γ-Aminobutyrate as carbon and nitrogen source for *Corynebacterium glutamicum* and regulation of the catabolic genes by GabR

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Abbreviations

Δ	Deletion
a.u.	Arbitrary units
ATCC	American Type Culture Collection
bp	Base pairs
BHI(S)	Brain Heart Infusion (+Sorbitol)
et al.	*et alii*
EMSA	Electrophoretic mobility shift assay
GABA	γ-Aminobutyric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
Kan^R	Kanamycin resistant
LB	Lysogeny broth
NCBI	National Center for Biotechnology Information
OD_{600}	Optical density at 600 nm
RBS	Ribosome binding site
rpm	Revolutions per minute
TCA	Tricarboxylic acid cycle
TSS	Transcriptional start site
v/v	Volume per volume
w/v	Weight per volume

Further abbreviations not included in this section were used according to international standards, for example listed in the author guidelines of the *Journal of Biological Chemistry*.

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1 Summary

1.1 Summary English

Corynebacterium glutamicum is a Gram-positive soil bacterium widely used in the industrial production of amino acids such as L-glutamate and L-lysine. *C. glutamicum* is able to use a variety of carbohydrates, alcohols and organic acids as single sources of carbon and energy for growth and some also for amino acid production. In this thesis, further potential carbon sources were investigated whether they can be used for growth by *C. glutamicum*. γ-Aminobutyric acid (GABA) is a non-proteinogenic amino acid and widespread in nature from microorganisms to plants and animals. *C. glutamicum* showed good growth with GABA as sole carbon and nitrogen source. Remarkably, ammonia and and to a lesser extent urea inhibited growth on GABA, whereas L-glutamine stimulated it. Possible reasons for these effects were analyzed. Genome-wide expression analysis revealed that the *gabTDP* genes encoding GABA transaminase, succinate semialdehyde dehydrogenase, and GABA permease, respectively, were highly induced in cells grown with GABA as carbon source compared to glucose-grown cells. The corresponding proteins catalyze GABA uptake, the transfer of the amino group to 2-oxoglutarate, and the oxidation of the resulting succinate semialdehyde to succinate. Transcriptional activation of the *gabTDP* genes was shown to be strictly dependent on the transcriptional regulator GabR, which is encoded upstream of and divergent to *gabT*. A Δ*gabR* mutant failed to grow on GABA, but not with other carbon sources. Growth of the Δ*gabR* mutant on GABA could be restored by plasmid-based expression of either *gabR* or *gabTDP*. Reporter gene studies confirmed that expression of *gabTDP* is dependent on GabR and GABA. Glucose caused reduced expression of *gabTDP* presumably via the cAMP-dependent global regulator GlxR. GabR belongs to the PucR family of transcriptional regulators. Purified GabR eluted as an octamer with an apparent mass of 420 kDa in size-exclusion chromatography and bound specifically to two binding sites in the *gabR-gabT* intergenic region extending from -36 to -56 and from -67 to -87 upstream of the *gabT* transcriptional start site. These results uncover new features of actinobacterial GABA utilization.

1.2 Summary German

Corynebacterium glutamicum ist ein Gram-positives Bodenbakterium, das für die industrielle Produktion von Aminosäuren wie L-Glutamat und L-Lysin genutzt wird. *C. glutamicum* kann eine Vielzahl von Kohlenhydraten, Alkoholen und organischen Säuren als einzige Kohlenstoff- und Energiequelle für das Wachstum und einige auch für die Aminosäureproduktion verwenden. In dieser Arbeit wurden weitere potenzielle Kohlenstoffquellen untersucht, ob sie für das Wachstum von *C. glutamicum* verwendet werden können. γ-Aminobuttersäure (GABA) ist eine nicht proteinogene Aminosäure und in der Natur von Mikroorganismen bis zu Pflanzen und Tieren weit verbreitet. *C. glutamicum* zeigte ein gutes Wachstum mit GABA als einziger Kohlenstoff- und Stickstoffquelle. Bemerkenswerterweise hemmten Ammoniak und in geringerem Maße Harnstoff das Wachstum von GABA, während L-Glutamin es stimulierte. Mögliche Gründe für diese Effekte wurden analysiert. Eine genomweite Expressionsanalyse ergab, dass die für GABA-Transaminase, Succinat-Semialdehyd-Dehydrogenase und GABA-Permease kodierenden *gabTDP*-Gene in Zellen, die mit GABA anstelle von Glucose als Kohlenstoffquelle kultiviert wurden, stark induziert waren. Die entsprechenden Proteine katalysieren die GABA-Aufnahme, den Transfer der Aminogruppe zu 2-Oxoglutarat und die Oxidation des entstandenen Succinatsemialdehyds zu Succinat. Es wurde gezeigt, dass die Transkriptionsaktivierung der *gabTDP*-Gene strikt von dem Transkriptionsregulator GabR abhängt, der stromaufwärts von und divergent zu *gabT* codiert ist. Eine Δ*gabR*-Mutante konnte auf GABA nicht wachsen, jedoch mit anderen Kohlenstoffquellen. Das Wachstum der Δ*gabR*-Mutante auf GABA konnte durch plasmidbasierte Expression von entweder *gabR* oder *gabTDP* wiederhergestellt werden. Reportergenstudien bestätigten, dass die Expression von *gabTDP* von GabR und GABA abhängt. Glucose verursachte eine verminderte Expression von *gabTDP*, vermutlich über den cAMP-abhängigen globalen Regulator GlxR. GabR gehört zur PucR-Familie der Transkriptionsregulatoren. Gereinigtes GabR wurde als Octamer mit einer apparenten Masse von 420 kDa in der Größenausschlusschromatographie eluiert und band spezifisch an zwei Bindungsstellen in der intergenen *gabR-gabT*-Region, die sich von -36 bis -56 und von -67 bis -87 stromaufwärts des *gabT*-Transkriptionsstarts erstreckten. Diese

