



OPEN ACCESS

Edited by:

Daniela De Biase,
Sapienza University of Rome, Italy

Reviewed by:

Nicolai Kallscheuer,
Radboud University Nijmegen,
Netherlands
Fernando Pérez-García,
Norwegian University of Science
and Technology, Norway
Koichi Toyoda,
Research Institute of Innovative
Technology for the Earth, Japan

***Correspondence:**

Meike Baumgart
m.baumgart@fz-juelich.de
Michael Bott
m.bott@fz-juelich.de

† Present address:

Lingfeng Zhu,
Center for Synthetic Biochemistry,
Institute of Synthetic Biology,
Shenzhen Institutes of Advanced
Technologies, Chinese Academy
of Sciences, Shenzhen, China

Specialty section:

This article was submitted to
Microbial Physiology and Metabolism,
a section of the journal
Frontiers in Microbiology

Received: 19 March 2020

Accepted: 17 September 2020

Published: 27 October 2020

Citation:

Zhu L, Mack C, Wirtz A, Kranz A,
Polen T, Baumgart M and Bott M
(2020) Regulation of γ -Aminobutyrate
(GABA) Utilization in *Corynebacterium
glutamicum* by the PucR-Type
Transcriptional Regulator GabR
and by Alternative Nitrogen
and Carbon Sources.
Front. Microbiol. 11:544045.
doi: 10.3389/fmicb.2020.544045

Regulation of γ -Aminobutyrate (GABA) Utilization in *Corynebacterium glutamicum* by the PucR-Type Transcriptional Regulator GabR and by Alternative Nitrogen and Carbon Sources

Lingfeng Zhu[†], Christina Mack, Astrid Wirtz, Angela Kranz, Tino Polen, Meike Baumgart* and Michael Bott*

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

γ -Aminobutyric acid (GABA) is a non-proteinogenic amino acid mainly formed by decarboxylation of L-glutamate and is widespread in nature from microorganisms to plants and animals. In this study, we analyzed the regulation of GABA utilization by the Gram-positive soil bacterium *Corynebacterium glutamicum*, which serves as model organism of the phylum *Actinobacteria*. We show that GABA usage is subject to both specific and global regulatory mechanisms. Transcriptomics revealed that the *gabTDP* genes encoding GABA transaminase, succinate semialdehyde dehydrogenase, and GABA permease, respectively, were highly induced in GABA-grown cells compared to glucose-grown cells. Expression of the *gabTDP* genes was dependent on GABA and the PucR-type transcriptional regulator GabR, which is encoded divergently to *gabT*. A Δ *gabR* mutant failed to grow with GABA, but not with glucose. Growth of the mutant on GABA was restored by plasmid-based expression of *gabR* or of *gabTDP*, indicating that no further genes are specifically required for GABA utilization. Purified GabR (calculated mass 55.75 kDa) formed an octamer with an apparent mass of 420 kDa and bound to two inverted repeats in the *gabR-gabT* intergenic region. Glucose, gluconate, and *myo*-inositol caused reduced expression of *gabTDP*, presumably via the cAMP-dependent global regulator GlxR, for which a binding site is present downstream of the *gabT* transcriptional start site. *C. glutamicum* was able to grow with GABA as sole carbon and nitrogen source. Ammonium and, to a lesser extent, urea inhibited growth on GABA, whereas L-glutamine stimulated it. Possible mechanisms for these effects are discussed.

Keywords: *Corynebacterium glutamicum*, *Actinobacteria*, γ -aminobutyrate, PucR-type regulator GabR, cAMP-dependent regulator GlxR, nitrogen metabolism, carbon metabolism

INTRODUCTION

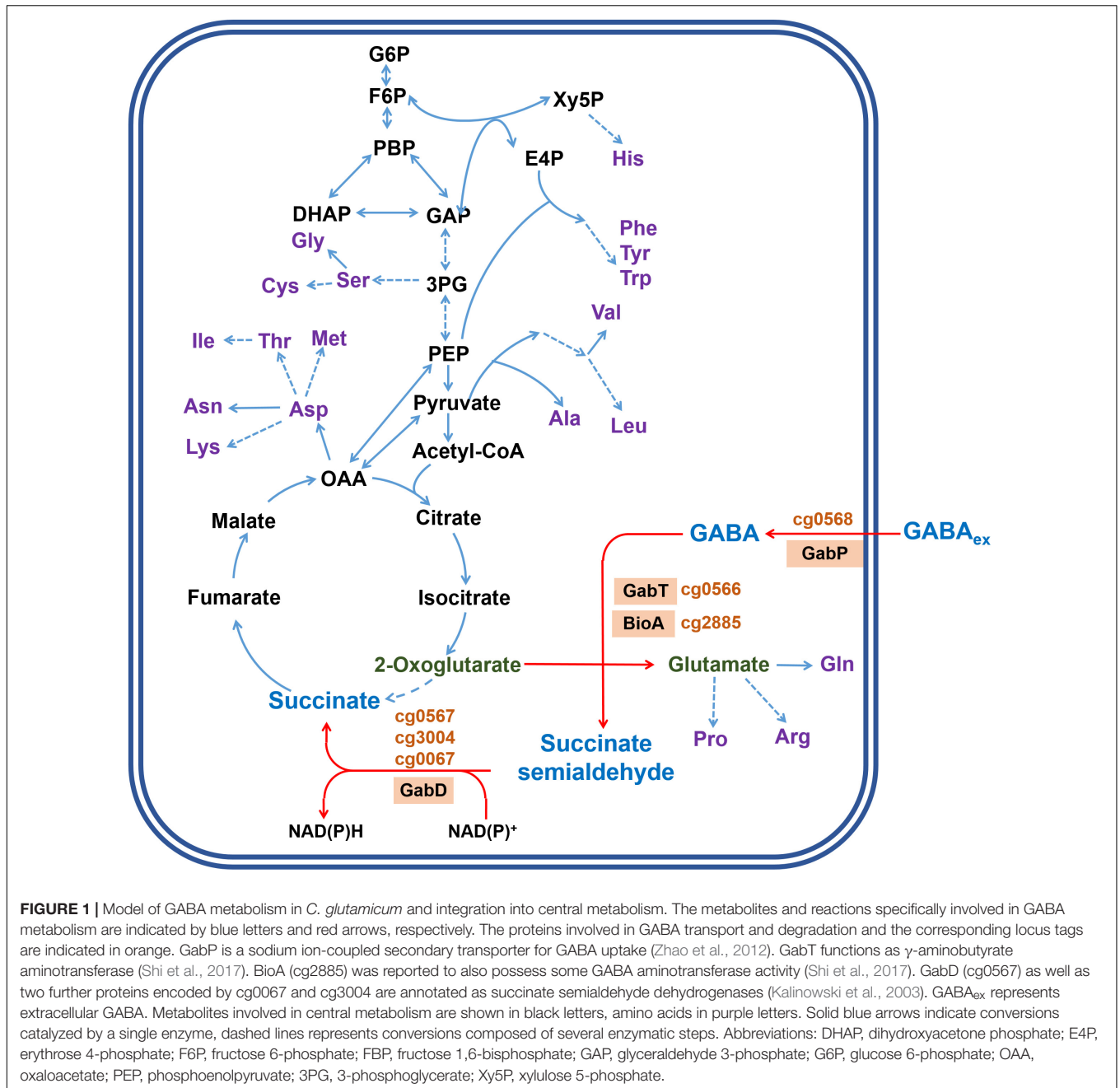
γ -Aminobutyric acid (GABA) is a non-proteinogenic amino acid, which is widespread in nature from microorganisms to plants and animals. GABA is formed by many gram-negative and gram-positive bacteria as part of the acid stress response (Cotter and Hill, 2003; Foster, 2004; Krulwich et al., 2011; De Biase and Pennacchietti, 2012). The irreversible decarboxylation of L-glutamate by glutamate decarboxylase results in the formation of GABA and carbon dioxide and consumes a proton within the cytoplasm, thereby acting against acid stress. An antiporter takes up L-glutamate and exports GABA. This acid stress response can also start from L-glutamine, which is first converted to L-glutamate and ammonia by a glutaminase (Pennacchietti et al., 2018). The human gut microbiome contains several GABA-producing or GABA-consuming species, and there might be a connection between GABA production in the gut and depressive disorders (Strandwitz et al., 2019). In plants, GABA plays a role in stress responses and at the interface of carbon and nitrogen metabolism (Michaeli and Fromm, 2015). In the brain of mammals, GABA is an important neurotransmitter (Abdou et al., 2006; Ben-Ari et al., 2007). GABA has also gained much attention as a building block for the synthesis of 2-pyrrolidone and the biodegradable polyamide nylon 4 (Kawasaki et al., 2005). Therefore, microbial synthesis of GABA has frequently been studied (Krulwich et al., 2011; Dhakal et al., 2012; Jorge et al., 2016; Xu et al., 2017).

Due to its widespread occurrence in nature, GABA can be used by many bacteria as carbon and nitrogen source. After uptake by specific transporters (usually named GabP), a transaminase (GabT) converts GABA and 2-oxoglutarate in a pyridoxal 5-phosphate (PLP)-dependent reaction to succinate semialdehyde and glutamate. Succinate semialdehyde is then oxidized by an NAD(P)⁺-dependent succinate semialdehyde dehydrogenase (GabD) to succinate, an intermediate of the central metabolism (Scott and Jakoby, 1958; Dover and Halpern, 1972; Feehily and Karatzas, 2013). Despite the fact that many bacteria possess the characteristic genes involved in GABA metabolism, their regulation has so far only been studied in a few species such as *Bacillus subtilis*, *Bacillus thuringiensis*, or *Escherichia coli*. The best-studied regulator is probably GabR of *B. subtilis* (designated here GabR_{Bs}), which belongs to the MocR/GabR subfamily of the GntR family of transcriptional regulators (Edayathumangalam et al., 2013; Wu et al., 2017). GabR_{Bs} consists of an N-terminal helix-turn-helix (HTH) domain and a C-terminal aminotransferase domain and activates its target genes *gabTD* in the presence of GABA and PLP (Belitsky, 2004). Surprisingly, in the close relative *B. thuringiensis*, the regulation of GABA metabolism is different. Here, the regulator GabR_{Bt} belongs to the family of bacterial enhancer binding proteins that function as activators of σ^{54} -dependent promoters. GabR_{Bt} is composed of a Per-ARNT-Sim (PAS) domain, a σ^{54} interaction domain, and a C-terminal HTH domain and activates its target gene *gabT* in the presence of GABA and succinate semialdehyde (Zhu et al., 2010; Peng et al., 2014). In *E. coli*, the genes *gabTDP*

are presumably organized in an operon *gabTDP* together with *gabC*, and activated under nitrogen limitation by the Nac regulator (Schneider et al., 2002). The product of *gabC* is a transcriptional regulator belonging to the FadR subfamily of the GntR family of transcriptional regulators and seems to repress this operon. However, the exact physiological function of GabC_{Ec} is unclear up to now (Schneider et al., 2002). GABA is also an intermediate of putrescine degradation to succinate in *E. coli*, which involves P_{uuE} as isoenzyme of GabT and P_{uuC} as isoenzyme of GabD (Kurihara et al., 2010; Schneider and Reitzer, 2012). In summary, the regulation of genes involved in GABA metabolism appears to be rather diverse in bacteria.

Corynebacterium glutamicum is a gram-positive soil bacterium widely used for the industrial production of amino acids, in particular the flavor enhancer L-glutamate and the feed additive L-lysine (Eggeling and Bott, 2005). The wild type is unable to synthesize GABA, but recombinant strains for GABA production were constructed (Shi and Li, 2011; Takahashi et al., 2012; Shi et al., 2013; Jorge et al., 2016). In the course of these studies, it became clear that *C. glutamicum* is able to degrade GABA and a number of proteins involved in this process were studied (Figure 1). GabP (Cg0568) is a secondary Na⁺-dependent transporter for GABA uptake with a K_m value of $34.2 \pm 1.1 \mu\text{M}$ and a V_{max} of $67.3 \pm 1.0 \text{ nmol min}^{-1} (\text{mg cell dry weight})^{-1}$ at pH 7.5 and essential for GABA utilization (Zhao et al., 2012). GabT (Cg0566) is a GABA transaminase and the purified enzyme has a specific activity of 1.34 U/mg protein and K_m values of 10.65 mM for GABA and 9.21 mM for 2-oxoglutarate (Shi et al., 2017). GabT was shown to be a tetramer and the crystal structure in complex with PLP-GABA allowed the identification of the key residues that contribute to the formation of the active site (Hong and Kim, 2019). Since a *gabT* deletion strain was still able to degrade GABA, the presence of another transaminase catalyzing this reaction was suggested (Ni et al., 2015). The protein encoded by *bioA* (cg2885) was recently reported to possess GABA transaminase activity, but the specific activity of the purified BioA enzyme was only 2% of the GabT activity (Shi et al., 2017). With respect to succinate semialdehyde dehydrogenase, three genes were annotated to encode this enzyme, namely *gabD* (cg0566), cg0067, and cg3004 (Figure 1) (Kalinowski et al., 2003). None of the corresponding proteins has been biochemically characterized to our knowledge.

In this study, our aim was to investigate the regulation of GABA utilization in *C. glutamicum*. We show that the *gabTDP* operon is regulated by a specific transcriptional regulator, GabR, which does not belong to the regulator families described above for GabR proteins of other bacteria, but to the PucR family, further confirming the heterogeneity in transcriptional regulation of the genes involved in GABA transport and metabolism. GabR presumably requires GABA for transcriptional activation, but not for DNA-binding. The *gabTDP* operon is probably also regulated by the cAMP-dependent global regulator GlxR, which is proposed to repress expression when alternative carbon sources are present. Besides alternative carbon sources, also alternative nitrogen sources have a profound



influence on GABA utilization, with ammonium and urea acting as inhibitors and glutamine serving as stimulator.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Growth Conditions

The bacterial strains and plasmids used in this study are listed in **Table 1**. The *C. glutamicum* ATCC 13032 type strain served as wild type (WT). *C. glutamicum* was routinely cultivated at 30°C. For precultivation of *C. glutamicum*, brain heart

infusion (BHI) medium (Becton Dickinson GmbH, Heidelberg, Germany) with 90 g L⁻¹ sorbitol was used. The cells of these precultures were harvested by centrifugation (5,000 g, 4°C, 10 min), and washed twice with phosphate buffer (100 mM KH₂PO₄/Na₂HPO₄ pH 7.0). Growth experiments were routinely performed in a BioLector microcultivation system (m2p-labs, Baesweiler, Germany) using 48-well FlowerPlates (m2p-labs) containing 750 μ L CGXII minimal medium (Keilhauer et al., 1993) supplemented with 30 mg L⁻¹ protocatechuic acid as iron chelator. In the BioLector, growth of the cells is measured as scattered light at 620 nm (Kensy et al., 2009a,b). Please note that the absolute backscatter values of the graphs in this manuscript

