work.**

Oligonucleotide	Sequence 5'→3'	Use
Primer for sequencing and strain verification		
A1-seq- <i>pyc</i> -fw	CTGCGCCACGGTTTTGTGAAG	Sequencing <i>pyc</i>
A2-seq- <i>pyc</i> -rv	CAACCACATCTGCACTGCGATC	Sequencing <i>pyc</i>
A14-seq- <i>ptsG</i> -flanks-fw	GGCTCACTGACGTTGACAGTG	Sequencing <i>ptsG</i>
A15-seq- <i>ptsG</i> -flanks-rv	GATATCGCGGCAACTTGGTC	Sequencing <i>ptsG</i>
A17-seq- <i>pgi</i> -fw	GTGATGGCACCTGCCGATTG	Sequencing <i>pgi</i>
A18-seq- <i>pgi</i> -rv	CGGAATCCACGAAATCGCCG	Sequencing <i>pgi</i>
104- <i>aceE</i> int-seq-fw	CCAGGGCTCCTTCTTTACCAATG	Sequencing <i>aceE</i>
105- <i>aceE</i> int-seq-rv	CCTTCTTCCCGGCACTGTG	Sequencing <i>aceE</i>
<i>ppc</i> del_fw	GGAATAGACTCGCTCGGC	Sequencing <i>ppc</i>
<i>ppc</i> del_rv	GTGAACAGGCTCTCGATGC	Sequencing <i>ppc</i>
<i>ureD</i> _E188*_fw	CGGACTCCCGATAGGGAAGTTTTG	Sequencing <i>ureD</i>
<i>ureD</i> _E188*_rv	CAAAACTTCCCTATCGGGAGTCCG	Sequencing <i>ureD</i>
596- <i>aceA</i> -Seq-fw	CGAGTTCCTTCTGGAACCTTAGCG	Sequencing <i>aceA</i>
599- <i>aceA</i> -Seq-rv2	CTTCGGTGCGTGCATTCATC	Sequencing <i>aceA</i>
597- <i>aceB</i> -Seq-fw	CTTCGAAGACTCCGTTGCAGC	Sequencing <i>aceB</i>
598- <i>aceB</i> -Seq-rv	TTGTTGAGCTCACGGGTGAAG	Sequencing <i>aceB</i>
600- <i>aceB</i> -Seq-rv2	GAGCAAGCAGCTCGCGTTG	Sequencing <i>aceB</i>
M19-M13-fw	CACTGACCCCTTTGGGACCGC	Sequencing of pK19 <i>mobsacB</i>
M20-M13-rv	AGCGGATAACAATTCACACAGGA	Sequencing of pK19 <i>mobsacB</i>
R12-pJC1-MCS-fw	CAGGGACAAGCCACCCGCACA	Sequencing of inserts in pJC1
R13-pJC1-MCS-rv	GGAAGCTAGAGTAAGTAGTTCGC	Sequencing of inserts in pJC1
Construction of plasmid pK19<i>mobsacB</i>-Δ<i>ptsG</i>-P3 used for deletion of <i>ptsG</i>		
A7- <i>ptsG</i> -leftflank-fw	<u>CAGGTCGACTCTAGAGGATCGCTTTTGGCGGGCCG</u> TTCGG	Left flank fw Δ <i>ptsG</i>
A8- <i>ptsG</i> -leftflank-rv	<u>GTCTGTAACCGAGCATCTCTCGTCAAACCTTTCTA</u> AACGTAGGGTCTG	Left flank rv Δ <i>ptsG</i>
A9- <i>ptsG</i> -rightflank-fw	<u>GAGAGATGCTCGGTTACAGACCAGCTAAGCCGAAG</u> CTGGCCG	Right flank fw Δ <i>ptsG</i>
A10- <i>ptsG</i> -rightflank-rv	<u>GTAAAACGACGGCCAGTGAATTGTTACTCGTTCTT</u> GCCGTTGACCTTG	Right flank rv Δ <i>ptsG</i>