Ergebnisse zeigen neue Charakteristika der Verwendung von GABA in Actinobakterien.

2 Scientific context and key results of this thesis

2.1 *Corynebacterium glutamicum*

2.1.1 Characteristics of *C. glutamicum*

Corynebacterium glutamicum is a Gram-positive soil bacterium, a member of the *Actinobacteria*, and is generally regarded as safe (GRAS status). It was first isolated in 1950s from the Ueno Zoo in Tokyo (Japan), during a search for natural L-glutamate producers (Kinoshita et al., 1957). *C. glutamicum* is used since decades been for the safe production of amino acids such as L-glutamate (Hashimoto, 2017; Hirasawa and Wachi, 2017) and L-lysine (Eggeling and Bott, 2015; Ikeda, 2017; Hermann, 2003). *C. glutamicum* is able to use different substrates such as carbohydrates (Blombach and Seibold, 2010), organic acids (Cocaign and et al., 1993; Wendisch et al., 2000; Brocker et al., 2009; Teramoto et al., 2008; Youn et al., 2009; Frunzke et al., 2008a), or alcohols (Arndt et al., 2008) as single sources of carbon and energy for growth and also for amino acid production. The availability of the complete genome sequences of *C. glutamicum* (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003) had a major impact on the improvement of the industrial production of chemicals (Becker and Wittmann, 2012; Wendisch et al., 2006). Investigation of the metabolism and its regulation in *C. glutamicum* has also made an important contribution toward optimization of strains and production processes, but knowledge is still restricted and needs to be extended. Therefore, a comprehensive understanding of the physiological and metabolic characteristics of *C. glutamicum* is important for developing new production strains or to improve the existing ones.

2.1.2 Substrates that can be used as sole carbon and nitrogen source by *C. glutamicum*

C. glutamicum is able to use a variety of substrates, but the use of a single substrate both as carbon and nitrogen source by *C. glutamicum* has so far been described only for a few compounds. According to a previous report, *C. glutamicum* wild type ATCC 13032 is able to grow with 70 mM L-glutamine as sole carbon and nitrogen source, but growth was quite slow (almost three days to reach the stationary phase) (Rehm et al., 2010). The additional presence of 150 mM ammonium sulfate had no influence

on glutamine-dependent growth (Rehm et al., 2010). Another publication showed that *C. glutamicum* wild type grew on minimal medium agar plates with glutamine as sole carbon and nitrogen source, whereas growth of a mutant lacking serine/threonine protein kinase G (PknG) was severely inhibited indicating its essential function in glutamine metabolism (Niebisch et al., 2006). PknG regulates 2-oxoglutarate dehydrogenase complex (ODH) activity via the phosphorylation status of OdhI (Niebisch et al., 2006). Furthermore, a spontaneous *C. glutamicum* mutant (M4) was isolated from a wild type culture in minimal medium with glucosamine as sole carbon source, which was able to grow on glucosamine as sole carbon and nitrogen source (Uhde et al., 2013). Characterization of the M4 mutant revealed a significantly increased expression of the *nagB* gene, which encodes the glucosamine-6-phosphate deaminase NagB involved in degradation of glucosamine, due to a single mutation in the *nagB* promoter region (Uhde et al., 2013). Some publications stated that glutamate can also be used as sole carbon and nitrogen source by *C. glutamicum* (Kronemeyer et al., 1995; Burkovski et al., 1996), but there is no published experimental evidence so far.

2.1.3 Products produced using *C. glutamicum*

Corynebacterium glutamicum is used since the 1960ies for the industrial production of amino acids. The flavour enhancer L-glutamate and the feed-additive L-lysine are produced in a scale of several million tons per annum with strains of *C. glutamicum* (Eggeling and Bott, 2005; Takeno et al., 2007; Wendisch et al., 2016). Besides the production of L-glutamate and L-lysine, many other amino acids can produced with engineered *C. glutamicum* (Becker and Wittmann, 2012; Wendisch et al., 2006). For example, L-serine-producing strains were generated by overexpression of key genes and feedback deregulation of the biosynthetic reactions as well as elimination of serine-consuming reactions (Peters-Wendisch et al., 2005; Stolz et al., 2007). The best strain produced up to 42.62 g L^{-1} L-serine (Zhu et al., 2015). For the fermentative production of L-alanine, an anaerobic process with growth-arrested cells was recently reported to produce 98 g L^{-1} L-alanine within 32 h (Jojima et al., 2010). *C. glutamicum* was also investigated as a host for synthesis of D-amino acids (Stäbler et al., 2011).