TABLE 1 | Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristics	Source or references
<i>E. coli</i>		
DH5 α	F ⁻ Φ 80 <i>dlac</i> Δ (<i>lacZ</i>)M15 Δ (<i>lacZYA-argF</i>) U169 <i>endA1 recA1 hsdR17</i> (<i>r</i> _K ⁻ , <i>m</i> _K ⁺) <i>deoR thi-1 phoA supE44</i> λ ⁻ <i>gyrA96 relA1</i> ; strain used for cloning procedures	(Hanahan, 1983)
BL21(DE3)	F- <i>ompT hsdS_B</i> (<i>r</i> _B ⁻ , <i>m</i> _B ⁻) <i>gal dcm</i> (DE3); host for protein production	(Studier and Moffatt, 1986)
<i>C. glutamicum</i>		
ATCC 13032 (Cg WT)	Biotin-auxotrophic wild type	(Kinoshita et al., 1957)
Δ <i>gabR</i>	WT derivative with an in-frame deletion of <i>gabR</i> (cg0565)	This work
Δ <i>gdh</i> (LN Δ GDH)	ATCC 13032 with deletion of <i>gdh</i> (cg2280)	(Müller et al., 2006)
Δ <i>glnA</i> (LN Δ GS)	ATCC 13032 with deletion of <i>glnA</i> (cg2429)	(Müller et al., 2006)
Δ <i>gltBD</i> (LN Δ <i>gltBD</i>)	ATCC 13032 with deletion of <i>gltB</i> (cg0229) and <i>gltD</i> (cg0230)	(Rehm et al., 2010)
Δ <i>glnA</i> Δ <i>glnA2</i> Δ <i>gdh</i> (DA-2)	ATCC 13032 with deletion of <i>gdh</i> (cg2280), <i>glnA</i> (cg2429) and <i>glnA2</i> (cg2477)	(Walter et al., 2008)
Plasmids		
pK19 <i>mobsacB</i>	Kan ^R ; plasmid for allelic exchange in <i>C. glutamicum</i> ; (pK18 <i>oriV_{E.c.}</i> , <i>sacB</i> , <i>lacZ</i> α)	(Schäfer et al., 1994)
pK19 <i>mobsacB</i> - Δ <i>gabR</i>	Kan ^R ; pK19 <i>mobsacB</i> derivative containing a PCR product covering the up- and downstream regions of <i>gabR</i> (cg0565)	This work
pAN6	Kan ^R ; <i>C. glutamicum</i> / <i>E. coli</i> shuttle vector for regulated gene expression using the P _{tac} promoter, derivative of pEKEx2.	(Frunzke et al., 2008)
pAN6- <i>gabR</i>	Kan ^R ; pAN6 derivative for expression of <i>gabR</i> (cg0565) under control of P _{tac}	This work
pAN6- <i>gabTDP</i>	Kan ^R ; pAN6 derivative for expression of <i>gabTDP</i> cluster	This work
pET-TEV	Kan ^R ; pET28b derivative for overexpression of genes in <i>E. coli</i> , adding an N-terminal decahistidine tag and a TEV protease cleavage site to the target protein (pBR322 <i>oriV_{E.c.}</i> , P _{T7} , <i>lacI</i>)	(Bussmann et al., 2010)
pET-TEV- <i>gabR</i>	Kan ^R ; pET-TEV derivative for overproduction of GabR (cg0565)	This work
pJC1-P _{tac} - <i>eyfp</i>	Kan ^R ; pJC1 derivative containing the <i>eyfp</i> gene under the control of P _{tac}	(Kortmann et al., 2015)
pJC1-venus-term	Kan ^R ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, pJC1 derivative carrying the venus coding sequence and additional terminators	(Baumgart et al., 2013)
pJC1-P _{gabT} -eYFP	Kan ^R ; pJC1-venus-term derivative carrying the promoter of <i>gabT</i> (cg0566) fused to <i>eyfp</i>	This work
pJM0462	Cm ^R ; pASK-IBA-3C derivative with the coding sequence of <i>gabT</i> (cg0566, NCgl0462)	(Marienhagen et al., 2005)

differ because of the use of different instruments. Due to this, reference cultures were always included to allow comparison between different experiments. For determination of GABA and glucose consumption, cells were cultured in 500-mL baffled shake flasks with 50 mL medium that were incubated at 30°C and 130 rpm. *E. coli* was grown at 37°C in lysogeny broth (Bertani, 1951). When required for plasmid maintenance, media were supplemented with kanamycin (50 μ g mL⁻¹ for *E. coli*, 25 μ g mL⁻¹ for *C. glutamicum*) or chloramphenicol (20 μ g mL⁻¹).

Recombinant DNA Work and Construction of Deletion Mutants

Routine methods such as PCR, DNA restriction and ligation were performed using standard protocols (Sambrook et al., 1989; van der Rest et al., 1999). All oligonucleotides used in this study are listed in **Supplementary Table 1** and were synthesized by Eurofins Genomics (Ebersberg, Germany). The enzymes for recombinant DNA work were obtained from New England Biolabs (Frankfurt, Germany) and Thermo Fisher Scientific (Vilnius, Lithuania). The correctness of the insert sequences of all recombinant plasmids was verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany).

The *C. glutamicum* Δ *gabR* strain was constructed by allelic exchange using the plasmid pK19*mobsacB* (Schäfer et al., 1994) as described before (Niebisch and Bott, 2001). For construction of pK19*mobsacB*- Δ *gabR*, the oligonucleotide pairs cg0565frontF/R and cg0565backF/R were used to amplify DNA regions of approximately 500 bp upstream and downstream of *gabR* using *C. glutamicum* WT genomic DNA as template. The resulting PCR products were fused by Gibson assembly (Gibson et al., 2009) with pK19*mobsacB* which had been digested with *Bam*HI and *Pst*I. After successful construction, pK19*mobsacB*- Δ *gabR* was used to transform competent *C. glutamicum* WT. The transformed cells were first selected for kanamycin resistance and subsequently for tolerance toward sucrose. Successful deletion of *gabR* was confirmed by colony PCR with the oligonucleotides cg0565checkF and cg0565checkR, which anneal outside of the deleted regions. Out of six analyzed clones, three showed the desired *gabR* deletion, while three clones represented the WT situation.

For construction of plasmid pAN6-*gabR*, the *gabR* coding region was amplified by PCR using the oligonucleotides 0565F and 0565R and chromosomal DNA of *C. glutamicum* WT as template. After digestion with *Nde*I and *Nhe*I, the PCR product was cloned into the expression plasmid pAN6 cut with

the same restriction enzymes using the Rapid DNA Ligation Kit (Roche Diagnostics, Mannheim, Germany). The plasmid pET-TEV-*gabR* was constructed similar to pAN6-*gabR*. The *gabR* gene was amplified with the oligonucleotides 0565F and petfhis0565R, the PCR product was digested with *NdeI* and *HindIII* and ligated with pET-TEV cut with the same restriction enzymes. For construction of plasmid pAN6-*gabTDP*, the genes *gabTDP*, were amplified by PCR using the oligonucleotide pair *gabtdpF/gabtdpR* and chromosomal DNA of *C. glutamicum* WT as template. The resulting PCR product was fused with pAN6 cut with *NdeI* and *NheI* by Gibson assembly.

For construction of plasmid pJC1-*P_{gabT}*-eYFP, a 500-bp fragment covering the *gabTDP* promoter region immediately upstream of the *gabT* start codon was amplified using the oligonucleotide pair *PgabTF/PgabTR* with genomic DNA of *C. glutamicum* WT as template. The eYFP-encoding gene was amplified with the oligonucleotide pair eYFPF/eYFP-termR and plasmid DNA of pJC1-*P_{tac}*-*eyfp* as template. The two PCR fragments were fused by Gibson assembly with pJC1-*venus-term*, which had been digested with *BamHI* and *SpeI*.

DNA Microarray Analysis

Comparative transcriptome analysis using DNA microarrays was performed as described previously (Vogt et al., 2014), except that $(\text{NH}_4)_2\text{SO}_4$ was omitted from the medium to exclude ammonium and 20 mM K_2SO_4 was added to avoid a possible sulfate limitation. The medium still contains 83 mM urea as nitrogen source. *C. glutamicum* WT was cultivated in this modified medium with either 62.5 mM GABA or with 41.7 mM glucose as carbon source, corresponding to 250 mM C. When an optical density at 600 nm (OD_{600}) of 5 was reached [measured with an Ultrospec 2100pro spectrophotometer (Biochrom, Berlin, Germany)], the cells were harvested by centrifugation (4000g, 10 min, 4°C). The cell pellet was subsequently frozen in liquid nitrogen and stored at -80°C . Total RNA isolation of *C. glutamicum* cells was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany), and the RNA was kept at -80°C until further use. Fluorescently labeled cDNA copies of total RNA of *C. glutamicum* were prepared using SuperScript III reverse transcriptase (Life Technologies, Darmstadt, Germany). To remove unincorporated fluorophores, the probes were purified using Amicon Centrifugal Filters (Merck Millipore, Darmstadt, Germany). The cDNA probes labeled with Cy3 and Cy5 were hybridized using Agilent's Gene Expression Hybridization Kit (Waldbronn, Germany), hybridization oven and hybridization chamber. After 16 h of hybridization at 65°C , the microarrays were washed using Agilent's Wash Buffer Kit according to the manufacturer's instructions. Signal acquisition was performed with a GenePix 4000B laser scanner and GenePix Pro 7.0 software (Molecular Devices, Sunnyvale, CA, United States). For background correction of spot intensities, ratio calculation and ratio normalization, data were processed using the BioConductor R-packages *limma* and *marray*¹. The full microarray data sets of this study have been deposited in the NCBI Gene

¹<http://www.bioconductor.org>

Expression Omnibus and can be found under the GEO accession number GSE138829.

Determination of the Transcriptional Start Sites (TSSs)

The TSS of *gabT* was initially determined using the 5'/3' RACE Kit (Roche Diagnostics, Mannheim, Germany). In brief, RNA was extracted from *C. glutamicum* WT cells cultivated with GABA as sole carbon and nitrogen source using an RNeasy Mini Kit (Qiagen). Then transcription of specific mRNA sequences into first-strand cDNA was performed using the extracted RNA and oligonucleotide GABTTS1R (Supplementary Table 1). A homopolymeric A-tail was added to the 3'-end of the first-strand cDNA using recombinant terminal transferase and dATP. Finally, the amplification of dA-tailed cDNA was performed using first and second round PCR using the primers GABTTS2R and GABTTS3R, respectively (Supplementary Table 1). The PCR products were sequenced to determine the TSS. The TSS determination was performed independently for three biological replicates.

In an alternative approach, the TSSs present within the *gabR-gabTDP* region were analyzed by RNAseq using RNA of WT cells cultivated with GABA as sole carbon and energy source. Three independent cultures (5 ml BHI) were inoculated with a single colony from a fresh agar plate and cultivated at 30°C and 170 rpm during the day. The cells were washed two times with 0.9% (w/v) NaCl and used to inoculate the second precultures of 20 ml CGXII medium with 2% (w/v) glucose in a 1:100 dilution. These cultures were cultivated at 30°C and 130 rpm overnight. For the main cultures, 50 ml CGXII medium without ammonium and urea but with 62.5 mM GABA as carbon and nitrogen source were inoculated with the precultures to an OD_{600} of 1. The main cultures were incubated at 30°C and 130 rpm and harvested after 6 h at an OD_{600} of 2.5–2.7. RNA isolation was performed with the RNeasy Mini Kit (Qiagen, Hilden, Germany). For the sequencing, RNA of all three cultures was mixed in equal amounts. Further RNA processing and sequencing was performed by Vertis Biotechnologie AG (Freising-Weihenstephan, Germany). Processing and mapping of the 10,117,524 single-end sequenced reads was performed with the CLC Genomics Workbench (Qiagen Aarhus A/S). Reads were trimmed by removing adapter sequences using the Trim Sequences tool and filtered for Phred quality scores < 30 (Ewing and Green, 1998). Trimmed reads (10,052,707) were mapped to the *C. glutamicum* reference sequence (BX927147). Automatic detection of TSSs was done with ReadXplorer (Hilker et al., 2014) using the following parameters: (i) Only single perfect mappings without errors were considered. (ii) Minimum percentage of coverage increase was set to 100% and minimum number of reads starts to 20. Every possible TSS for cg0565-cg0568 was manually checked including TSS with less than 20 reads.

Protein Production and Purification

For GabR production and purification, *E. coli* BL21(DE3) harboring pET-TEV-*gabR* was first cultivated in 5 mL LB medium

with 50 $\mu\text{g mL}^{-1}$ kanamycin in 20 mL glass tubes at 37°C with shaking overnight. Subsequently, 2 mL preculture was used to inoculate 200 mL LB medium with kanamycin in a 500 mL shake flask, which was cultivated at 37°C and 120 rpm. When the culture reached an OD_{600} of 0.6, 0.2 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) was added to induce *gabR* expression. After induction, the cells were further cultivated at 16°C for 8 h and harvested by centrifugation (4,000g, 10 min, 4°C). The harvested cells were suspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, 10 mM imidazole, pH 7.8) and disrupted by sonication for 20 minutes while cooling on ice with a UP 200 S ultrasonic device (Hielscher, Germany) using a power amplitude of 55% and a pulse cycle of 0.3. The resulting cell extract was centrifuged at 12,000g for 20 min to remove cell debris and the supernatant was subjected to Ni^{2+} affinity chromatography using Ni-NTA Superflow (Qiagen, Hilden, Germany). The column was equilibrated with binding buffer. After adding the cell-free extract, the column was washed first with three column volumes of binding buffer and then with three column volumes of wash buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, 80 mM imidazole, pH 7.8). *GabR* protein with an N-terminal decahistidine tag was eluted with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM DTT, and 300 mM imidazole, pH 7.8). Subsequently, the His-tag was cleaved off with tobacco etch virus (TEV) protease (Kapust and Waugh, 1999) by incubation of 12 mg His-tagged *GabR* with 0.24 mg His-tagged TEV protease overnight at 4°C in TEV buffer (25 mM Tris-HCl, pH 8.0, 0.25 mM EDTA, 1 mM DTT). Subsequently, *GabR* was further purified by gel filtration with a SuperdexTM 200 increase 10/300 GL column (GE Healthcare, Freiburg, Germany) connected to an ÄktaTM Pure25 system (GE Healthcare) using 100 mM phosphate buffer pH 7.0 with 1 mM DTT and a flow rate of 0.75 mL min^{-1} . The elution volumes of the standard proteins were 9.41 mL for thyroglobulin (669 kDa), 10.87 mL for apoferritin (443 kDa), 12.06 mL for amylase (200 kDa), 12.85 mL for alcohol dehydrogenase (150 kDa), 14.27 mL for BSA (66 kDa), 16.26 mL for carbonic anhydrase (29 kDa), and 17.34 mL for cytochrome *c* (12.4 kDa).

Overproduction and purification of the transaminase *GabT* were performed according to a previous publication (Marienhagen et al., 2005). *E. coli* BL21(DE3) carrying plasmid pJM0462 was precultivated in 5 mL LB medium with 20 $\mu\text{g mL}^{-1}$ chloramphenicol in 20 mL tubes at 37°C and 120 rpm overnight. Subsequently, 2 mL preculture was used to inoculate 200 mL LB medium with 20 $\mu\text{g mL}^{-1}$ chloramphenicol in a 500 mL shake flask, which was incubated at 37°C and 120 rpm. Induction of target gene expression was triggered by addition of 20 μL anhydrotetracycline (2 mg (mL ethanol)⁻¹) when the cultures had reached an OD_{600} of 0.4 to 0.6 and the cultures were further incubated for 8 h at 16°C. Crude extracts were obtained by sonication while cooling on ice. After removal of the cellular debris by centrifugation (15 min, 16,000g, 4°C), protein purification was performed by affinity chromatography on ice using Strep-TactinXT Sepharose (IBA, Göttingen, Germany). Buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA) was used for equilibration and washing, and buffer BXT (buffer W containing 50 mM biotin) was used for elution.

The purified proteins were analyzed by SDS-PAGE. Protein concentrations were determined using the BC Protein Assay Kit (Interchim Uptima, Montlucon Cedex, France) with bovine serum albumin (BSA) as standard.

Electrophoretic Mobility Shift Assays (EMSA)

Electrophoretic mobility shift assays were performed as described previously (Wennerhold and Bott, 2006). The DNA fragments (100 ng, 30 – 500 bp) were incubated with purified *GabR* protein (0 – 2.6 μM monomer) in binding buffer (10 mM Tris-HCl pH 7.5, 50 mM NaCl, 5% (v/v) glycerol, 0.005% (v/v) Triton X-100) for about 20 min at room temperature. Electrophoresis was performed using 6% or 8% (w/v) native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl pH 8.2, 89 mM boric acid) as running buffer. A pre-run without samples was carried out, so that buffer differences between the gel and the running buffer were adjusted (180 V, 1.5 h). After the pre-run, the samples were mixed with sample buffer (0.01% (w/v) xylene cyanol, 0.01% (w/v) bromophenol blue, 20% (v/v) glycerol, in 1 \times TB buffer) and loaded onto the gel. Electrophoresis was performed with the same conditions as the pre-run for 40 minutes and the gels were subsequently stained with SYBR green (Sigma-Aldrich, Darmstadt, Germany).