Construction of plasmid pK19*mobsacB*- Δ *pgi* used for deletion of *pgi*

A21- <i>pgi</i> -leftflank-fw	<u>CAGGTCGACTCTAGAGGATCCAGCACCCGACAAACA</u> CGATC	Left flank fw Δ <i>pgi</i>
A22- <i>pgi</i> -leftflank-rv	GTCTGTAAACCGAGCATCTCTCGAAAACCTCCTTTAT TGTCTGTTAAATAAC	Left flank rv Δ <i>pgi</i>
A23- <i>pgi</i> -rightflank-fw	GAGAGATGCTCGGTTACAGACCCTGTTCCACTGG CACTG	Right flank fw Δ <i>pgi</i>
A24- <i>pgi</i> -rightflank-rv	GTAAAACGACGGCCAGTGAATTGCTACCTATTTGC GCGGTACC	Right flank rv Δ <i>pgi</i>

Construction of plasmid pJC1-*pyc* used for overexpression of *pyc*

A31- <i>pyc</i> -pJC1-fw	<u>ACCGACGCCGACGGGGATCCGGATTGCTTTGTGC</u> ACTCCTGG	<i>pyc</i> fw, including promoter region
A32- <i>pyc</i> -pJC1-rv	<u>AAAACGACGGCCAGTACTAGCAGAAAGGTTAGGA</u> AACGACGAC	<i>pyc</i> rv

Construction of plasmid pJC1-*pyc* used for overexpression of *aceA* and *aceB*

588- <i>aceA</i> -fw	<u>CTGAACTTAAGAAGGAGATATCATATGTCAAACG</u> TTGGAAGCCACG	Insert 1 <i>aceA</i> fw
589- <i>aceA</i> -linker-rv	CATATGTATATCTCCTTCTTAAAGTTAAACAAAAT TATTTCTAGTCTAGTTGTGGAAGTGGCCTTCTTC	Insert 1 <i>aceA</i> rv, including linker
590- <i>aceB</i> -linker-fw	GTTTAACTTTAAGAAGGAGATATACATATGACTGA ACAGGAAGTGTGTCTG	Insert 2 <i>aceB</i> fw, including linker
591- <i>aceB</i> -rv	CTGTAAAACGACGGCCAGTACTAGTTAGTTTTTTG CTTTGAAGTCCGCGC	Insert 2 <i>aceB</i> rv

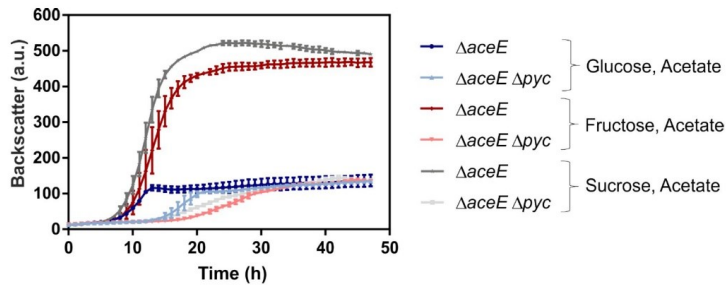
Construction of plasmid pJC1-*pyc* used for overexpression of *aceB* and *aceA*

584- <i>aceB</i> -fw	<u>CTGAACTTAAGAAGGAGATATCATATGACTGAAC</u> AGGAAGTGTGTCTG	Insert 1 <i>aceB</i> fw
585- <i>aceB</i> -linker-rv	ATGTATATCTCCTTCTTAAAGTTAAACAAAATAT TTCTAGTTAGTTTTTTGCTTTGAACTCGCGG	Insert 1 <i>aceB</i> rv, including linker
586- <i>aceA</i> -linker-fw	GTTTAACTTTAAGAAGGAGATATACATATGTCAA CGTTGGAAGCCACG	Insert 2 <i>aceA</i> fw, including linker
587- <i>aceA</i> -rv	<u>CTGTAAAACGACGGCCAGTACTAGTTGTGGA</u> ACTGGCCTTCTTC	Insert 2 <i>aceA</i> rv