Since *C. glutamicum* is regarded as a robust and easily manageable production host, recent studies showed that genetically modified *C. glutamicum* strains are also

promising biocatalysts for the production of at least some organic acids. More than 500 mM (45 g L^{-1}) pyruvate was produced using engineered *C. glutamicum* Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA* with a $Y_{P/S}$ of 0.97 mol pyruvate per mol of glucose (Wieschalka et al., 2012). Furthermore, *C. glutamicum* was reported to produce L-lactate with concentrations of more than 1 M (97.5 g L^{-1}) (Okino et al., 2005) and 1.34 M D-lactate (i.e. 120 g L^{-1}) within 30 h with an optical purity of 99.9% and a $Y_{P/S}$ of 1.73 mol per mol of glucose (Okino et al., 2008b). A *C. glutamicum* strain (Δ*ldhA*-pCRA717) that overexpresses the *pyc* gene encoding pyruvate carboxylase while simultaneously carrying a disrupted *ldhA* gene encoding L-lactate dehydrogenase, produced 1.24 M (146 g L^{-1}) succinic acid with intermittent addition of sodium bicarbonate and glucose (Okino et al., 2008a). Litsanov et al. reported an engineered strain which was transformed with plasmid pVWEx1-*glpFKD* coding for the glycerol utilization genes of *Escherichia coli* and formed 79 mM (9.3 g L^{-1}) succinate from 375 mM glycerol (Litsanov et al., 2013).

In addition, efficient *C. glutamicum* strains for production of fuels have been developed for future commercial usage. A *C. glutamicum* strain harboring genes for pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) was constructed to produce ethanol under oxygen deprivation (Jojima et al., 2015). Overexpression of four glycolytic genes (*pgi*, *pfkA*, *gapA*, and *pyk*) and the gene *tpi*, encoding triosephosphate isomerase in *C. glutamicum,* significantly increased the rate of ethanol production. Fed-batch fermentation using an optimized strain resulted in ethanol production of 119 g L^{-1} from 245 g L^{-1} glucose with a yield of 95 % of the theoretical maximum (Jojima et al., 2015).

Furthermore, *C. glutamicum* has been engineered to produce aromatic compounds (Kogure et al., 2016; Kitade et al., 2018; Kubota et al., 2016). Kogure et al. constructed an engineered strain that achieved production of 141 g/L shikimate from glucose with a yield of 51% (mol/mol) after 48 h in minimal medium (Kogure et al., 2016). Recently, some publications proved that engineered *C. glutamicum* is also suitable as a platform to produce plant polyphenols (Sun et al., 2016; Kallscheuer et al., 2016; Kallscheuer et al., 2017). Carotenoids such as lycopene, native and non-native C50 carotenoids decaprenoxanthin, and sarcinaxanthin, as well as non-native C40 carotenoids β-carotene and zeaxanthin were also produced in the milligrams per cell dry weight (CDW) range with *C. glutamicum* engineered strains (Heider et al.,

2012; Heider et al., 2014). Fatty acids (Takeno et al., 2013; Nickel et al., 2010) and proteins (Freudl, 2017) were reported to be produced with *C. glutamicum* strains, too.

2.1.4 Nitrogen metabolism and regulation in *C. glutamicum*

Ammonium is a preferred nitrogen source of *C. glutamicum* and a standard component of defined growth media. The Amt and AmtB permeases transport ammonium into the cell (Burkovski, 2003). There are two main pathways for ammonium assimilation in *C. glutamicum* (**Fig. 1**) (Burkovski, 2003). When ammonium is present in concentrations above 5 mM, it is primarily assimilated via glutamate dehydrogenase (GDH). GDH assimilates ammonium by the reductive amination of 2-oxoglutarate to L-glutamate (Tesch et al., 1999). Alternatively, ammonium is assimilated via the glutamine synthetase/glutamate synthase (GS/GOGAT) pathway, whereby ammonium is used to amidate L-glutamate under consumption of ATP to form L-glutamine by glutamine synthetase (GS). The amide group is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT), resulting in the net conversion of 2-oxoglutarate to L-glutamate (Tesch et al., 1999).