HPLC Analysis of GABA and Glucose

For the determination of GABA and glucose in culture supernatants, 1-mL culture aliquots were taken at selected time points, centrifuged for 5 min at 13,000g, and the supernatant was stored at -20°C. Thawed samples were filtered (0.2 μm syringe filter, WhatmanTM, GE Healthcare, Freiburg, Germany) prior to HPLC analysis. The GABA concentration was quantified as *ortho*-phthalaldehyde derivative by reverse phase chromatography using an Agilent 1290 Infinity I LC system (Agilent, Santa Clara, CA, United States) equipped with a 5-micron 4.6 \times 12.5 mm protective column and a Zorbax Eclipse AAA 3.5 micron 4.6 \times 75 mm separating column. A gradient of sodium borate buffer (A: 10 mM Na_2HPO_4 , 10 mM $\text{Na}_2\text{B}_4\text{O}_7$, pH 8.2) and methanol (B) was used as eluent. Starting initially with 100% A, B was increased within 9.8 min from 0% to 57%. At 10 min, B was set to 100% and hold for 2.5 min. Re-equilibration to 100% A was started after 12.5 min and terminated after 14 min. The *ortho*-phthalaldehyde derivative was detected by a fluorescence detector using an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The flow rate was 2 mL/min and the temperature was kept constant at 40°C.

Glucose was quantified essentially as described (Richhardt et al., 2012) using an Agilent LC-1100 system (Agilent, Santa Clara, CA, United States) equipped with a Carbo-Ca Guard Cartridge (Phenomenex, Aschaffenburg, Germany) and a RezexTM RCM-Monosaccharide 300 \times 7.8 mm column (Phenomenex, Aschaffenburg, Germany). Separation was performed at 80°C with water as eluent at a flow rate of 0.6 mL/min. Glucose was detected with a refraction index detector (35°C). Glucose was calibrated in a range of 0.1 to 8 g/L, with a retention time of 11.2 min.

Assay for GABA Transaminase Activity

The activity of GABA transaminase was measured as described (Marienhagen et al., 2005). Briefly, the assay buffer contained 200 mM Tris-HCl pH 8, 0.25 mM pyridoxal 5'-phosphate, 20 mM GABA, and 20 mM 2-oxoglutaric acid. To test if ammonium affects GABA transaminase activity, 30 mM $(\text{NH}_4)_2\text{SO}_4$ was added. The reaction mixture (initial volume 10 ml) was preincubated for 2 min at 30°C and started by the addition of purified protein at a final concentration of 3 $\mu\text{g mL}^{-1}$. Six 500 μl samples were collected over a period of 26 min and the reaction was immediately terminated by mixing each sample with 300 μL of 5% (v/v) perchloric acid and 38% (v/v) ethanol. After this, the sample was neutralized by addition of 200 μL of 20 mM Tris-HCl (pH 8) with 23 mM K_2CO_3 . The precipitated salts were removed by centrifugation (10 min, 16,000g). Subsequently, the glutamate concentration in the sample was measured by HPLC (Agilent 1260 series) equipped with an Agilent Eclipse XDB-C18 column using a variable wavelength detector and a fluorescence detector. Elution was performed with a mixture of 43% (v/v) buffer A [10 mM Na_2HPO_4 , 10 mM $\text{Na}_2\text{B}_4\text{O}_7$ (pH 8.2)] and 57% (v/v) methanol at a flow rate of 2 mL/min for 14 minutes. Before chromatographic separation, amino acids were derivatized with *o*-phthalaldehyde (Lindroth and Mopper, 1979). 25–1000 μM sodium glutamate was used as standard.

RESULTS

Transcriptome Comparison of WT Cells Grown With GABA or Glucose

To analyze the influence of GABA on global gene expression, we compared the transcriptomes of cells cultivated in modified CGXII minimal medium lacking $(\text{NH}_4)_2\text{SO}_4$ and containing either GABA or glucose as carbon source. In GABA-grown cells, 163 genes showed a ≥ 2 -fold increased mRNA level and 71 genes a ≥ 2 -fold lowered mRNA level compared to glucose-grown cells (Supplementary Table 2). The genes showing the by far strongest upregulation in GABA-grown cells were *gabTDP* (87-, 78-, and 65-fold, respectively) (Figure 2 and Supplementary Table 2). Interestingly, also expression of the *gabR* gene located upstream and divergent to *gabTDP* was four-fold increased in GABA-grown cells. The *gabR* gene encodes a transcriptional regulator (see below). Among the other genes upregulated in GABA-grown cells, only cg0083 encoding a putative nicotinamide mononucleotide transporter showed a more than 10-fold increased expression. In general, the upregulated genes belonged to a large variety of functional categories and the same holds true for the genes downregulated in GABA-cultivated cells. The latter group included at least 30 genes for proteins involved in transport, including those for phosphate uptake (*pitA*, *pstSCAB*), genes involved in amino acid biosynthesis (*leuCD*, *serA*, *metE*, *argC*, *aroG*, *glnA*), and genes involved in lactate metabolism (cg3226, *lldD*). In summary, this experiment demonstrated that the *gabTDP* genes were strongly induced in the presence of GABA, indicating that they are subject to transcriptional control. All other

differentially expressed genes were significantly less changed under this condition.

Genomic Location and Conservation of the *gabR-gabTDP* Gene Cluster

The *gabR* gene is located upstream and divergent to *gabTDP* and encodes a transcriptional regulator, which might regulate these genes. To check whether this genomic organization is conserved in related species, which is a hint for a regulatory relationship between these genes, we analyzed the presence and organization of the *gabR-gabTDP* gene cluster in other organisms. As shown in Figure 3, similar gene clusters were found in a number of *Corynebacterium* species and, in a different organization, also in some *Rhodococcus* and *Mycobacterium* species. Interestingly, additional copies of *gabR* and *gabT* are present immediately adjacent to the *gabR-gabTDP* gene cluster in *Corynebacterium aurimucosum*. *GabR* and its homologs from *Actinobacteria* belong to the PucR protein family (Pfam PF07905) and have a C-terminal DNA-binding HTH motif. The best characterized representative of this family is PucR of *B. subtilis*, which is involved in the regulation of purine catabolism (Schultz et al., 2001; Beier et al., 2002).

Relevance of *gabR* and the *gabTDP* Operon for Growth With GABA

To investigate whether *GabR* is involved in the regulation of GABA metabolism, we generated the deletion mutant *C. glutamicum* ΔgabR and compared it with the WT. Both strains grew identically with glucose, but the ΔgabR mutant had lost the ability to grow with GABA as sole carbon and nitrogen source (Figure 4A). The growth defect of the ΔgabR strain with GABA was abolished after transformation with the expression plasmid pAN6-*gabR* harboring *gabR* under control of a leaky *tac* promoter (Figure 4B), which confirmed that the loss of *GabR* was responsible for the observed phenotype. Whereas full complementation was achieved by basal *gabR* expression in the absence of IPTG, induction of plasmid-borne *gabR* expression with 50 μM IPTG had a negative effect on growth of the ΔgabR mutant and the WT in comparison to the non-induced cultures (Supplementary Figure 1). This might be a consequence of too strong expression of the *gabTDP* operon causing e.g., membrane-stress by overexpression of the permease-encoding gene *gabP* or metabolic disturbances by an excessive GABA metabolism.

In a further experiment, we tested whether growth of the ΔgabR strain on GABA can be restored by plasmid-based expression of *gabTDP*. *C. glutamicum* WT and the ΔgabR mutant were transformed with pAN6-*gabTDP* and cultivated with GABA as sole carbon and nitrogen source and different IPTG concentrations (Figure 4C). Without IPTG addition, the ΔgabR strain with pAN6-*gabTDP* grew very slowly. Addition of 10 μM IPTG significantly improved the growth of this strain and with 100 μM IPTG it grew comparably to the WT carrying pAN6-*gabTDP*. These results suggest that *gabTDP*

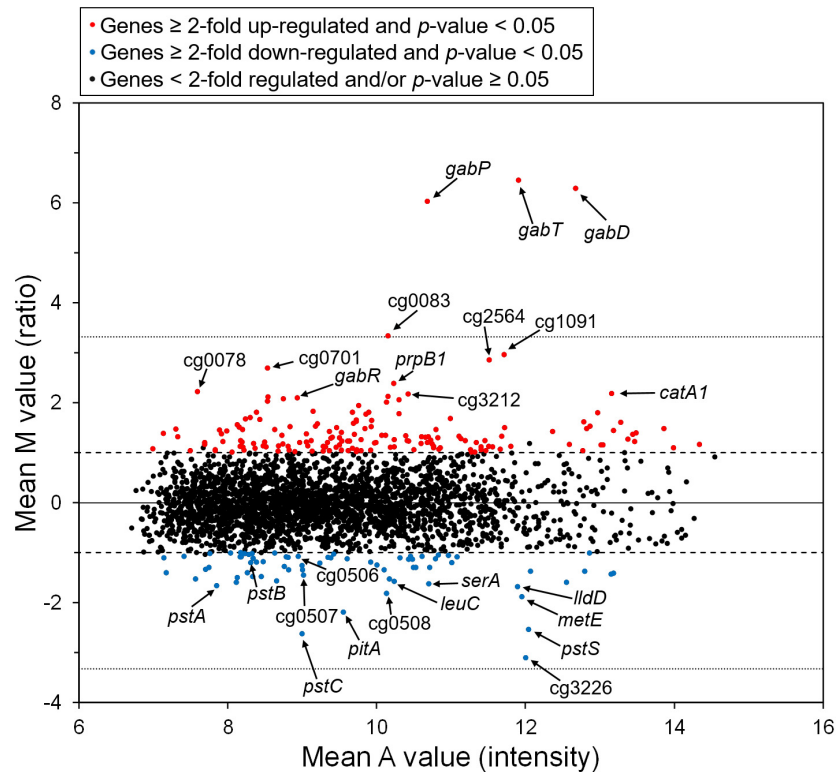


FIGURE 2 | M/A plot showing differential gene expression in *C. glutamicum* WT cells grown with GABA and urea compared to WT cells grown with glucose and urea. The data shown are based on four two-channel DNA microarray hybridizations starting with cDNA from four independent biological replicates. The dashed lines indicate a 2-fold altered mRNA ratio, the dotted lines a 10-fold altered mRNA ratio.

are the only genes required for growth with GABA that are controlled by GabR.

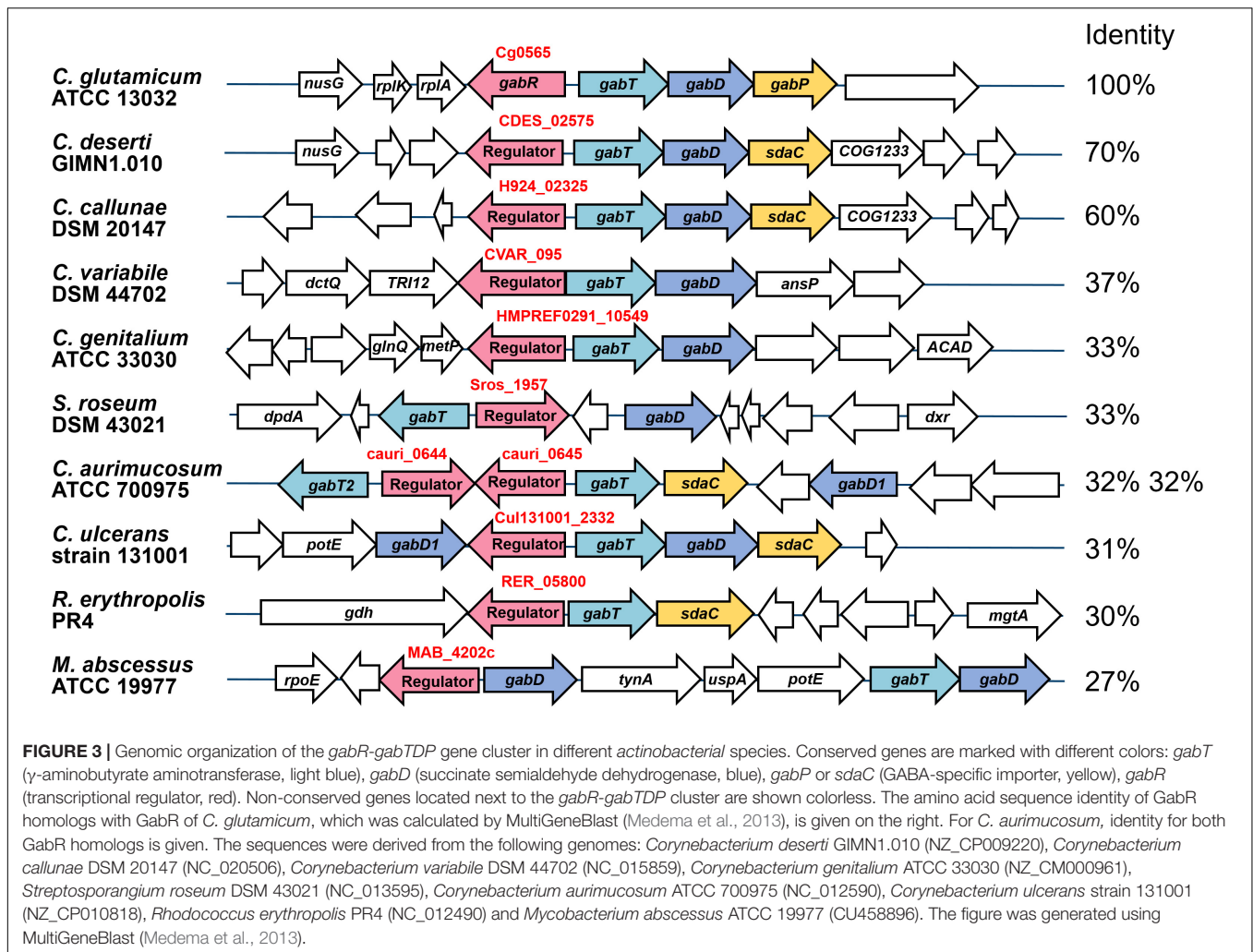
Purification of GabR and Determination of the Native Size

The growth experiments with the $\Delta gabR$ mutant showed that GabR activates *gabTDP* expression. To get further insights into the regulatory mechanism, we purified GabR for interaction studies with the P_{gabTDP} promoter by EMSAs. GabR was overproduced in *E. coli* BL21(DE3) and purified by means of an N-terminal decahistidine tag and Ni-NTA affinity chromatography (Figure 5A). The tag was cleaved off with TEV protease followed by size exclusion chromatography to further purify GabR and determine its native size and oligomeric state (Figure 5B). The peak of GabR appeared at an elution volume of 10.67 mL. Based on the calibration curve (K_{av} versus $\log M_r$) derived from the standard proteins (Figure 5C), the native size of GabR was calculated to be 420 kDa. Since the theoretical mass of GabR is 55.75 kDa, the native size suggests that *C. glutamicum* GabR forms an octamer (theoretical mass 446 kDa).