60 Underlined = complementary to backbone, **red** = linker sequence; fw = forward, rv = reverse

61

62



63

64 **Figure S1: Effect of different PTS-sugars on growth of *C. glutamicum* $\Delta aceE$ and**65 **$\Delta aceE \Delta pyc$.** The strains $\Delta aceE$ and $\Delta aceE \Delta pyc$ were inoculated to an OD_{600} of 1 in

66 CGXII containing 154 Mm acetate and 222 mM of varying PTS sugars: glucose (shades

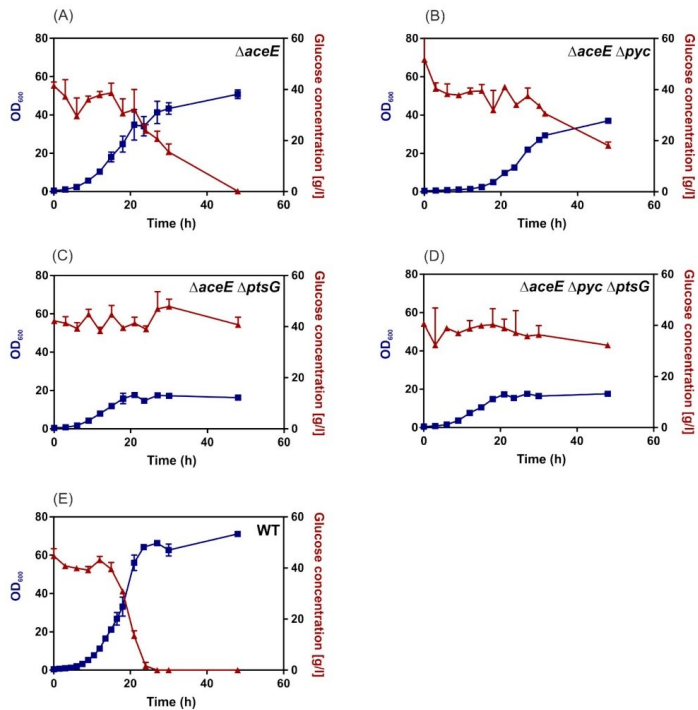
67 of blue), fructose (shades of red), or sucrose (shades of grey). Growth curves shown are

68 based on the backscatter measurements in a microtiter cultivation system. Symbols

69 represent the backscatter means and error bars their standard deviations of biological

70 triplicates.

71



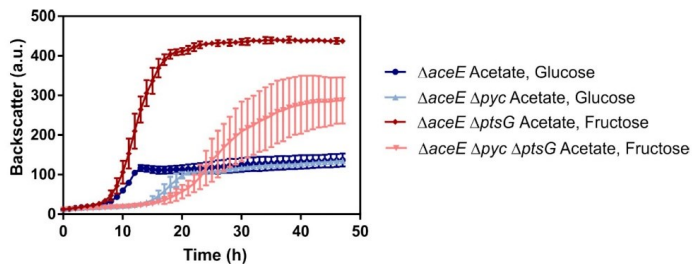
72

73 **Figure S2: Growth and glucose consumption of different *C. glutamicum* PDHC-**
 74 **deficient strains.** The PDHC-deficient strains $\Delta aceE$, $\Delta aceE \Delta pyc$, $\Delta aceE \Delta ptsG$, $\Delta aceE$
 75 $\Delta pyc \Delta ptsG$ as well as the wild type (WT) as control were inoculated to an OD₆₀₀ of 1 each
 76 in 50 ml CGXII containing 154 mM acetate and 222 mM glucose. OD₆₀₀ measurements
 77 (blue graphs) and sampling for glucose measurements were performed every 3 h until 33
 78 h and once additionally at 48 h. (A-E) Measurement of glucose concentration was
 79 performed with the D-Glucose UV-Test Kit (r-biopharm, Darmstadt, Germany) (red

80 graphs). Symbols represent the OD_{600} means and error bars their standard deviations of
81 biological triplicates.