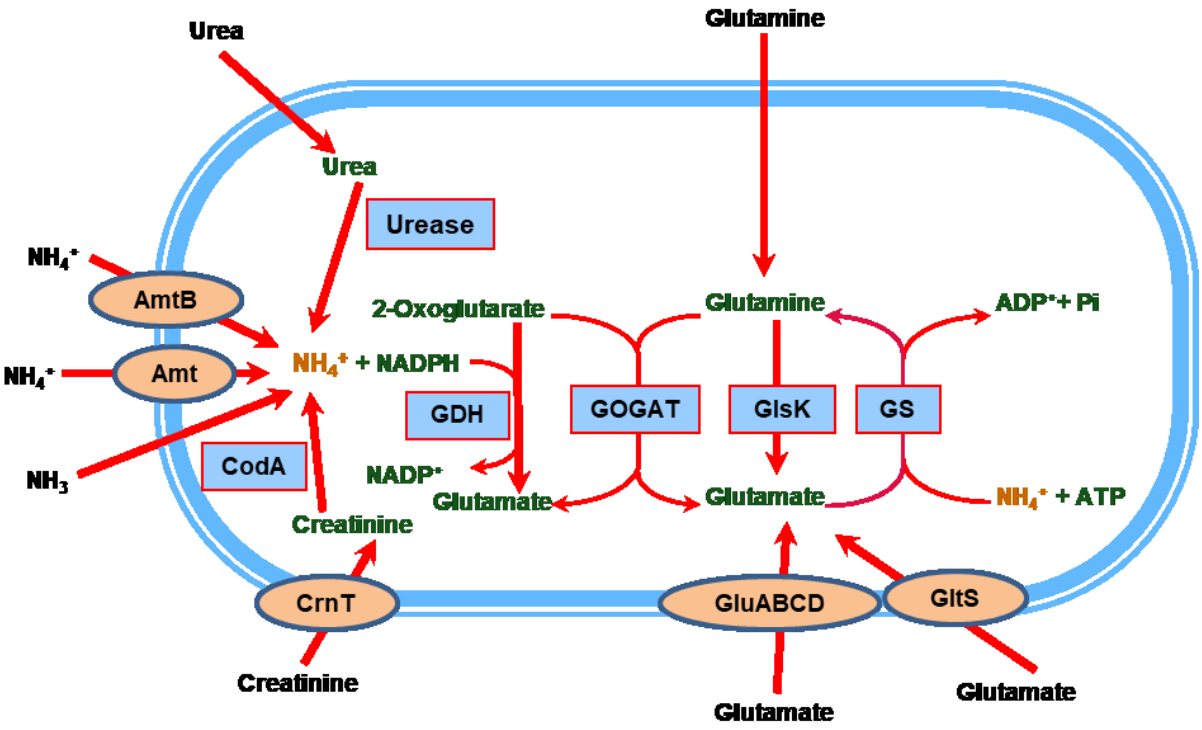

Fig. 1 Nitrogen metabolism in *C. glutamicum*. Rectangular boxes: enzymes, round boxes: importers. Amt, AmtB: ammonium uptake system; GluABCD: glutamate uptake system; GDH:

Glutamate dehydrogenase; GS: glutamine synthetase; GOGAT: glutamate synthase; CrnT: creatinine permease; CodA: creatinine deaminase; GlsK: glutaminase.

C. glutamicum can also use other nitrogen sources such as urea, creatinine and different amino acids (**Fig. 1**) (Burkovski, 2008). Urea can easily pass the cell membrane by diffusion and is then hydrolyzed to ammonium and carbon dioxide by urease which is encoded by *ureABCEFGD* (Beckers et al., 2004). Creatinine is taken up by a permease encoded by *crnT* and then metabolized by creatinine deaminase encoded by *codA* (Bendt et al., 2004). *C. glutamicum* can use glutamate (Burkovski et al., 1996) and glutamine as nitrogen source (Rehm et al., 2010), too. Glutamate is taken up in *C. glutumicum* via a binding protein-dependent transport system encoded by the *gluABCD* gene cluster (Burkovski et al., 1996) or via the membrane-potential-driven GltS system (Trötschel et al., 2003). *C. glutamicum* took up glutamine by a sodium-dependent secondary transport system, and GOGAT is the main glutamine-metabolizing enzyme in *C. glutamicum* (Rehm et al., 2010). A *glsK* (encoded glutaminase) deletion strain showed impaired growth with L-glutamine as carbon and energy source (Buerger et al., 2016).

In *C. glutamicum*, the master regulator AmtR governs the ammonium starvation-dependent gene expression (Burkovski, 2003). AmtR is a member of the TetR family of transcriptional regulators (Beckers et al., 2005). Subsequent studies showed that AmtR regulates transcription of at least 35 genes involved in nitrogen metabolism encoding transporters of ammonium (*amt amtB*), enzymes for ammonium assimilation (*gltBD*, *gdh*, *dapD* and *glnA*), enzymes for urea metabolism (*ureABCEFGD*) and nitrogen signal transduction proteins (*glnK*, *glnD*) (Beckers et al., 2005; Buchinger et al., 2009). An AmtR binding site consensus motif was deduced from bioinformatic analyses of available genome sequence information and competitive gel retardation assays (Beckers et al., 2005). The resulting AmtR box with the nucleotide sequence tttCTATN6AtAGat/aA (with bases represented by capital letters being highly conserved) is a more or less palindromic sequence and can be located in the promoter region either on the sense or antisense strand. AmtR activity is mediated by the regulatory protein GlnK (Beckers et al., 2005). Under conditions of nitrogen limitation, GlnK is adenylylated by the GlnD protein at Tyr51 (Strösser et al., 2004). In its adenylylated form GlnK binds to AmtR and prevents binding of AmtR to its target genes (Strösser et al., 2004). The crystal structures of AmtR in apo- and DNA-bound forms have been published (Palanca and Rubio, 2016).