Determination of Transcriptional Start Sites

In a previous RNAseq study, a TSS of P_{gabR} was identified, but no TSS of P_{gabTDP} , presumably because the latter genes were not

expressed in the absence of GABA (Pfeifer-Sancar et al., 2013). Therefore, we determined the TSS of P_{gabTDP} initially using a 5'/3'-RACE kit and identified a single TSS for *gabT* located 36 bp upstream of the *gabT* start codon (Figure 6A). The -10 region (TACTCA) and the -35 region (TTAACT) of P_{gabTDP} and the RBS of *gabT* (AGGAG) were predicted according to known consensus sequences (Pfeifer-Sancar et al., 2013). In an alternative approach, we performed RNAseq of GABA-grown WT cells to identify TSSs. For *gabT*, the transcriptional start site found by 5'/3'-RACE was confirmed with 43906 reads starting at this position. Interestingly, another TSS was identified within the *gabD* coding region. 28 reads started 210 bp upstream of the *gabP* start codon, suggesting the presence of an additional weak promoter for *gabP* expression. The deduced -10 region (AACAAAT) and -35 region (TTCGGC) showed reasonable similarity to the reported consensus sequences (Pfeifer-Sancar et al., 2013). This promoter might allow GabR-independent weak expression of *gabP* and could enable initial GABA uptake to allow GabR-dependent expression of the *gabTDP* operon. With respect to the TSS of *gabR*, the RNAseq data revealed only two reads that started at the position 59 bp upstream of the *gabR* start codon (Figure 6A) that was previously reported (Pfeifer-Sancar et al., 2013). Four reads started 11 bp upstream of the *gabR* start codon, suggesting either another very weak promoter for *gabR* expression with weakly conserved -10 region (TAATTG) and -35 region (TTGTAG)



or processing of the transcript starting 59 bp upstream of the *gabR* start codon.

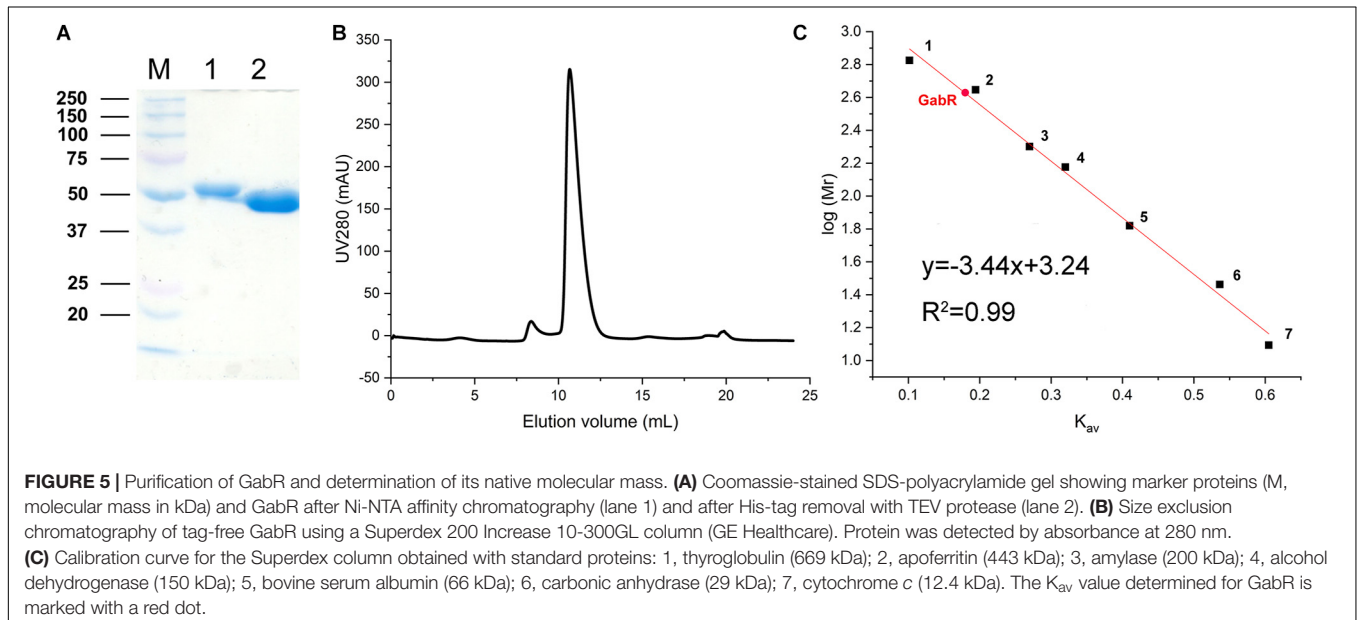
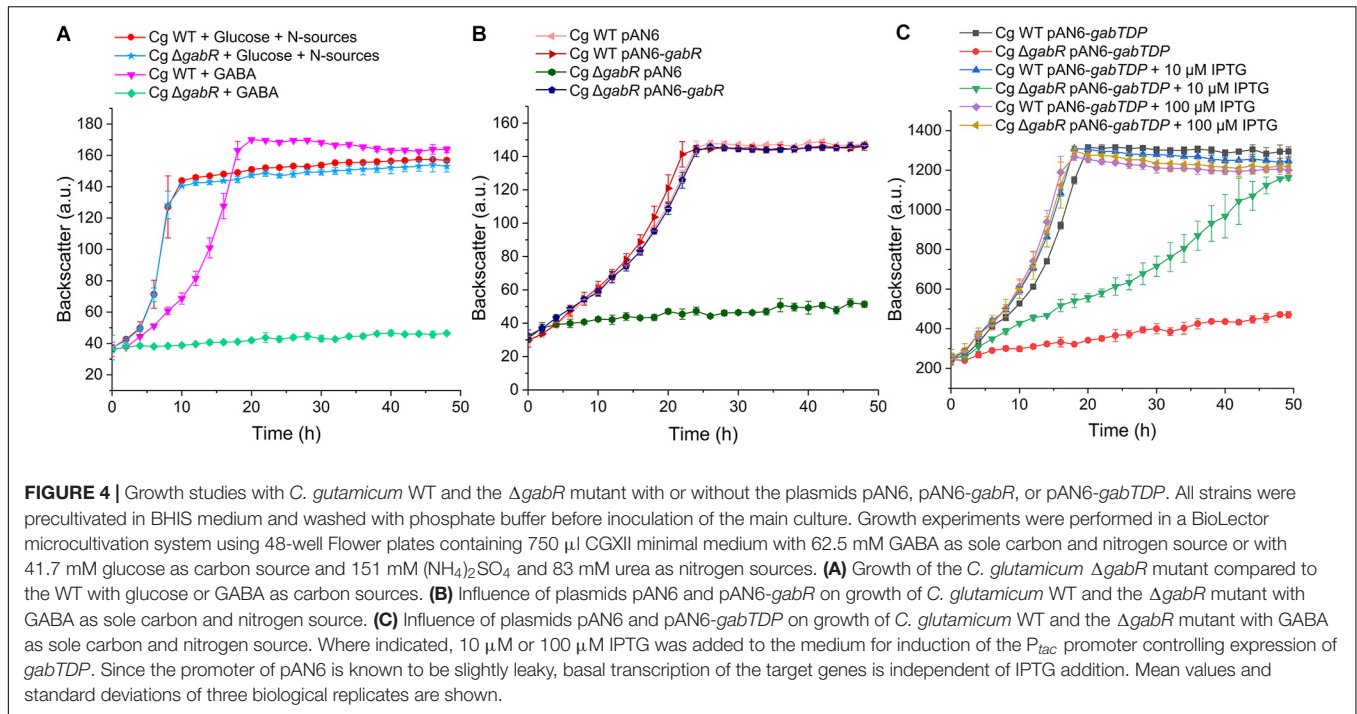
Determination of the GabR Binding Site(s) in the *gabTDP* Promoter Region

For the determination of the GabR binding site, EMSAs were performed with purified GabR. In the first experiment, binding of GabR to a 500 bp fragment covering the entire *gabR-gabT* intergenic region was analyzed using a DNA fragment of similar size of the *ldhA* gene of *C. glutamicum* as negative control. The *gabR-gabT* intergenic region was shifted partially with 80 nM GabR (monomer) and fully with 240 nM GabR (Supplementary Figure 2), whereas no shift was observed for the control fragment up to 320 nM GabR (Supplementary Figure 2). This suggests that GabR binds specifically to the *gabTDP* promoter region. In the following, we reduced the size of the DNA fragments step by step to localize the binding site(s) of GabR using the above mentioned fragments as positive and negative controls. As shown in Supplementary Figure 3, a full shift was observed for fragments FA1 and FA2, a partial shift for FA3, and a very poor shift for FA4 and FA5. For the fragments starting

at the other side of the promoter region, clear shifts were observed for fragments FB1, FB2 and FB3, weak shifts for FB4 and FB5, and no shift for FB6. These results suggested that GabR binds between position -87 and -23 with respect to the TSS of *gabT*.

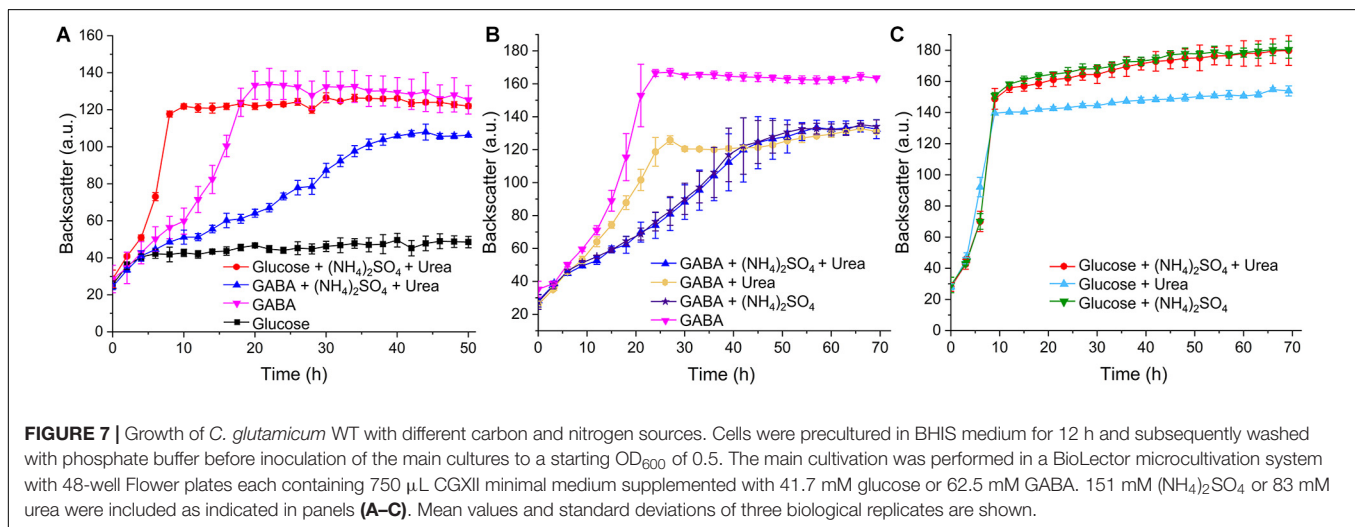
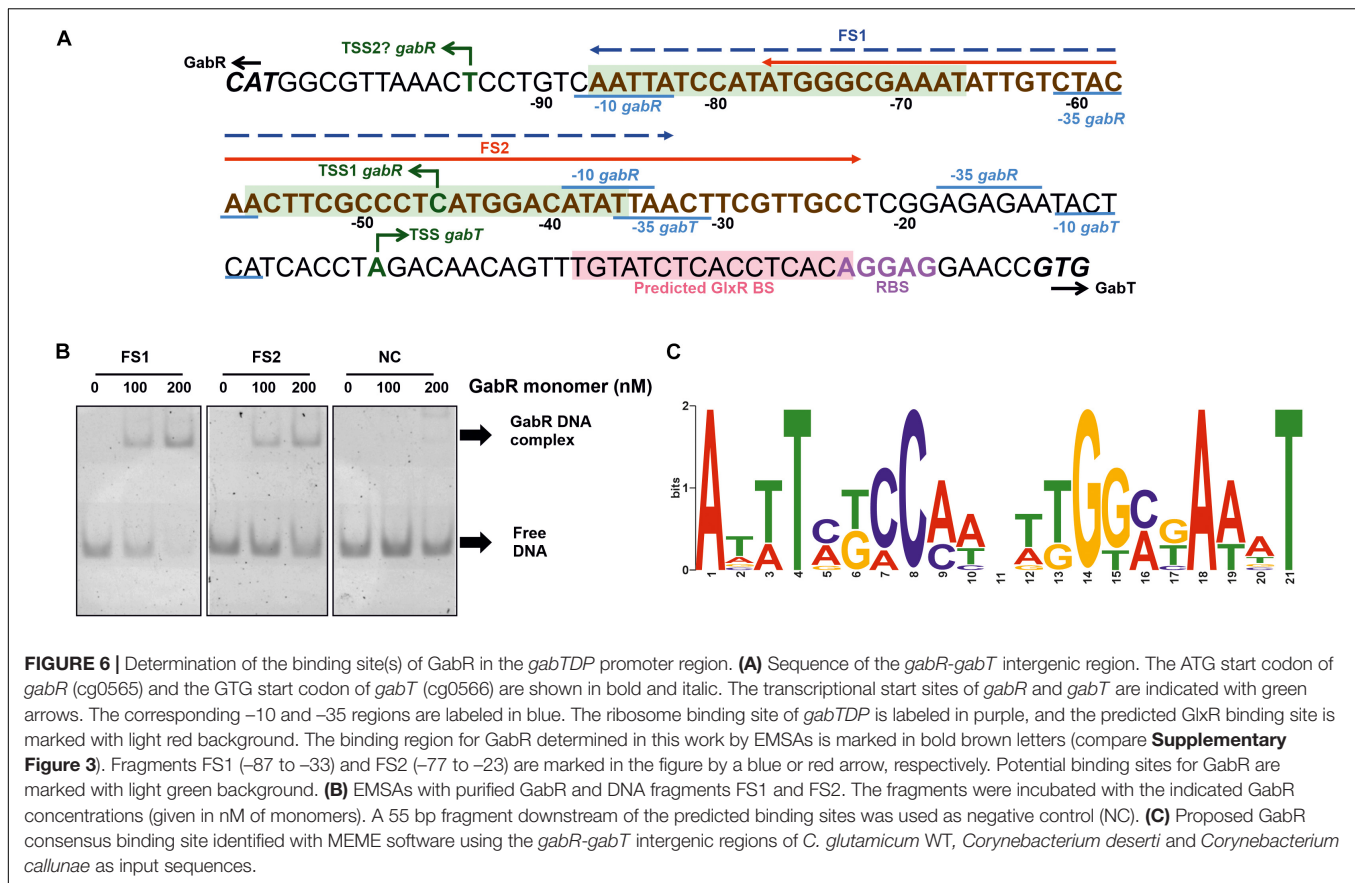
Regulator binding sites are often conserved among closely related species, which is useful to identify a binding motif when only a single target promoter is known (Wennerhold et al., 2005). We searched for a binding motif in the *gabR-gabT* intergenic regions of *C. glutamicum*, *Corynebacterium deserti*, and *Corynebacterium callunae* using MEME software (Bailey et al., 2006). The search uncovered a 21 bp inverted repeat that was present 1-2 times in each input sequence (Figure 6C). An alignment of the input sequences (Supplementary Figure 4) revealed that the regions encompassing the two proposed GabR binding motifs belong to the more conserved regions, which is a further hint that these represent GabR binding sites.

In further EMSAs, we used 55 bp fragments to verify the localization of the GabR binding sites in the *C. glutamicum gabTDP* promoter. FS1 is a fragment covering both of the predicted binding sites, whereas FS2 covers half of the first



binding site and the entire second binding site (**Figure 6A**). A 55 bp fragment downstream of the predicted binding sites was used as negative control. Binding of GabR was observed for both FS1 and FS2, but it was stronger for FS1 (**Figure 6B**). This suggests that one complete binding site is sufficient for weak binding, but two sites are required for strong binding by GabR. In another experiment, binding of GabR to two 31 bp fragments (FS3 and FS4) covering one binding site each was tested. A 31 bp fragment downstream of the predicted binding sites was used as negative control. Reasonable binding was observed for FS3

containing the first binding site and weak binding, not much stronger compared to the negative control, was observed for FS4 (**Supplementary Figure 5**). The band of the complex did not run far into the gel, presumably because of the large size of the GabR octamer. We assume that this band represents the complex, as it was absent from the negative control and from the DNA samples without protein. Our results suggest that each of the two binding sites can be bound separately by GabR, but binding is much stronger when both binding sites are present.