82

83



84

85 **Figure S3: Effect of PTS-sugar fructose on growth of *ptsG*-deficient *C. glutamicum***86 **$\Delta aceE$ and $\Delta aceE \Delta pyc$.** Growth curves shown are based on the backscatter87 measurements in a microtiter cultivation system, inoculated at an OD_{600} of 1. Symbols

88 represent the backscatter means and error bars their standard deviations of biological

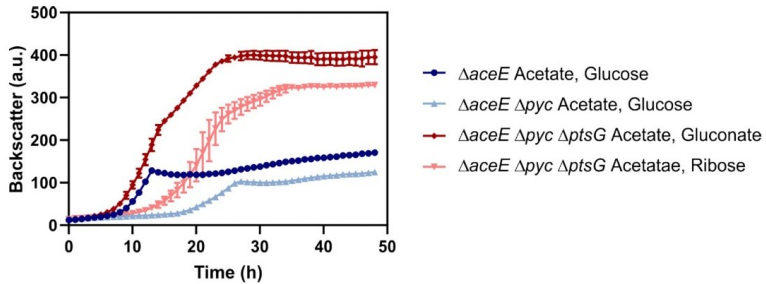
89 triplicates. Deletion of the *ptsG* gene was accomplished using the pK19*mobsacB*-system90 for both strains $\Delta aceE$ and $\Delta aceE \Delta pyc$. These $\Delta ptsG$ strains (shades of red) were

91 cultivated in a microtiter cultivation system in CGXII containing 154 mM acetate and 222

92 mM fructose. Strains $\Delta aceE$ and $\Delta aceE \Delta pyc$ cultivated in 154 mM acetate and 222 mM

93 glucose served as control, and are shown in shades of blue.

94



95

96 **Figure S4: Effect of gluconate and ribose on growth of *ptsG*-deficient *C. glutamicum***97 **$\Delta aceE \Delta pyc$.** Growth curves shown are based on the backscatter measurements in a98 microtiter cultivation system, inoculated at an OD_{600} of 1. Symbols represent the

99 backscatter means and error bars their standard deviations of biological

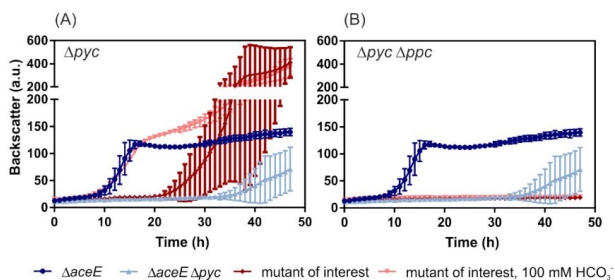
100 triplicates. Deletion of the *ptsG* gene was accomplished using the pK19*mobsacB*-system101 for both strains $\Delta aceE$ and $\Delta aceE \Delta pyc$. These $\Delta ptsG$ strains (shades of red) were

102 cultivated in a microtiter cultivation system in CGXII containing 154 mM acetate and 222

103 mM gluconate (dark red) or 154 mM acetate and 222 mM ribose (light red). Strains $\Delta aceE$ 104 and $\Delta aceE \Delta pyc$ cultivated in 154 mM acetate and 222 mM glucose served as control,

105 and are shown in shades of blue.