AmtR has an extra C-terminal helix, a large extended external loop that resembles the flexible tranducer T-loop of GlnK in sequence, and a large open cavity towards the intersubunit region that changes shape upon DNA binding (Palanca and Rubio, 2016).

2.1.5 Gene regulation in *C. glutamicum*

For the rational improvement of biotechnological production strains, it is important to know the metabolic pathways and the regulatory functions of the transcription factors. Since the genome sequence of *C. glutamicum* was first published (Ikeda and Nakagawa, 2003; Kalinowski et al., 2003), its versatile metabolic pathways and their genetic components and regulatory mechanisms have been extensively studied (Brinkrolf et al., 2010; Toyoda and Inui, 2016; Bott, 2007; Bott and Brocker, 2012; Teramoto et al., 2011; Schröder and Tauch, 2010). Knowledge of the regulatory system in *C. glutamicum* is very useful for engineering the producer strain. Previous studies on transcription factors, including two-component signal transduction systems (Bott and Brocker, 2012) and σ factors (Toyoda and Inui, 2016), in *C. glutamicum* have revealed that so many transcriptional regulatory systems form a complex transcriptional regulatory network (Brinkrolf et al., 2010; Schröder and Tauch, 2010). According to Brinkrolf et al., a collection of 159 genes encoding DNA-binding transcriptional regulators, including 13 two-component signal transduction systems and seven σ factors, can be regarded as the minimal regulatory repertoire used for transcriptional regulation in the type strain ATCC13032 (Brinkrolf et al., 2010). The knowledge of the transcription factors, such as their target genes (regulons), DNA sequence motifs for recognition, and effector molecules controlling their activities is important for understanding the regulatory functions (Toyoda and Inui, 2016).

C. glutamicum has multi-layered, well-established regulatory systems for efficient utilization of multiple carbon sources, such as SugR (Engels and Wendisch, 2007), RamB (Gerstmeir et al., 2004), RamA (Cramer et al., 2006), GlxR (Kim et al., 2004), GntR1/2 (Frunzke et al., 2008b), or LldR (Georgi et al., 2008). For example, GlxR, a homologue of *Escherichia coli* CRP, plays a global regulatory role in *C. glutamicum* (Kohl et al., 2008). GlxR was first identified as a regulator repressing the promoter activity of *aceB,* encoding malate synthase, a glyoxylate shunt enzyme (Kim et al., 2004), and has subsequently been supposed to be involved in regulation of a number

of genes (Jungwirth et al., 2013). Jungwirth et al. reported that GlxR targets are involved in energy production and conversion, carbohydrate transport and metabolism, amino acid transport and metabolism, inorganic ion transport and metabolism (Jungwirth et al., 2013). The crystal structures of apo-GlxR and holo-GlxR indicated that a distinctive conformational change occurs upon cyclic AMP (cAMP) binding (Townsend et al., 2014). Another report showed that cAMP binding to GlxR also protects the protein from cleavage (Hong et al., 2014). A single membrane-bound class III adenylate cyclase, CyaB, forming cAMP from ATP was found in *C. glutamicum* (Cha et al., 2010). Furthermore, a class II cAMP phosphodiesterase named CpdA also plays a key role in the control of cAMP concentration and GlxR activity (Schulte et al., 2017).

Some other regulators are regulating genes belonging to the tricarboxylic acid (TCA) cycle (Krug et al., 2005; Beckers et al., 2005; Wennerhold et al., 2005). AcnR, one of these regulators, is a TetR-type repressor whose gene is located immediately downstream of the aconitase structural gene. In an *acnR* deletion mutant, the aconitase activity was increased fivefold on glucose, threefold on citrate, twofold on acetate and fivefold on propionate, supporting a repressor function of AcnR (Krug et al., 2005). The structure of AcnR has also been reported, and AcnR is regulated by citrate-Mg^{2+} through a non-canonical binding site (García-Nafría et al., 2013). *C. glutamicum* ATCC 13032 also possesses 13 two component systems involved in processes such as citrate utilization (CitAB), osmoregulation and cell wall homeostasis (MtrAB), adaptation to phosphate starvation (PhoSR), adaptation to copper stress (CopSR), and heme homeostasis (HrrSA) (Bott and Brocker, 2012).

2.2 γ-Aminobutyric acid (GABA) production in *C. glutamicum*

γ-Aminobutyric acid (GABA), a four-carbon non-proteinogenic amino acid, has its amino group on the γ- instead of the α-carbon, and a relative molecular weight of 103.1. GABA is a zwitterion at physiological pH, having both a positive and negative charge with pK_a values of 10.56 and 4.03, respectively (Shelp et al., 1999). It is widespread in nature from microorganisms to plants and animals. It has hypotensive, anti-anxiety, tranquilizing, analgesic and diuretic functions, and it can be applied to treat various neurological disorders (Sen et al., 2016; Ben-Ari et al., 2007; Abdou et al., 2006). In microorganisms, GABA is an important component for acidic pH

resistance in *E. coli* (Tramonti et al., 2006), and is required for the oxidative stress tolerance of *Saccharomyces cerevisiae* (Coleman et al., 2001). GABA has also gained much attention as a building block for the synthesis of 2-pyrrolidone and the biodegradable polyamide nylon 4, further expanding its application area into industrial biotechnology of bulk chemicals (Kawasaki et al., 2005; Park et al., 2013).