To verify that both binding sites are relevant for good binding, we mutated four conserved bases of both motifs either separately or in combination in fragment FS1 and analyzed the binding by GabR (**Supplementary Figure 6**). Mutation of one of the binding sites reduced the affinity to GabR, and with both binding sites mutated, the binding was almost completely abolished. This verifies that both sites are relevant for GabR binding.

Influence of Ammonium and Urea on Growth of *C. glutamicum* With GABA

In our initial growth studies using the BioLector microcultivation system, *C. glutamicum* WT was cultivated in standard CGXII minimal medium containing either glucose (41.7 mM corresponding to 250 mM carbon) or GABA (62.5 mM corresponding to 250 mM carbon) as carbon sources. In

glucose-containing medium, a growth rate μ of 0.4 h^{-1} was observed, whereas it was only 0.05 h^{-1} in GABA-containing medium. Interestingly, a growth rate of 0.13 h^{-1} was obtained when the standard nitrogen sources of CGXII medium, ammonium sulfate (151 mM) and urea (83 mM), were omitted and GABA served as sole carbon and nitrogen source (Figure 7A). Measurement of the final OD₆₀₀ in a spectrophotometer after 50 h revealed no significant difference between the cultures with glucose, (NH₄)₂SO₄ and urea on the one hand (OD₆₀₀ = 12.17 ± 0.54) and the cultures with GABA without additional nitrogen sources on the other hand (OD₆₀₀ = 11.81 ± 0.96). Further growth experiments revealed that (NH₄)₂SO₄ alone was sufficient to exert the same inhibitory effect on growth with GABA ($\mu = 0.04 \text{ h}^{-1}$) as in combination with urea ($\mu = 0.05 \text{ h}^{-1}$) (Figure 7B). Urea alone was also inhibitory, but to a lesser extent ($\mu = 0.08 \text{ h}^{-1}$). Urea is converted by urease to ammonium and carbon dioxide (Siewe et al., 1998), but the ammonia levels formed in this way are probably lower than in media containing (NH₄)₂SO₄, which could explain the weaker inhibitory effect of urea. When similar experiments were performed with glucose as carbon source, all cultures grew with the same rate (Figure 7C). A further experiment revealed that the inhibitory effect of (NH₄)₂SO₄ on growth with GABA was concentration-dependent and weak inhibition was observed already with 10 mM (NH₄)₂SO₄ (Supplementary Figure 7). The negative influence of 151 mM (NH₄)₂SO₄ on the final backscatter during growth with GABA as sole carbon source varied throughout our study. Sometimes the same final backscatter was reached in the presence of (NH₄)₂SO₄ as in its absence, whereas in other experiments the final backscatter was much lower in the presence of (NH₄)₂SO₄. The reason for this variability is currently unknown. In summary, *C. glutamicum* grew quite well in minimal medium with GABA as sole carbon and nitrogen source and the additional presence of ammonium was inhibitory. In the following studies, we searched for the basis of ammonium inhibition.

Influence of Ammonium on GABA Consumption

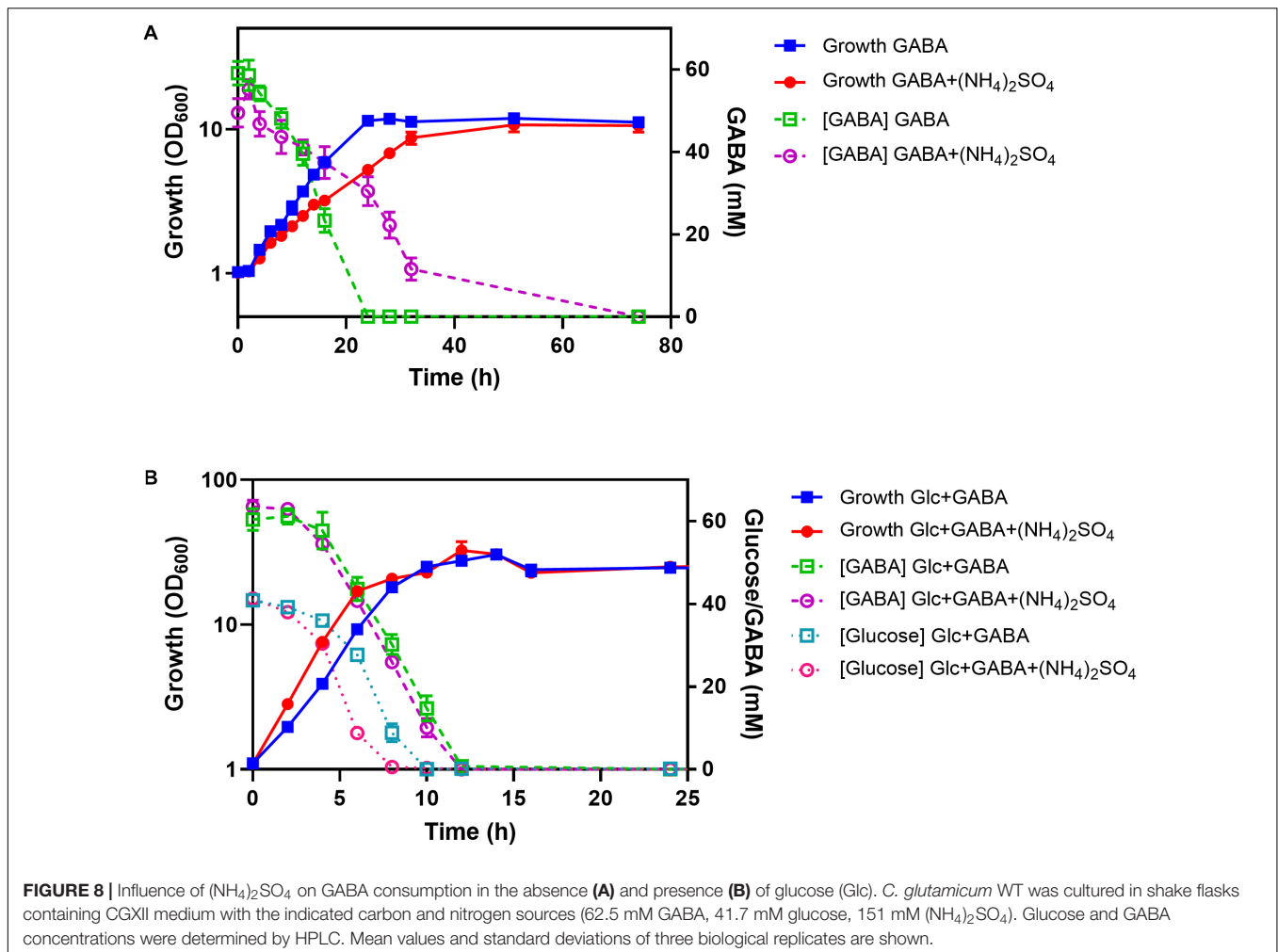
To test the influence of ammonium on GABA consumption, the WT was cultured in shake flasks with minimal medium containing either GABA or GABA plus (NH₄)₂SO₄. With GABA as sole carbon and nitrogen source, a growth rate of 0.12 h^{-1} was observed, which was reduced to 0.08 h^{-1} in the presence of 151 mM (NH₄)₂SO₄ (Figure 8A). In the absence of (NH₄)₂SO₄, GABA (62.5 mM) was completely consumed after 24 h and only after about 36 h in the presence of (NH₄)₂SO₄. Both cultures reached a final OD₆₀₀ of about 11, corresponding to 2.75 g cell dry weight/L (Kabus et al., 2007). In a further experiment, cells were cultivated in minimal medium containing either GABA and glucose or GABA, glucose, and (NH₄)₂SO₄. In this case, the cultures with (NH₄)₂SO₄ grew slightly faster ($\mu = 0.46 \text{ h}^{-1}$) than the ones without (NH₄)₂SO₄ ($\mu = 0.39 \text{ h}^{-1}$) and also consumed glucose faster than the cultures without (NH₄)₂SO₄ (Figure 8B). Complete glucose consumption was observed after 8 h and 10 h, respectively. Interestingly, GABA consumption was comparable

for the cultures with and without (NH₄)₂SO₄, in contrast to the cultures without glucose, and complete GABA consumption was observed after 12 h. It is obvious from Figure 8B that glucose and GABA are used simultaneously, also in the presence of (NH₄)₂SO₄.

Relevance of Ammonia-Assimilating Enzymes for Growth With GABA

During growth in minimal medium with GABA as sole carbon and nitrogen source, L-glutamate is formed from 2-oxoglutarate by the GABA transaminase GabT. L-Glutamine synthesis is dependent on glutamine synthetase encoded by the *glnA* gene (Jakoby et al., 1997). A second putative glutamine synthetase is encoded by *glnA2*, but this gene cannot complement the glutamine auxotrophy of a Δ *glnA* mutant and the function of the GlnA2 protein is not known yet (Nolden et al., 2001; Rehm and Burkowski, 2011). Glutamine synthetase requires L-glutamate, ATP and NH₄⁺ as substrates. Therefore, the question arises which reactions lead to ammonia release and thereby allow glutamine synthesis during growth with GABA as sole nitrogen source. A reasonable candidate is glutamate dehydrogenase (Gdh), which catalyzes the reductive amination of 2-oxoglutarate with ammonia and NADPH to L-glutamate and NADP⁺, and also the reverse reaction (Shiio and Ozaki, 1970). In order to determine whether Gdh is important as provider of ammonium during growth on GABA, we tested a Δ *gdh* mutant and found that it grew similarly as the wild type with GABA as sole carbon and nitrogen source (data not shown). This does not exclude the Gdh reaction as a source of ammonium, but shows that alternative ways for ammonium generation exist.

Besides the Δ *gdh* mutant, we also tested the growth behavior on GABA of other mutants with defects in nitrogen assimilation, which are mutants lacking either glutamate synthase (Δ *gltBD*), or glutamine synthetase (Δ *glnA*), or a triple mutant devoid of GlnA, GlnA2, and Gdh (Δ *glnADglnA2Dgdh*). Whereas the Δ *gltBD* mutant grew like the WT, the other two mutants were unable to grow with GABA as sole carbon and nitrogen source, irrespective of the absence or presence of ammonium. In Figure 9, the data for the triple mutant are shown. The result corresponds to the expectation that GltBD is not required for glutamate synthesis, whereas GlnA is essential for glutamine synthesis. This was further confirmed by supplementing GABA medium with L-glutamine, which rescued growth of the triple mutant (Figure 9). The addition of L-glutamine also improved the growth rate of the WT with GABA by about 50% ($\mu = 0.15 \text{ h}^{-1}$ vs. $\mu = 0.10 \text{ h}^{-1}$ without glutamine) and the final backscatter by about 25% (Figure 9). *C. glutamicum* is able to use L-glutamine as sole carbon and nitrogen source (Rehm et al., 2010). Whereas the increased final backscatter can be explained by the use of L-glutamine as additional carbon source, the increased growth rate suggests a deficiency in glutamine availability when GABA serves as sole nitrogen source. Interestingly, when (NH₄)₂SO₄ was added to medium with GABA and glutamine, it again had a negative effect on growth of both the WT and the triple mutant (Figure 9). This suggests that the inhibitory effect of ammonium



on growth with GABA alone is not due to a negative effect on glutamine synthesis.

Influence of Growth Conditions and GabR on the Promoter Activity of P_{gabTDP}

To analyze the influence of ammonia and other conditions on the expression of the *gabTDP* operon, we constructed the reporter plasmid pJC1- P_{gabT} -eYFP containing a transcriptional fusion between P_{gabTDP} (500 bp upstream of the *gabT* start codon) and the eYFP-encoding gene. Thereby the promoter activity can be monitored by measuring the fluorescence of the culture. Cell density (backscatter) and eYFP fluorescence of WT cells with pJC1- P_{gabT} -eYFP cultivated in different media is shown in **Figures 10A,B**. As the cells reached different final backscatter values, the specific fluorescence (representing the ratio of fluorescence/backscatter) was calculated for the cultures in the stationary phase at 40 h, which represents the activity of P_{gabTDP} over the entire cultivation (**Figure 10C**). We also checked the specific fluorescence of the cultures in the exponential growth phase, which was very similar to the values after 40 h (data not shown). Maximal specific fluorescence (2.81 ± 0.07) was observed for the cultures grown with GABA alone, whereas

it was almost zero (0.03 ± 0.00) for the cultures grown with glucose alone, indicating that P_{gabTDP} was active only in the presence of GABA. The specific fluorescence of the cultures grown with GABA, ammonium sulfate and urea (1.84 ± 0.10) was reduced by 35% in comparison to the cultures with GABA alone, indicating that ammonium inhibited transcription of *gabTDP*. Addition of glutamine to a culture with GABA and ammonium sulfate did not enhance *gabTDP* expression (data not shown), suggesting that glutamine does not influence transcription of *gabTDP*.

The specific fluorescence of cells cultivated with GABA, glucose, ammonium sulfate and urea (0.90 ± 0.02) was reduced by even 68% compared to growth on GABA alone, indicating that not only ammonium, but also glucose had a negative effect on *gabTDP* transcription. This effect was not specific for glucose, as it was also observed with gluconate or *myo*-inositol as additional carbon sources (**Supplementary Figure 8**).

In further experiments, we analyzed the activity of P_{gabTDP} in the $\Delta gabR$ mutant (**Figures 10D,E**). In medium with only GABA as carbon and nitrogen source, the mutant showed neither growth nor fluorescence, as expected. In medium with glucose and GABA, the mutant was able to grow, but no fluorescence was

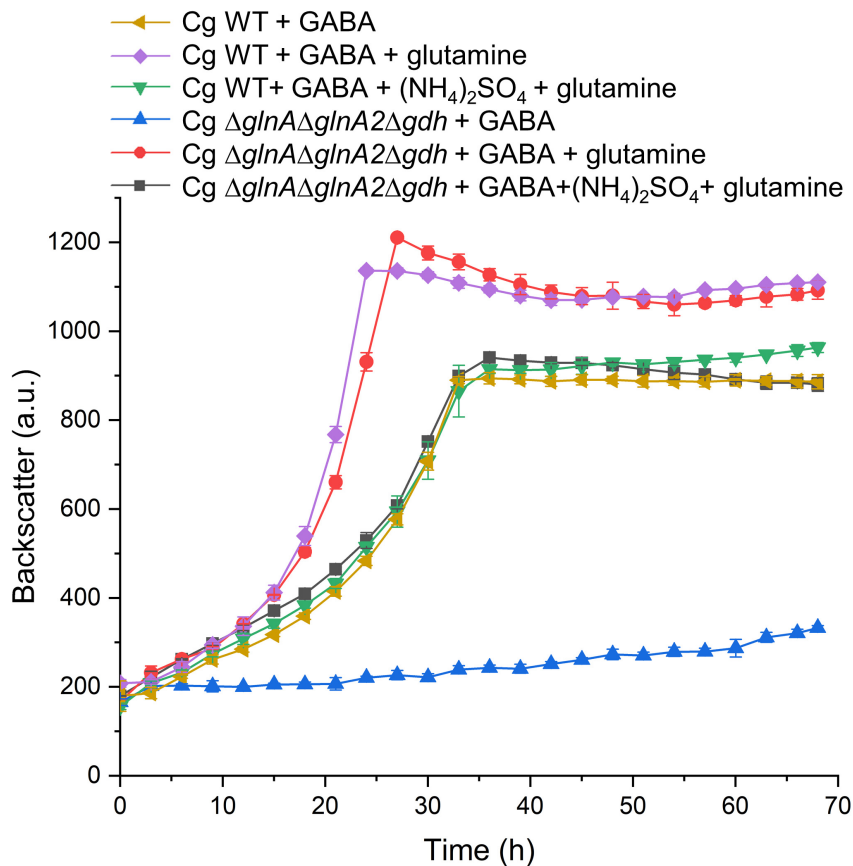


FIGURE 9 | Influence of L-glutamine supplementation on growth of *C. glutamicum* WT and the $\Delta glnA\Delta glnA2\Delta gdh$ mutant in CGXII medium with GABA. Cells were precultured in BHIS medium for 12 h and subsequently washed with phosphate buffer before inoculation of the main cultures to a starting OD_{600} of 0.5. The main cultivation was performed in a BioLector microcultivation system with 48-well Flower plates each containing 750 μ L CGXII minimal medium supplemented with 62.5 mM GABA. 20 mM L-glutamine and 151 mM $(NH_4)_2SO_4$ were included as indicated. For the WT mean values and standard deviations of three biological replicates are shown, for the mutant mean values and standard deviations of two biological replicates.

observed. This result confirms that besides GABA also GabR is essential for expression of the *gabTDP* genes.