106



107

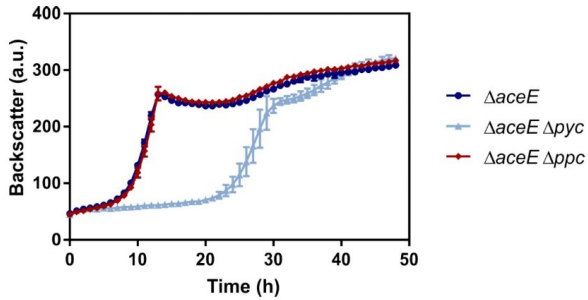
108 **Figure S5: Addition of HCO_3^- restored growth defects of *pyc*-deficient**109 ***C. glutamicum* strains.** The strains (A) Δpyc and (B) $\Delta pyc \Delta ppc$ were inoculated to an110 OD_{600} of 1 in CGXII media containing 154 mM acetate and 222 mM glucose (shown in111 dark red) or additionally 100 mM $KHCO_3^-$ (shown in light red). Further, $\Delta aceE$ and $\Delta aceE$ 112 Δpyc in CGXII media containing 154 mM acetate and 222 mM glucose were cultivated as

113 controls (shown in shades of blue). Growth curves shown are based on the backscatter

114 measurements in a microtiter cultivation system. Symbols represent the backscatter

115 means and error bars their standard deviations of biological triplicates.

116



117

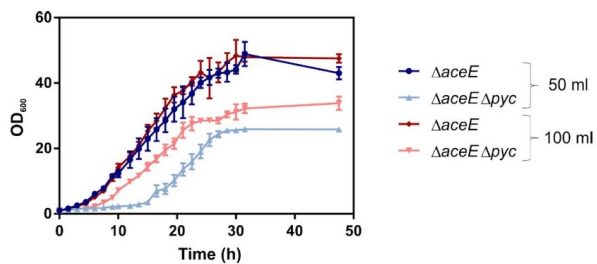
118 **Figure S6: Growth of the PEPCx-deficient strain $\Delta aceE \Delta ppc$.** The strain $\Delta aceE \Delta ppc$ 119 was inoculated to an OD_{600} of 1 in CGXII media containing 154 mM acetate and 222 mM120 glucose (red). Further, $\Delta aceE$ and $\Delta aceE \Delta pyc$ in CGXII media containing 154 mM

121 acetate and 222 mM glucose were cultivated as controls (shown in shades of blue).

122 Growth curves shown are based on the backscatter measurements in a microtiter

123 cultivation system. Symbols represent the backscatter means and error bars the standard

124 deviations of biological triplicates.



125

126 **Figure S7: Effect of culture volume on the growth of *C. glutamicum* $\Delta aceE$ and**127 **$\Delta aceE \Delta pyc$.** Main cultures of $\Delta aceE$ and $\Delta aceE \Delta pyc$ were inoculated to an OD₆₀₀ of 1

128 in CGXII containing 154 mM acetate and 222 mM glucose once in 50 ml in a 500 ml

129 shaking flask (shades of blue) and once in 100 ml in a 1 l shaking flask (shades of red).

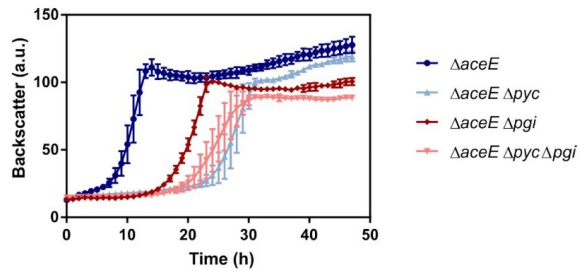
130 Cultivation was performed in an orbital shaker. Growth curves shown are based on OD₆₀₀

131 measurements which were performed every 90 minutes until 33 h, and an additional

132 measurement after 48 h. Symbols represent the OD₆₀₀ means and error bars their

133 standard deviations of biological triplicates.