In recent years, a number of studies have focused on the efficient production of GABA (Xu et al., 2017; Dhakal et al., 2012), e.g. with *E. coli* (Zhao et al., 2017; Pham et al., 2015), *C. glutamicum* (Choi et al., 2015; Zhang et al., 2014; Baritugo et al., 2018) and other organisms (Irla et al., 2017; Renes et al., 2017). The major pathway of GABA biosynthesis is the irreversible α-decarboxylation of L-glutamate, which is catalyzed by the glutamate decarboxylase (GAD) (**Fig. 2**) (Choi et al., 2015; Pham et al., 2015). *C. glutamicum* is known as an excellent glutamate producer, which is the direct precursor of GABA. Although it does not possess an endogenous GAD, it is a promising candidate for the large scale production of GABA, because strains producing large amounts of glutamate are already available (Schultz et al., 2007). For example, 26.32 g L^{-1} GABA was produced with the recombinant *C. glutamicum* co-expressing two GADs from *L. brevis* LB85 after 60 h fed-batch fermentation under optimal urea supplementation (Shi et al., 2013). Moreover, Zhang et al. developed an efficient direct biosynthesis route from glucose based on recombinant *C. glutamicum* expressing *L. plantarum* GAD, without the need for exogenous supplementation of the expensive PLP cofactor, which achieved the production of 70.6 g/L of GABA after 70 h fermentation (Zhang et al., 2014). An alternative route for the production of GABA via putrescine was established by Jorge et al. in *C. glutamicum* (**Fig. 2**) (Jorge et al., 2016). A putrescine-producing recombinant *C. glutamicum* strain was converted into a GABA-producing strain by heterologous expression of putrescine transaminase (PatA) and γ-aminobutyraldehyde dehydrogenase (PatD) genes of *E. coli*, and the resulting strain produced 8 g L^{-1} of GABA (Jorge et al., 2016).

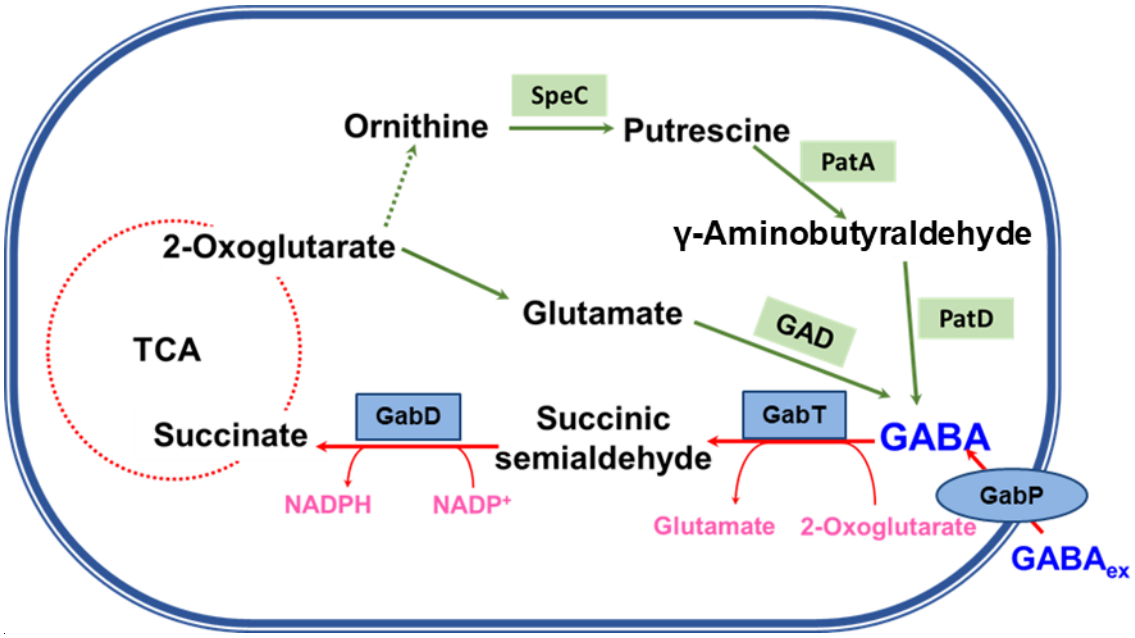

Fig. 2 Overview of GABA degradation in *C. glutamicum* wild type as well as GABA synthesis pathways in engineered strains. Enzymatic reactions and the involved proteins are depicted. *Dashed arrows* represent more than one enzymatic reaction. Rectangular boxes: enzymes, round box: importer protein. Blue boxes: enzymes for GABA catabolism in *C. glutamicum*, green boxes: enzymes for GABA production. SpeC: ornithine decarboxylase, PatA: putrescine transaminase, PatD: γ-aminobutyraldehyde dehydrogenase, GAD: glutamate decarboxylase, GabT: γ-aminobutyrate aminotransferase, GabD: succinate semialdehyde dehydrogenase, and GabP: GABA-specific importer.