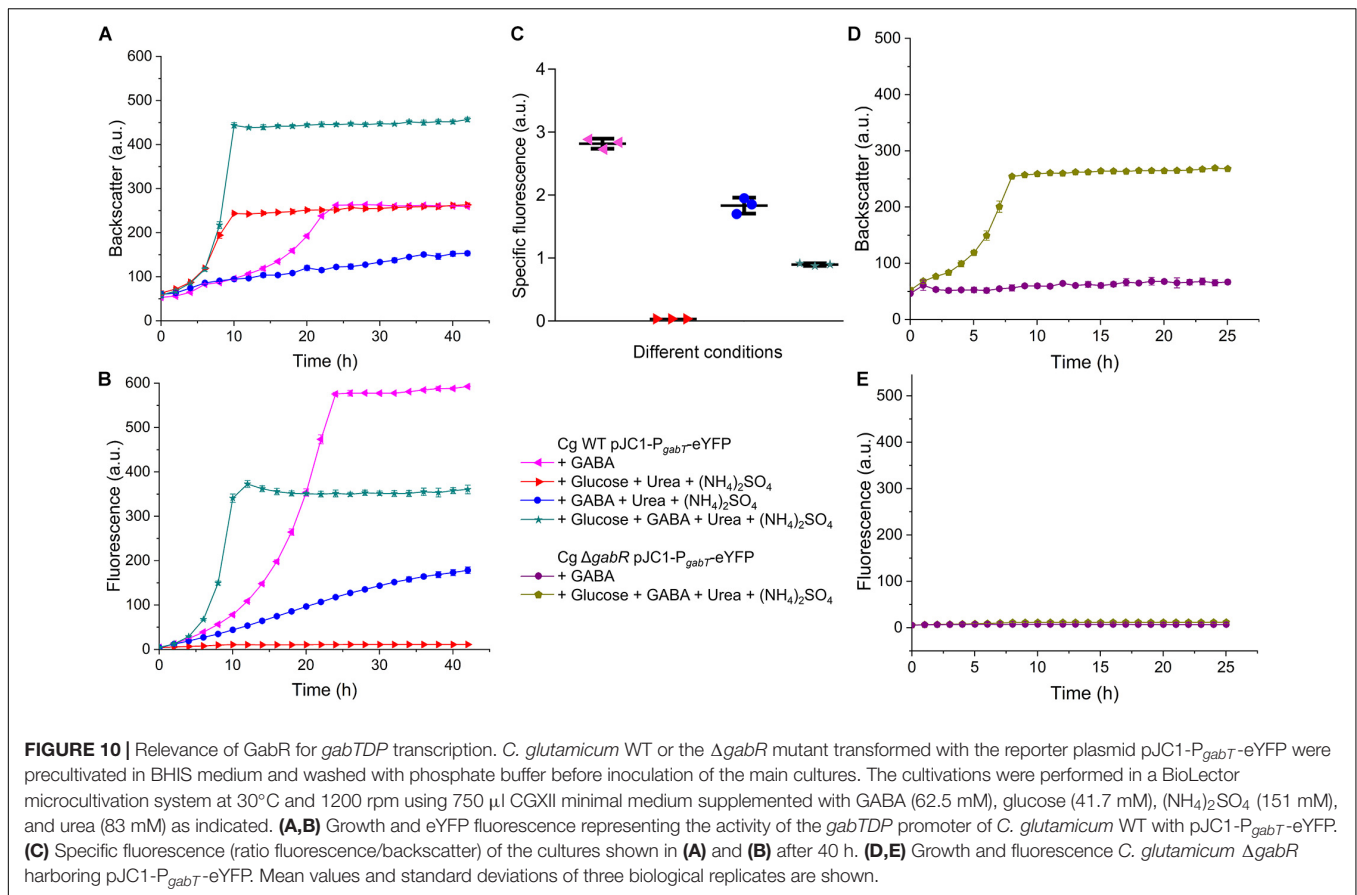
Influence of Plasmid-Based *gabTDP* Expression on the Effect of Ammonium

The reporter gene assays had revealed that ammonium reduces transcription of *gabTDP* (Figure 10C) by about 35%. To test whether this effect is sufficient to explain the growth inhibition by ammonium, we analyzed the consequences of plasmid-based overexpression of *gabTDP*. *C. glutamicum* WT was transformed with pAN6-*gabTDP* or pAN6 as control. The two strains were cultivated with GABA alone or with GABA plus $(NH_4)_2SO_4$ and expression of plasmid-borne *gabTDP* was induced with 10 μ M IPTG (Figure 11). With GABA alone, the WT containing pAN6-*gabTDP* grew comparably to the control strain with pAN6, suggesting that under these conditions chromosomal *gabTDP* expression was not limiting growth. In medium with GABA and $(NH_4)_2SO_4$, the strain with pAN6-*gabTDP* grew faster than the control strain with pAN6, although still not as good as with GABA alone, even when *gabTDP* expression was induced

with 100 μ M IPTG (data not shown). The results indicate that increased expression of *gabTDP* can partially overcome the growth inhibition by $(NH_4)_2SO_4$.

DISCUSSION

In this study, we analyzed the regulation of GABA utilization in the actinobacterial model organism *C. glutamicum*. The fact that *C. glutamicum* possesses the genes/proteins required for GABA uptake and metabolism suggests that GABA occurs in its natural soil habitat, where it could be derived e.g., from decomposed plant material or formed by other microorganisms via glutamate decarboxylation. We show that expression of the *gabTDP* operon encoding the proteins involved in GABA uptake and catabolism is transcriptionally activated by the divergently encoded regulator GabR. GabR is essential for growth of *C. glutamicum* on GABA. The observation that growth of a $\Delta gabR$ mutant could be restored by plasmid-based, GabR-independent expression of the *gabTDP* genes indicated that no additional genes activated by GabR



are required for GABA utilization, characterizing GabR as a local regulator.

GabR belongs to the PucR family of transcriptional regulators (Pfam PF07905). According to Pfam, more than 95% of the PucR sequences in the database are derived from *Actinobacteria* or *Firmicutes*. About half of the PucR family members in the database contain a GGDEF-like domain. Only few PucR-type regulators were studied to date, all of which contain the GGDEF-like domain, but a specific function of this domain has not been reported. These regulators are *B. subtilis* PucR involved in purine catabolism (Beier et al., 2002), *B. subtilis* PutR involved in proline utilization (Belitsky, 2011), *B. subtilis* AdeR involved in alanine catabolism (Lin et al., 2012), *Streptomyces ambofaciens* Srm22 (SrmR) controlling expression of a polyketide synthase gene for the synthesis of an antibiotic (Geistlich et al., 1992; Karray et al., 2010), and *Escherichia coli* CdaR (SdaR), which regulates genes involved in the uptake and metabolism of galactarate and glucarate (Monterrubio et al., 2000). All of these PucR regulators are activators, except for *B. subtilis* PucR, which acts both as activator and as repressor. GabR from *C. glutamicum* is the first characterized member of the PucR family that does not contain a GGDEF-like domain. Our results suggest that GabR binds to two 21 bp inverted repeats in the *gabTDP* promoter region. For AdeR, a 24 bp inverted repeat and for PutR, a 17 bp inverted repeat have been determined as binding sites, which is similar to our findings (Belitsky, 2011; Lin et al., 2012). The binding site of PucR is not

palindromic (Beier et al., 2002) and the binding sites for Srm22 and CdaR have not been determined yet.

Gel filtration of GabR indicated that it forms octamers. As the oligomeric state of other PucR-like regulators has not been determined yet, we do not know whether this is a specific feature of this group of regulators. We searched for other octameric transcriptional regulators to compare their binding patterns and function. The LysR-type transcriptional regulator (LTTR) CrgA of *Neisseria meningitidis* forms octameric rings and is proposed to bind to two binding sites in its target promoter with two stacked octameric rings (Sainsbury et al., 2009). The two CrgA binding sites cover a region of 63 bp, which is quite similar in size to the region covered by our two proposed motifs. CrgA also binds to DNA fragments containing just one of the binding sites (Sainsbury et al., 2009), but with strongly decreased affinity, which is also comparable to our data. GabR might work in a similar manner. Regulators belonging to the Lrp family are also frequently forming octamers, for example Lrp of *E. coli* (de los Rios and Perona, 2007), LrpA of *Pyrococcus furiosus* (Leonard et al., 2001), LrpA of *Mycobacterium tuberculosis* (Reddy et al., 2008; Song et al., 2016), AldR of *M. tuberculosis* (Dey et al., 2016), or BarR of the archaeum *Sulfolobus acidocaldarius* (Liu et al., 2014).

Transcriptional activators for catabolic pathways often require the corresponding substrate or one of its degradation products as co-activator. In the case of GabR from *C. glutamicum*, binding

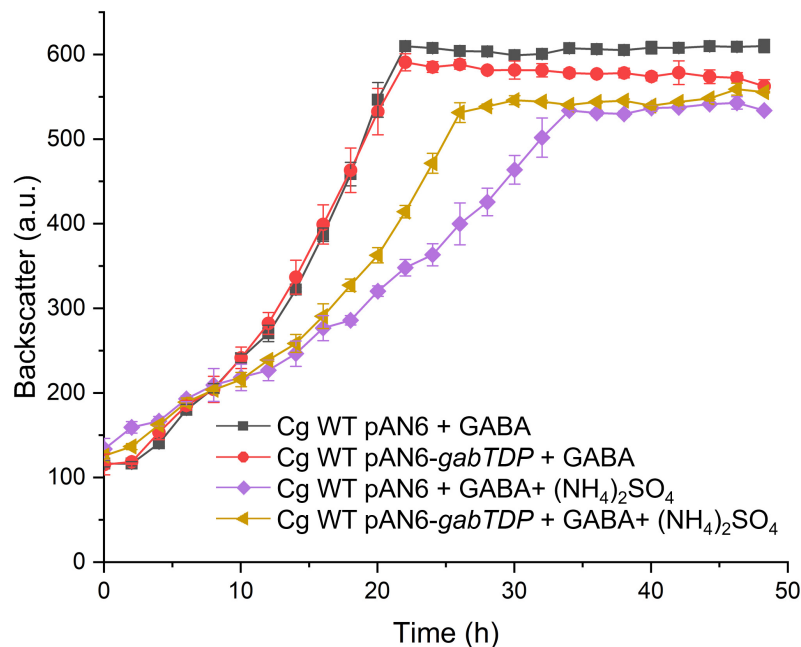


FIGURE 11 | Growth of *C. glutamicum* WT harboring pAN6-*gabTDP* or pAN6 with different substrates in the presence of 10 μ M IPTG. Growth experiments were performed in a BioLector microcultivation system with 48-well Flower plates containing 750 μ L CGXII minimal medium supplemented with GABA (62.5 mM) and $(\text{NH}_4)_2\text{SO}_4$ (151 mM) as indicated. BHIS medium was used for precultures and the cells were washed with phosphate buffer before inoculation of the main culture. Mean values and standard deviations of three biological replicates are shown.

to its target DNA did not require the presence of GABA and the presence of GABA had no obvious influence on the binding affinity (**Supplementary Figure 9**). GabR might bind to its target DNA both in the apo-state and in the ligand-bound state, but requires binding of GABA or another effector metabolite to trigger a conformational change that is necessary to activate transcription of *gabTDP*. Such a situation was found e.g., for *B. subtilis* GabR_{BS}, which binds to its target promoter independent of GABA, but transcription activation of the target genes requires binding of GABA and PLP (Wu et al., 2017). However, GabR_{BS} belongs to a different protein family than GabR of *C. glutamicum* and also PLP had no influence on GabR binding to DNA, neither alone nor in combination with GABA (**Supplementary Figure 9**). In case of *B. subtilis* PutR, the presence of proline increased the affinity for its target DNA about 10-fold (Belitsky, 2011), whereas for *B. subtilis* AdeR, L-alanine did not increase the affinity for the target promoter (Lin et al., 2012). Further studies are required to identify the co-activator(s) of GabR and the mechanism of transcriptional activation.

The GabR binding site is located immediately upstream of the -35 region of P_{gabTDP} (**Figure 6**), which is a typical position for transcriptional activators. However, in the transcriptome analysis, not only the *gabTDP* mRNA levels were increased during growth with GABA, but also the *gabR* mRNA level, although to a much lesser extent (4-fold) compared to *gabTDP* (65- to 87-fold). As the GabR binding site is located downstream of the -10 region of the *gabR* promoter identified in previous RNAseq studies (Pfeifer-Sancar et al., 2013), the question arises how the apparent positive autoregulation of

gabR is accomplished. One possibility could be an alternative transcriptional start site, leading e.g., to a leaderless transcript, which is found quite often in *C. glutamicum*. Our RNAseq analysis indeed identified a possible alternative TSS located 11 bp upstream of the *gabR* start codon (**Figure 6A**), but also in this case the promoter region overlaps with one of the two GabR binding sites.

When GABA is used as sole carbon source by *C. glutamicum*, growth requires the formation of PEP for gluconeogenesis. This task is fulfilled by PEP carboxykinase encoded by the *pck* gene (Riedel et al., 2001). Expression of the *pck* gene was found to be slightly upregulated in cells grown with GABA (1.68-fold, *p*-value 0.02), supporting its requirement in gluconeogenesis. Transcription of *pck* is subject to regulation by at least four transcriptional regulators and upregulation during growth on GABA might be due to derepression by RamA or activation by GntR1/GntR2 (Klaffl et al., 2013).

We observed that ammonium inhibits growth with GABA, with 10 mM already showing slight growth retardation. This negative effect is caused at least in part by an influence on *gabTDP* transcription, which was reduced by about 35% in the presence of 151 mM ammonium and 83 mM urea (**Figure 10**). The molecular basis for the diminished expression is not known yet. Control of nitrogen metabolism has been extensively studied in *C. glutamicum* and AmtR was identified as global regulator (Jakoby et al., 2000; Beckers et al., 2005; Buchinger et al., 2009). In the presence of sufficient nitrogen, AmtR represses a large set of genes involved in uptake and

utilization of various nitrogen sources. However, the *gabTDP* operon or *gabR* were never identified as members of the AmtR regulon, arguing against AmtR as mediator of reduced *gabTDP* expression in the presence of ammonia. In our EMSA studies with purified GabR, ammonia did not influence binding to the *gabT* promoter region (**Supplementary Figure 10**), but this does not exclude an effect of ammonia on GabR-activated transcription initiation. For example, binding of ammonium to GabR might inhibit binding of GABA or another effector and thus transcriptional activation. Future studies are required to clarify the mechanism by which ammonium inhibits *gabTDP* expression.

Growth with GABA as sole carbon and nitrogen sources requires the formation of ammonium as substrate for glutamine synthetase. Oxidative deamination of glutamate by glutamate dehydrogenase might contribute to ammonium provision, although deletion of the *gdh* gene had no effect on growth with GABA. An alternative possibility is the reaction catalyzed by aspartate ammonia-lyase, which catalyzes the conversion of aspartate to fumarate and ammonia (Dover and Halpern, 1972). The corresponding gene *aspA* (cg1697) was 2.50-fold upregulated in cells grown with GABA and urea compared to cells grown with glucose and urea. The observation that glutamine supplementation improved the growth rate with GABA suggests that glutamine availability in the absence of supplementation is limited, presumably by limited availability of the ammonia for glutamine synthetase. However, glutamine stimulated growth also in the presence of ammonium. In this situation, glutamine synthetase activity might be limiting due to limited expression of the *glnA* gene in the presence of ammonium (Nolden et al., 2001; Strösser et al., 2004) and due to limited activity of the enzyme due to adenylation by GlnE (Amon et al., 2010; Rehm and Burkovski, 2011). We also tested whether ammonium inhibits the activity of the GabT transaminase activity using purified GabT, but observed only a slight reduction by 6% in the presence of 30 mM (NH₄)₂SO₄ (9.07 ± 0.33 μmol min⁻¹ mg⁻¹ vs. 9.69 ± 0.12 μmol min⁻¹ mg⁻¹ in the absence of ammonium sulfate).