134

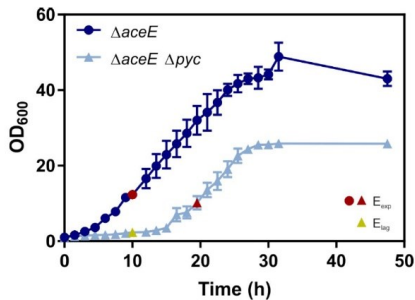


135

136 **Figure S8: Deletion of *pgi* in PDHC-deficient strains to reroute flux through the**
 137 **pentose phosphate pathway.** Deletion of the *pgi* gene was accomplished using the
 138 pK19*mobsacB* system for both strains $\Delta aceE$ and $\Delta aceE \Delta pyc$. Generated strains were
 139 inoculated in a microtiter cultivation system to an OD_{600} of 1 in CGXII containing 154 mM
 140 acetate and 222 mM glucose. Additionally, the parental strains $\Delta aceE$ and $\Delta aceE \Delta pyc$
 141 served as control, and are shown in shades of blue, while $\Delta aceE \Delta pgi$ and $\Delta aceE \Delta pyc$
 142 Δpgi are shown in shades of red. Growth curves shown are based on the backscatter
 143 measurements in a microtiter cultivation system. Symbols represent the backscatter
 144 means and error bars their standard deviations of biological triplicates.

145

146



147

148 **Figure S9: Growth phase dependent sampling of *C. glutamicum* $\Delta aceE$ and $\Delta aceE$** 149 **Δpyc for DNA microarrays and GC-ToF analysis.** The strains $\Delta aceE$ and $\Delta aceE \Delta pyc$ 150 were cultivated. The main culture was inoculated to an OD₆₀₀ of 1 in 50 ml CGXII
151 containing 154 mM acetate and 222 mM glucose. Cultivation was performed in 500 ml152 shaking flasks. Growth curves shown are based on OD₆₀₀ measurements which were

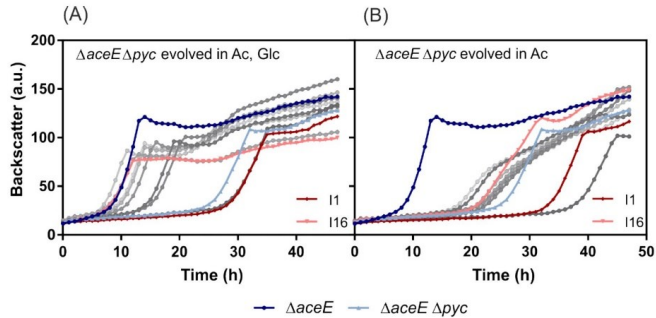
153 performed every 90 minutes until 33 h, and an additional measurement after 48 h. Colored

154 data points indicate the time where samples were taken: red = sampling time point during

155 exponential phase (E_{exp}) used for DNA microarrays and GC-ToF analysis, yellow =156 sampling time point during lag phase of $\Delta aceE \Delta pyc$ (E_{lag}) additionally used for GC-ToF

157 experiments.

158



159

160 **Figure S10: Adaptive laboratory evolution of *C. glutamicum* $\Delta aceE \Delta pyc$ on acetate.**

161 Growth analysis of single inoculation steps obtained from the adaptive laboratory evolution
 162 (ALE) approach which was performed with the strain $\Delta aceE \Delta pyc$ (A) with selection
 163 pressure in CGXII containing 154 mM acetate and 222 mM glucose and (B) without
 164 selection pressure in CGXII containing solely 154 mM acetate. Growth curves are shown
 165 based on the backscatter measurements in a microtiter cultivation system. For growth
 166 analysis on population level, glycerol stocks which were prepared after batch number 1,
 167 4, 6, 7, 8, 9, 10, 11, 13 and 16 during ALE were directly used for the inoculation of a first
 168 pre-culture in BHI supplemented with 51 mM acetate. The second pre-culture in CGXII
 169 containing 154 mM acetate was then used for inoculation of the main culture in CGXII
 170 containing 154 mM acetate and 222 mM glucose (start OD_{600} of 1). The first and last
 171 inoculations are shown in shades of red. The parental strains $\Delta aceE$ and $\Delta aceE \Delta pyc$
 172 (shades of blue) served as controls. Symbols represent the backscatter means. I =
 173 Inoculation.

174

175 **References**

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184

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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