2.3 GABA catabolism

For the generation of superior biotechnological production strains, measures to prevent product degradation are often a major issue. GABA is metabolized to succinic semialdehyde by GABA aminotransferase, and the succinic semialdehyde is subsequently oxidized to succinate by succinic semialdehyde dehydrogenase. In *Escherichia coli*, there are duplicate GABA aminotransferases (GabT and PuuE) and duplicate GABA aminotransferase (GabD and YneI) (Kurihara et al., 2010a). The *Bacillus subtilis ycnG* (*gabT*) and *ycnH* (*gabD*) genes were shown to encode GABA aminotransferase and succinic semialdehyde dehydrogenase, respectively, and to form a GABA-inducible operon (Belitsky and Sonenshein, 2002). Null mutation in *gabT* or *gabD* gene blocked the utilization of GABA as sole nitrogen source (Belitsky and Sonenshein, 2002). GABA degradation by *C. glutamicum* has also been reported in previous studies, but the catabolic pathway (**Fig. 2**) and its regulation was just proposed and not fully experimentally proven (Jorge et al., 2016; Ni et al., 2015; Shi

et al., 2017). GabP (Cg0568) is a GABA-specific importer and essential for the use of GABA as sole carbon or sole nitrogen source (Ni et al., 2015; Zhao et al., 2012). Deletion of GabP can effectively prevent GABA decomposition in *C. glutamicum* (Ni et al., 2015). The gene *gabT* (cg0566) encodes a γ-aminobutyrate aminotransferase that catalyzes the first step in GABA metabolism and has recently been crystallized (Hong and Kim, 2019; Ni et al., 2015). A *gabT* deletion strain was still able to degrade GABA, which suggested the presence of at least one additional enzyme able to catalyze this reaction (Ni et al., 2015; Shi et al., 2017). This enzyme was reported to be BioA (Cg2885) and showed very low γ-aminobutyrate aminotransferase activity (approximately 0.03 U mg^{-1}) only when coupled with the succinic semialdehyde dehydrogenase (SSADH), GabD (Shi et al., 2017). GABA decomposition was effectively prevented in the *gabT*/*bioA* deleted strain (Shi et al., 2017). For the succinate semialdehyde dehydrogenase, the situation is even more complex, as three genes have been annotated in the *C. glutamicum* genome to catalyze this reaction, *gabD* (cg0566), cg0067 and cg3004. But there is almost no information of these three enzymes available in previous publications.

2.4 Regulation of GABA metabolism

2.4.1 Regulators of GABA metabolism

Regulation of GABA metabolism has so far only been studied in a few organisms. GabR of *B. subtilis* ($GabR_{Bs}$) belongs to the MocR/GabR subfamily of the GntR family of transcriptional regulators and has been investigated in several publications (Edayathumangalam et al., 2013; Belitsky, 2004; Wu et al., 2017). $GabR_{Bs}$ binds to its own promoter and represses *gabR* transcription in the absence of GABA (Edayathumangalam et al., 2013). In the presence of pyridoxal-5'-phosphate (PLP) and GABA, $GabR_{Bs}$ activates the *gabTD* operon (Belitsky, 2004). The crystal structure of full-length $GabR_{Bs}$ has been published (Edayathumangalam et al., 2013). The structure of $GabR_{Bs}$ showed that PLP bound to the GabR effector-binding/oligomerization domain reacts with the effector GABA to form a stable external aldimine, which apparently triggers the transcription from the *gabT* promoter in *B. subtilis* (Wu et al., 2017). A second metabolite pair of pyridoxamine-5'-phosphate (PMP) and succinate semialdehyde could also cause transcriptional activation upon binding to GabR (Belitsky, 2004). Surprisingly, even in the close

relative *Bacillus thuringiensis*, the regulation of GABA metabolism is different. Here $GabR_{Bt}$ is a regulator containing a Per-ARNT-Sim (PAS) domain, a σ^{54} interaction domain and a C-terminal HTH-domain (Peng et al., 2014; Zhu et al., 2010). It activates its target genes *gabT* and *gabRD* in a GABA dependent-manner (Zhu et al., 2010; Peng et al., 2014). In *E. coli*, the genes *gabTDPC* are organized in an operon and activated under nitrogen limitation (Schneider et al., 2002). GabC belongs to the FadR subfamily of the GntR family of transcriptional regulators and seems to repress this operon, but the exact physiological function is unclear up to now (Schneider et al., 2002). The isozymes of GabT and GabD, PuuE and YneI, are induced by putrescine and repressed by succinate (Kurihara et al., 2010b). The transcriptional regulator PuuR seems to be involved in the regulation of *puuE* but not of *yneI* (Kurihara et al., 2010b). In summary, regulation of GABA metabolizing genes appears to be rather diverse.