Besides ammonium, also the presence of alternative carbon sources had a negative influence on the expression of the *gabTDP* operon. The inhibition by glucose, gluconate, and myo-inositol is presumably caused by repression of *gabTDP* by the global cAMP-dependent transcriptional regulator GlxR. GlxR can function both as activator and repressor, depending on the localization of the binding site. A GlxR binding site (5'-TGTATCTCACCTCAC-3') has been detected in the *gabR-gabT* intergenic region (Toyoda et al., 2011; Jungwirth et al., 2013), which is located downstream of the *gabT* TSS (**Figure 6A**), indicating a repressor function of GlxR for *gabTDP*. GlxR-binding to DNA is controlled by cAMP, which is formed by the membrane-bound adenylate cyclase CyaB (Cha et al., 2010; Wolf et al., 2020). The tested additional carbon sources might lead to elevated cAMP levels and thus to enhanced binding of GlxR to the *gabT* promoter and repression. In contrast to e.g., *E. coli*, *C. glutamicum* usually shows parallel consumption of carbon sources rather than diauxic growth.

This has been demonstrated e.g., for glucose and acetate (Wendisch et al., 2000), glucose and gluconate (Frunzke et al., 2008), or glucose and citrate (Brocker et al., 2009). GlxR is presumably involved in coordinating the consumption of carbon sources in *C. glutamicum* to avoid an overload of the metabolic capacity of the cells. In the case of GABA, a similar situation can be envisaged, where the presence of an alternative carbon source causes a reduction of the GABA utilization rate to avoid an overload of the TCA cycle at the stage of succinate.

In summary, we characterized GabR as the first PucR-type transcriptional regulator without a GGDEF-like domain and showed that GabR is essential for activating expression of the GABA catabolic genes *gabTDP*. Although GABA did not influence the binding affinity of purified GabR to its target promoter, we assume it to be the most likely effector molecule, as the *gabTDP* genes were not expressed in the absence of GABA. The inhibitory effect of ammonium on GABA utilization is caused via inhibition of *gabTDP* transcription via a yet unknown mechanism and presumably via a negative effect on glutamine synthetase expression and activity. The negative effect on *gabTDP* transcription by alternative carbon sources is likely caused by the global cAMP-dependent regulator GlxR. Many of the results obtained here will probably also be relevant for other *Actinobacteria* capable of utilizing GABA as carbon and nitrogen source.

ORCID

Lingfeng Zhu
 orcid.org/0000-0002-9145-5419
 Christina Mack
 orcid.org/0000-0002-3495-3796
 Astrid Wirtz
 orcid.org/0000-0003-2159-3717
 Angela Kranz
 orcid.org/0000-0002-8000-0400
 Tino Polen
 orcid.org/0000-0002-0065-3007
 Meike Baumgart
 orcid.org/0000-0002-9874-1151
 Michael Bott
 orcid.org/0000-0002-4701-8254

DATA AVAILABILITY STATEMENT

The microarray datasets generated for this study can be found in the NCBI Gene Expression Omnibus under the GEO accession number GSE138829. The RNAseq data are available in the GEO database with the accession number GSE156688.

AUTHOR CONTRIBUTIONS

LZ performed the majority of the experimental work presented in this study, wrote the first draft of the manuscript and

provided most of the figures and tables. CM performed several growth experiments in shake flasks and the BioLector microcultivation system and she was responsible for the RNA isolation for RNAseq analysis. AW established and performed the HPLC method for measuring the GABA concentration in supernatants. AK evaluated the RNAseq data and TP supported the DNA microarray analysis. MBa and MBo designed the study, supervised the experimental work, and evaluated the data together with LZ, AK, and TP. MBa prepared figures added during revision. MBa and MBo revised the manuscript and MBo was responsible for the final version. All authors contributed to the article and approved the submitted version.

FUNDING

LZ was supported by a Ph.D. fellowship of the China Scholarship Council. Parts of this publication are derived from the Ph.D. thesis of LZ (Zhu, 2020).

REFERENCES

- Abdou, A. M., Higashiguchi, S., Horie, K., Kim, M., Hatta, H., and Yokogoshi, H. (2006). Relaxation and immunity enhancement effects of γ -aminobutyric acid (GABA) administration in humans. *BioFactors* 26, 201–208. doi: 10.1002/biof.5520260305
- Amon, J., Titgemeyer, F., and Burkovski, A. (2010). Common patterns - unique features: nitrogen metabolism and regulation in Gram-positive bacteria. *FEMS Microbiol. Rev.* 34, 588–605. doi: 10.1111/j.1574-6976.2010.00216.x
- Bailey, T. L., Williams, N., Mischel, C., and Li, W. W. (2006). MEME: discovering and analyzing DNA and protein sequence motifs. *Nucleic Acids Res.* 34, W369–W373. doi: 10.1093/nar/gkl198
- Baumgart, M., Luder, K., Grover, S., Gätgens, C., Besra, G. S., and Frunzke, J. (2013). IpsA, a novel LacI-type regulator, is required for inositol-derived lipid formation in *Corynebacteria* and *Mycobacteria*. *BMC Biol.* 11:122. doi: 10.1186/1741-7007-11-122
- Beckers, G., Strösser, J., Hildebrandt, U., Kalinowski, J., Farwick, M., Krämer, R., et al. (2005). Regulation of AmtR-controlled gene expression in *Corynebacterium glutamicum*: mechanism and characterization of the AmtR regulon. *Mol. Microbiol.* 58, 580–595.
- Beier, L., Nygaard, P., Jarmer, H., and Saxild, H. H. (2002). Transcription analysis of the *Bacillus subtilis* PucR regulon and identification of a *cis*-acting sequence required for PucR-regulated expression of genes involved in purine catabolism. *J. Bacteriol.* 184, 3232–3241. doi: 10.1128/jb.184.12.3232-3241.2002
- Belitsky, B. R. (2004). *Bacillus subtilis* GabR, a protein with DNA-binding and aminotransferase domains, is a PLP-dependent transcriptional regulator. *J. Mol. Biol.* 340, 655–664. doi: 10.1016/j.jmb.2004.05.020
- Belitsky, B. R. (2011). Indirect repression by *Bacillus subtilis* CodY via displacement of the activator of the proline utilization operon. *J. Mol. Biol.* 413, 321–336. doi: 10.1016/j.jmb.2011.08.003
- Ben-Ari, Y., Gaiarsa, J. L., Tyzio, R., and Khazipov, R. (2007). GABA: a pioneer transmitter that excites immature neurons and generates primitive oscillations. *Physiol. Rev.* 87, 1215–1284. doi: 10.1152/physrev.00017.2006
- Bertani, G. (1951). Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *J. Bacteriol.* 62, 293–300.
- Brocker, M., Schaffer, S., Mack, C., and Bott, M. (2009). Citrate utilization by *Corynebacterium glutamicum* is controlled by the CitAB two-component system through positive regulation of the citrate transport genes *citH* and *tctCBA*. *J. Bacteriol.* 191, 3869–3880. doi: 10.1128/jb.00113-09
- Buchinger, S., Strösser, J., Rehm, N., Hänssler, E., Hans, S., Bathe, B., et al. (2009). A combination of metabolome and transcriptome analyses reveals new targets of the *Corynebacterium glutamicum* nitrogen regulator AmtR. *J. Biotechnol.* 140, 68–74.

ACKNOWLEDGMENTS

The authors are most grateful to Andreas Burkovski (Erlangen, Germany) for providing the *C. glutamicum* strains LN Δ GDH (*C. glutamicum* Δ gdh), LN Δ GS (*C. glutamicum* Δ glnA), LN Δ gltBD (*C. glutamicum* Δ gltBD) and ATCC 13032 DA-2 (*C. glutamicum* Δ glnA Δ glnA2 Δ gdh) and information on nitrogen metabolism in *C. glutamicum*. In addition we thank Karen Wohlers for the help with glucose determination via HPLC and Jan Marienhagen for providing plasmid pJM0462.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.544045/full#supplementary-material>

- Bussmann, M., Baumgart, M., and Bott, M. (2010). RosR (Cg1324), a hydrogen peroxide-sensitive MarR-type transcriptional regulator of *Corynebacterium glutamicum*. *J. Biol. Chem.* 285, 29305–29318. doi: 10.1074/jbc.M110.156372
- Cha, P. H., Park, S. Y., Moon, M. W., Subhadra, B., Oh, T. K., Kim, E., et al. (2010). Characterization of an adenylate cyclase gene (*cybB*) deletion mutant of *Corynebacterium glutamicum* ATCC 13032. *Appl. Microbiol. Biotechnol.* 85, 1061–1068.
- Cotter, P. D., and Hill, C. (2003). Surviving the acid test: responses of gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* 67, 429–453. doi: 10.1128/Mmbr.37.3.429-453.2003
- De Biase, D., and Pennacchietti, E. (2012). Glutamate decarboxylase-dependent acid resistance in orally acquired bacteria: function, distribution and biomedical implications of the *gadBC* operon. *Mol. Microbiol.* 86, 770–786. doi: 10.1111/mmi.12020
- de los Rios, S., and Perona, J. J. (2007). Structure of the *Escherichia coli* leucine-responsive regulatory protein Lrp reveals a novel octameric assembly. *J. Mol. Biol.* 366, 1589–1602. doi: 10.1016/j.jmb.2006.12.032
- Dey, A., Shree, S., Pandey, S. K., Tripathi, R. P., and Ramachandran, R. (2016). Crystal structure of *Mycobacterium tuberculosis* H37Rv AldR (Rv2779c), a regulator of the *ald* gene: DNA binding and identification of small molecule inhibitors. *J. Biol. Chem.* 291, 11967–11980. doi: 10.1074/jbc.M115.700484
- Dhakal, R., Bajpai, V. K., and Baek, K. H. (2012). Production of GABA (γ -aminobutyric acid) by microorganisms: a review. *Braz. J. Microbiol.* 43, 1230–1241. doi: 10.1590/S1517-83822012000400001
- Dover, S., and Halpern, Y. S. (1972). Utilization of γ -aminobutyric acid as the sole carbon and nitrogen source by *Escherichia coli* K-12 mutants. *J. Bacteriol.* 109, 835–843.
- Edayathumangalam, R., Wu, R., Garcia, R., Wang, Y., Wang, W., Kreinbring, C. A., et al. (2013). Crystal structure of *Bacillus subtilis* GabR, an autorepressor and transcriptional activator of *gabT*. *Proc. Natl. Acad. Sci. U.S.A.* 110, 17820–17825. doi: 10.1073/pnas.1315887110
- Eggeling, L., and Bott, M. (eds) (2005). *Handbook of Corynebacterium glutamicum*. Boca Raton, FL: CRC Press.
- Ewing, B., and Green, P. (1998). Base-calling of automated sequencer traces using phred. II. Error probabilities. *Genome Res.* 8, 186–194.
- Feehily, C., and Karatzas, K. A. (2013). Role of glutamate metabolism in bacterial responses towards acid and other stresses. *J. Appl. Microbiol.* 114, 11–24. doi: 10.1111/j.1365-2672.2012.05434.x
- Foster, J. W. (2004). *Escherichia coli* acid resistance: tales of an amateur acidophile. *Nat. Rev. Microbiol.* 2, 898–907. doi: 10.1038/nrmicro1021
- Frunzke, J., Engels, V., Hasenbein, S., Gätgens, C., and Bott, M. (2008). Co-ordinated regulation of gluconate catabolism and glucose uptake in

- Corynebacterium glutamicum* by two functionally equivalent transcriptional regulators, GntR1 and GntR2. *Mol. Microbiol.* 67, 305–322.
- Geistlich, M., Losick, R., Turner, J. R., and Rao, R. N. (1992). Characterization of a novel regulatory gene governing the expression of a polyketide synthase gene in *Streptomyces ambofaciens*. *Mol. Microbiol.* 6, 2019–2029. doi: 10.1111/j.1365-2958.1992.tb01374.x
- Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A., and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Meth.* 6, 343–345. doi: 10.1038/Nmeth.1318
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580.
- Hilker, R., Stadermann, K. B., Doppmeier, D., Kalinowski, J., Stoye, J., Straube, J., et al. (2014). ReadXplorer—visualization and analysis of mapped sequences. *Bioinformatics* 30, 2247–2254. doi: 10.1093/bioinformatics/btu205
- Hong, J., and Kim, K. J. (2019). Crystal structure of γ -aminobutyrate aminotransferase in complex with a PLP-GABA adduct from *Corynebacterium glutamicum*. *Biochem. Biophys. Res. Commun.* 514, 601–606. doi: 10.1016/j.bbrc.2019.04.194
- Jakoby, M., Nolden, L., Meier-Wagner, J., Krämer, R., and Burkovski, A. (2000). AmtR, a global repressor in the nitrogen regulation system of *Corynebacterium glutamicum*. *Mol. Microbiol.* 37, 964–977.
- Jakoby, M., Tesch, M., Sahm, H., Krämer, R., and Burkovski, A. (1997). Isolation of the *Corynebacterium glutamicum* *glnA* gene encoding glutamine synthetase I. *FEMS Microbiol. Lett.* 154, 81–88.
- Jorge, J. M., Leggewie, C., and Wendisch, V. F. (2016). A new metabolic route for the production of gamma-aminobutyric acid by *Corynebacterium glutamicum* from glucose. *Amino Acids* 48, 2519–2531. doi: 10.1007/s00726-016-2272-6
- Jungwirth, B., Sala, C., Kohl, T. A., Uplekar, S., Baumbach, J., Cole, S. T., et al. (2013). High-resolution detection of DNA binding sites of the global transcriptional regulator GlxR in *Corynebacterium glutamicum*. *Microbiology* 159, 12–22. doi: 10.1099/mic.0.062059-0
- Kabus, A., Niebisch, A., and Bott, M. (2007). Role of cytochrome *bd* oxidase from *Corynebacterium glutamicum* in growth and lysine production. *Appl. Environ. Microbiol.* 73, 861–868.
- Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., et al. (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* 104, 5–25.
- Kapust, R. B., and Waugh, D. S. (1999). *Escherichia coli* maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci.* 8, 1668–1674. doi: 10.1110/ps.8.8.1668
- Karray, F., Darbon, E., Nguyen, H. C., Gagnat, J., and Pernodet, J. L. (2010). Regulation of the biosynthesis of the macrolide antibiotic spiramycin in *Streptomyces ambofaciens*. *J. Bacteriol.* 192, 5813–5821. doi: 10.1128/JB.00712-10
- Kawasaki, N., Nakayama, A., Yamano, N., Takeda, S., Kawata, Y., Yamamoto, N., et al. (2005). Synthesis, thermal and mechanical properties and biodegradation of branched polyamide 4. *Polymer* 46, 9987–9993. doi: 10.1016/j.polymer.2005.06.092
- Keilhauer, C., Eggeling, L., and Sahm, H. (1993). Isoleucine synthesis in *Corynebacterium glutamicum*: molecular analysis of the *ilvB-ilvN-ilvC* operon. *J. Bacteriol.* 175, 5595–5603.
- Kensy, F., Engelbrecht, C., and Büchs, J. (2009a). Scale-up from microtiter plate to laboratory fermenter: evaluation by online monitoring techniques of growth and protein expression in *Escherichia coli* and *Hansenula polymorpha* fermentations. *Microb. Cell Fact.* 8:68. doi: 10.1186/1475-2859-8-68
- Kensy, F., Zang, E., Faulhammer, C., Tan, R. K., and Büchs, J. (2009b). Validation of a high-throughput fermentation system based on online monitoring of biomass and fluorescence in continuously shaken microtiter plates. *Microb. Cell Fact.* 8:31. doi: 10.1186/1475-2859-8-31
- Kinoshita, S., Uda, S., and Shimono, M. (1957). Studies on amino acid fermentation. Part I. Production of L-glutamic acid by various microorganisms. *J. Gen. Appl. Microbiol.* 3, 193–205.
- Klaffl, S., Brocker, M., Kalinowski, J., Eikmanns, B. J., and Bott, M. (2013). Complex regulation of the phosphoenolpyruvate carboxykinase gene *pck* and characterization of its GntR-Type regulator IolR as a repressor of *myo*-inositol utilization genes in *Corynebacterium glutamicum*. *J. Bacteriol.* 195, 4283–4296. doi: 10.1128/JB.00265-13
- Kortmann, M., Kuhl, V., Klaffl, S., and Bott, M. (2015). A chromosomally encoded T7 RNA polymerase-dependent gene expression system for *Corynebacterium glutamicum*: construction and comparative evaluation at the single-cell level. *Microb. Biotechnol.* 8, 253–265. doi: 10.1111/1751-7915.12236
- Krulwich, T. A., Sachs, G., and Padan, E. (2011). Molecular aspects of bacterial pH sensing and homeostasis. *Nat. Rev. Microbiol.* 9, 330–343. doi: 10.1038/nrmicro2549
- Kurihara, S., Kato, K., Asada, K., Kumagai, H., and Suzuki, H. (2010). A putrescine-inducible pathway comprising PuuE-YneI in which γ -aminobutyrate is degraded into succinate in *Escherichia coli* K-12. *J. Bacteriol.* 192, 4582–4591. doi: 10.1128/JB.00308-10
- Leonard, P. M., Smits, S. H., Sedelnikova, S. E., Brinkman, A. B., de Vos, W. M., van der Oost, J., et al. (2001). Crystal structure of the Lrp-like transcriptional regulator from the archaeon *Pyrococcus furiosus*. *EMBO J.* 20, 990–997. doi: 10.1093/emboj/20.5.990
- Lin, T. H., Wei, G. T., Su, C. C., and Shaw, G. C. (2012). AdeR, a PucR-type transcription factor, activates expression of L-alanine dehydrogenase and is required for sporulation of *Bacillus subtilis*. *J. Bacteriol.* 194, 4995–5001. doi: 10.1128/JB.00778-12
- Lindroth, P., and Mopper, K. (1979). High-performance liquid chromatographic determination of subpicomole amounts of amino acids by precolumn fluorescence derivatization with o-phthalaldehyde. *Anal. Chem.* 51, 1667–1674.
- Liu, H., Orell, A., Maes, D., van Wolferen, M., Lindas, A. C., Bernander, R., et al. (2014). BarR, an Lrp-type transcription factor in *Sulfolobus acidocaldarius*, regulates an aminotransferase gene in a β -alanine responsive manner. *Mol. Microbiol.* 92, 625–639. doi: 10.1111/mmi.12583
- Marienhagen, J., Kennerknecht, N., Sahm, H., and Eggeling, L. (2005). Functional analysis of all aminotransferase proteins inferred from the genome sequence of *Corynebacterium glutamicum*. *J. Bacteriol.* 187, 7639–7646.
- Medema, M. H., Takano, E., and Breitling, R. (2013). Detecting sequence homology at the gene cluster level with MultiGeneBlast. *Mol. Biol. Evol.* 30, 1218–1223. doi: 10.1093/molbev/mst025
- Michaeli, S., and Fromm, H. (2015). Closing the loop on the GABA shunt in plants: are GABA metabolism and signaling entwined? *Front. Plant Sci.* 6:419. doi: 10.3389/fpls.2015.00419
- Monterrubio, R., Baldoma, L., Obradors, N., Aguilar, J., and Badia, J. (2000). A common regulator for the operons encoding the enzymes involved in D-galactarate, D-glucarate, and D-glycerate utilization in *Escherichia coli*. *J. Bacteriol.* 182, 2672–2674. doi: 10.1128/jb.182.9.2672-2674.2000
- Müller, T., Strösser, J., Buchinger, S., Nolden, L., Wirtz, A., Krämer, R., et al. (2006). Mutation-induced metabolite pool alterations in *Corynebacterium glutamicum*: towards the identification of nitrogen control signals. *J. Biotechnol.* 126, 440–453. doi: 10.1016/j.jbiotec.2006.05.015
- Ni, Y., Shi, F., and Wang, N. (2015). Specific γ -aminobutyric acid decomposition by *gabP* and *gabT* under neutral pH in recombinant *Corynebacterium glutamicum*. *Biotechnol. Lett.* 37, 2219–2227. doi: 10.1007/s10529-015-1897-y
- Niebisch, A., and Bott, M. (2001). Molecular analysis of the cytochrome *bc₁-aa₃* branch of the *Corynebacterium glutamicum* respiratory chain containing an unusual diheme cytochrome *c₁*. *Arch. Microbiol.* 175, 282–294.
- Nolden, L., Farwick, M., Krämer, R., and Burkovski, A. (2001). Glutamine synthetases of *Corynebacterium glutamicum*: transcriptional control and regulation of activity. *FEMS Microbiol. Lett.* 201, 91–98.
- Peng, Q., Yang, M., Wang, W., Han, L., Wang, G., Wang, P., et al. (2014). Activation of *gab* cluster transcription in *Bacillus thuringiensis* by γ -aminobutyric acid or succinic semialdehyde is mediated by the sigma-54-dependent transcriptional activator GabR. *BMC Microbiol.* 14:306. doi: 10.1186/s12866-014-0306-3
- Pennacchietti, E., D'Alonzo, C., Freddi, L., Occhialini, A., and De Biase, D. (2018). The glutaminase-dependent acid resistance system: qualitative and quantitative assays and analysis of its distribution in enteric bacteria. *Front. Microbiol.* 9:2869. doi: 10.3389/fmicb.2018.02869
- Pfeifer-Sancar, K., Mentz, A., Rückert, C., and Kalinowski, J. (2013). Comprehensive analysis of the *Corynebacterium glutamicum* transcriptome using an improved RNAseq technique. *BMC Genomics* 14:888. doi: 10.1186/1471-2164-14-888
- Reddy, M. C., Gokulan, K., Jacobs, W. R. Jr., Ioerger, T. R., and Sacchettini, J. C. (2008). Crystal structure of *Mycobacterium tuberculosis* LrpA, a leucine-responsive global regulator associated with starvation response. *Protein Sci.* 17, 159–170. doi: 10.1110/ps.073192208

- Rehm, N., and Burkovski, A. (2011). Engineering of nitrogen metabolism and its regulation in *Corynebacterium glutamicum*: influence on amino acid pools and production. *Appl. Microbiol. Biotechnol.* 89, 239–248. doi: 10.1007/s00253-010-2922-7
- Rehm, N., Georgi, T., Hiery, E., Degner, U., Schmiedl, A., Burkovski, A., et al. (2010). L-Glutamine as a nitrogen source for *Corynebacterium glutamicum*: derepression of the AmtR regulon and implications for nitrogen sensing. *Microbiology* 156, 3180–3193. doi: 10.1099/Mic.0.040667-0
- Richardt, J., Bringer, S., and Bott, M. (2012). Mutational analysis of the pentose phosphate and Entner-Doudoroff pathways in *Gluconobacter oxydans* reveals improved growth of a Δ edd Δ eda mutant on mannitol. *Appl. Environ. Microbiol.* 78, 6975–6986. doi: 10.1128/AEM.01166-12
- Riedel, C., Rittmann, D., Dangel, P., Möckel, B., Petersen, S., Sahm, H., et al. (2001). Characterization of the phosphoenolpyruvate carboxykinase gene from *Corynebacterium glutamicum* and significance of the enzyme for growth and amino acid production. *J. Mol. Microbiol. Biotechnol.* 3, 573–583.
- Sainsbury, S., Lane, L. A., Ren, J., Gilbert, R. J., Saunders, N. J., Robinson, C. V., et al. (2009). The structure of CrgA from *Neisseria meningitidis* reveals a new octameric assembly state for LysR transcriptional regulators. *Nucleic Acids Res.* 37, 4545–4558. doi: 10.1093/nar/gkp445
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multipurpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19 - Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145, 69–73.
- Schneider, B. L., and Reitzer, L. (2012). Pathway and enzyme redundancy in putrescine catabolism in *Escherichia coli*. *J. Bacteriol.* 194, 4080–4088. doi: 10.1128/JB.05063-11
- Schneider, B. L., Ruback, S., Kiupakis, A. K., Kasbarian, H., Pybus, C., and Reitzer, L. (2002). The *Escherichia coli* gabDTPC operon: specific γ -aminobutyrate catabolism and nonspecific induction. *J. Bacteriol.* 184, 6976–6986. doi: 10.1128/jb.184.24.6976-6986.2002
- Schultz, A. C., Nygaard, P., and Saxild, H. H. (2001). Functional analysis of 14 genes that constitute the purine catabolic pathway in *Bacillus subtilis* and evidence for a novel regulon controlled by the PucR transcription activator. *J. Bacteriol.* 183, 3293–3302. doi: 10.1128/JB.183.11.3293-3302.2001
- Scott, E. M., and Jakoby, W. B. (1958). Pyrrolidine metabolism - soluble γ -aminobutyric transaminase and semialdehyde dehydrogenase. *Science* 128, 361–362. doi: 10.1126/science.128.3320.361-a
- Shi, F., Jiang, J., Li, Y., Li, Y., and Xie, Y. (2013). Enhancement of γ -aminobutyric acid production in recombinant *Corynebacterium glutamicum* by co-expressing two glutamate decarboxylase genes from *Lactobacillus brevis*. *J. Ind. Microbiol. Biotechnol.* 40, 1285–1296. doi: 10.1007/s10295-013-1316-0
- Shi, F., and Li, Y. (2011). Synthesis of γ -aminobutyric acid by expressing *Lactobacillus brevis*-derived glutamate decarboxylase in the *Corynebacterium glutamicum* strain ATCC 13032. *Biotechnol. Lett.* 33, 2469–2474. doi: 10.1007/s10529-011-0723-4
- Shi, F., Si, H., Ni, Y. L., Zhang, L., and Li, Y. F. (2017). Transaminase encoded by NCgl2515 gene of *Corynebacterium glutamicum* ATCC 13032 is involved in γ -aminobutyric acid decomposition. *Process Biochem.* 55, 55–60. doi: 10.1016/j.procbio.2017.01.016
- Shiio, I., and Ozaki, H. (1970). Regulation of nicotinamide adenine dinucleotide phosphate-specific glutamate dehydrogenase from *Brevibacterium flavum*, a glutamate-producing bacterium. *J. Biochem.* 68, 633–647.
- Siewe, R. M., Weil, B., Burkovski, A., Eggeling, L., Krämer, R., and Jahns, T. (1998). Urea uptake and urease activity in *Corynebacterium glutamicum*. *Arch. Microbiol.* 169, 411–416.
- Song, N., Cui, Y., Li, Z., Chen, L., and Liu, S. (2016). New targets and cofactors for the transcription factor LrpA from *Mycobacterium tuberculosis*. *DNA Cell Biol.* 35, 167–176. doi: 10.1089/dna.2015.3040
- Strandwitz, P., Kim, K. H., Terekhova, D., Liu, J. K., Sharma, A., Levering, J., et al. (2019). GABA-modulating bacteria of the human gut microbiota. *Nat. Microbiol.* 4:396.
- Strösser, J., Lüdke, A., Schaffer, S., Krämer, R., and Burkovski, A. (2004). Regulation of GlnK activity: modification, membrane sequestration and proteolysis as regulatory principles in the network of nitrogen control in *Corynebacterium glutamicum*. *Mol. Microbiol.* 54, 132–147.
- Studier, F. W., and Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* 189, 113–130.
- Takahashi, C., Shirakawa, J., Tsuchida, T., Okai, N., Hatada, K., Nakayama, H., et al. (2012). Robust production of gamma-amino butyric acid using recombinant *Corynebacterium glutamicum* expressing glutamate decarboxylase from *Escherichia coli*. *Enzyme Microb. Technol.* 51, 171–176. doi: 10.1016/j.enzmictec.2012.05.010
- Toyoda, K., Teramoto, H., Inui, M., and Yukawa, H. (2011). Genome-wide identification of in vivo binding sites of GlxR, a cyclic AMP receptor protein-type regulator in *Corynebacterium glutamicum*. *J. Bacteriol.* 193, 4123–4133. doi: 10.1128/JB.00384-11
- van der Rest, M. E., Lange, C., and Molenaar, D. (1999). A heat shock following electroporation induces highly efficient transformation of *Corynebacterium glutamicum* with xenogeneic plasmid DNA. *Appl. Microbiol. Biotechnol.* 52, 541–545.
- Vogt, M., Haas, S., Klaffl, S., Polen, T., Eggeling, L., van Ooyen, J., et al. (2014). Pushing product formation to its limit: metabolic engineering of *Corynebacterium glutamicum* for L-leucine overproduction. *Metab. Eng.* 22, 40–52. doi: 10.1016/j.ymben.2013.12.001
- Walter, B., Kuspert, M., Ansorge, D., Krämer, R., and Burkovski, A. (2008). Dissection of ammonium uptake systems in *Corynebacterium glutamicum*: mechanism of action and energetics of AmtA and AmtB. *J. Bacteriol.* 190, 2611–2614.
- Wendisch, V. F., De Graaf, A. A., Sahm, H., and Eikmanns, B. J. (2000). Quantitative determination of metabolic fluxes during cointilization of two carbon sources: comparative analyses with *Corynebacterium glutamicum* during growth on acetate and/or glucose. *J. Bacteriol.* 182, 3088–3096.
- Wennerhold, J., and Bott, M. (2006). The DtxR regulon of *Corynebacterium glutamicum*. *J. Bacteriol.* 188, 2907–2918.
- Wennerhold, J., Krug, A., and Bott, M. (2005). The AraC-type regulator RipA represses aconitase and other iron proteins from *Corynebacterium* under iron limitation and is itself repressed by DtxR. *J. Biol. Chem.* 280, 40500–40508.
- Wolf, N., Bussmann, M., Koch-Koerfges, A., Katcharava, N., Schulte, J., Polen, T., et al. (2020). Molecular basis of growth inhibition by acetate of an adenylate cyclase-deficient mutant of *Corynebacterium glutamicum*. *Front. Microbiol.* 11:87. doi: 10.3389/fmicb.2020.00087
- Wu, R., Sanishvili, R., Belitsky, B. R., Juncosa, J. I., Le, H. V., Lehrer, H. J., et al. (2017). PLP and GABA trigger GabR-mediated transcription regulation in *Bacillus subtilis* via external aldimine formation. *Proc. Natl. Acad. Sci. U.S.A.* 114, 3891–3896. doi: 10.1073/pnas.1703019114
- Xu, N., Wei, L., and Liu, J. (2017). Biotechnological advances and perspectives of γ -aminobutyric acid production. *World J. Microbiol. Biotechnol.* 33:64. doi: 10.1007/s11274-017-2234-5
- Zhao, Z., Ding, J. Y., Ma, W. H., Zhou, N. Y., and Liu, S. J. (2012). Identification and characterization of γ -aminobutyric acid uptake system GabP_{Cg} (NCgl0464) in *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 78, 2596–2601. doi: 10.1128/AEM.07406-11
- Zhu, L. (2020). γ -Aminobutyrate as Carbon and Nitrogen Source for *Corynebacterium glutamicum* and Regulation of the Catabolic Genes by GabR. Doctoral thesis, University of Düsseldorf, Düsseldorf.
- Zhu, L., Peng, Q., Song, F., Jiang, Y., Sun, C., Zhang, J., et al. (2010). Structure and regulation of the gab gene cluster, involved in the γ -aminobutyric acid shunt, are controlled by a σ^{54} factor in *Bacillus thuringiensis*. *J. Bacteriol.* 192, 346–355. doi: 10.1128/JB.01038-09

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Zhu, Mack, Wirtz, Kranz, Polen, Baumgart and Bott. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.