2.4.2 Regulators belonging to the PucR family

According to the previous publication, *Bacillus subtilis* GabR belongs to the (MocR)/GabR (the *Rhizobium meliloti* rhizopine catabolism regulator) subfamily in the GntR (repressor of the gluconate operon) family of bacterial transcription regulators (Wu et al., 2017). However, *C. glutamicum* GabR belongs to the PucR family (Pfam PF07905). PucR is a transcriptional regulator of the purine-degradation genes (Brandenburg et al., 2002). PucR serves as a positive regulator of *pucF* (allantoate amidohydrolase), *pucH* (allantoinase), *pucI* (allantoin permease), *pucJK* (uric acid transport), *pucLM* (uricase) and *gde* (guanine deaminase) while it represses the expression *pucABCDE* (xanthine dehydrogenase) and is autoregulated (Beier et al., 2002). According to Pfam, more than 95% of the members of the PucR family in the database are derived from *Actinobacteria* or *Firmicutes* and, interestingly, about half of the sequences in the database contain a GGDEF-like domain, which suggests that they are diguanylate cyclases.

All PucR-type regulators characterized to date contain the GGDEF-like domain. These 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 a polyketide synthase for the assembly 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). PutR of *Bacillus subtilis* is an activator required for the expression of the *putBCP* operon which is essential for proline utilization (Belitsky, 2011). Purified PutR binds to the *putB* regulatory region and the absence of its putative effector Pro reduced the affinity of PutR about 10-fold (Belitsky, 2011). AdeR is another transcriptional activator which belongs to the PucR family and mediates *ald* (alanine dehydrogenase) expression in response to alanine availability in *B. subtilis* (Lin et al., 2012). The addition of L-alanine in an EMSA did not affect the affinity of AdeR for the operator DNA. L-alanine may instead induce a conformational change in AdeR, which does not alter its DNA-binding ability, to increase the affinity of RNA polymerase for the *ald* promoter and stimulate *ald* transcription (Lin et al., 2012).

2.5 Aims of this thesis

Corynebacterium glutamicum is an intensively studied model organism in white biotechnology and used industrially for the production of amino acids, organic acids and proteins. The first aim of this doctoral thesis was the investigation of the catabolic potential of *C. glutamicum*. Potential new carbon sources should be chosen based on literature analysis and commercial availability and tested whether they can be metabolized by *C. glutamicum*. GABA was identified as an interesting carbon source that at the same time could serve as nitrogen source. The second aim of this thesis was to characterize GABA utilization in detail and elucidate its regulation in *C. glutamicum*, as knowledge on this substrate is scarce in Actinobacteria.

2.6 Key results

2.6.1 Investigation of carbon metabolism in *C. glutamicum*

After literature analysis, we summarized the known substrates that can be used as carbon sources by *C. glutamicum* and alternative substrates that can be used by engineered *C. glutamicum* strains as carbon sources. To further explore the catabolic potential of *C. glutamicum*, a set of potential new carbon sources were investigated whether they can be used for growth by *C. glutamicum*. The growth experiments

revealed that *C. glutamicum* can grow on GABA or 4-cresol as sole carbon source. The subsequent studies focused on GABA utilization.

2.6.2 GABA can be used as sole carbon and nitrogen source in *C. glutamicum*

In initial growth studies, *C. glutamicum* WT was cultivated in minimal medium with glucose or GABA as carbon source and with or without $(NH_4)_2SO_4$ and urea as nitrogen source. When comparing growth with glucose or GABA in the presence of the standard nitrogen sources of CGXII medium, i.e. 151 mM $(NH_4)_2SO_4$ and 83 mM urea, growth with GABA was much slower and the cultures reached a lower final backscatter (parameter representing cell density) compared to the cultures with glucose. Surprisingly, cultures lacking $(NH_4)_2SO_4$ and urea and containing GABA as sole carbon and nitrogen source grew much better than the cultures with GABA and additional nitrogen sources and to a comparable final backscatter as the glucose-grown cultures. In summary, *C. glutamicum* grew rather well in minimal medium with GABA as sole carbon and nitrogen source and the presence of additional nitrogen sources was inhibitory.

2.6.3 GabR is a regulator that activates the GABA metabolic pathway

To analyze the influence of GABA on global gene expression, we compared the transcriptomes of cells cultivated either with GABA and urea or with glucose and urea. In the presence of GABA, 163 genes showed a ≥2-fold increased mRNA level and 71 genes a ≥2-fold decreased mRNA level. The genes showing the by far strongest upregulation in GABA-grown cells were *gabTDP* (87-, 78-, and 65-fold, respectively). Furthermore, *gabR* expression was increased 4-fold in GABA-grown cells.

To investigate whether GabR is involved in the regulation of GABA metabolism, we generated the deletion mutant *C. glutamicum* Δ*gabR* and compared its growth with the parental strain. Both strains grew identically with glucose, but the Δ*gabR* strain had lost the ability to grow with GABA. This suggests that GabR is required for induction of the *gabTDP* genes in the presence of GABA. The growth defect of the Δ*gabR* strain with GABA was abolished after transformation with the expression

plasmid pAN6-*gabR,* harboring *gabR* under control of a leaky *tac* promoter, which confirmed that the loss of GabR was responsible for the observed phenotype.

In a further experiment, we tested whether growth of the Δ*gabR* strain on GABA can be restored by plasmid-based expression of *gabTDP.* Without IPTG addition, the Δ*gabR* 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 GabR is an activator of the *gabTDP* genes and that transcription is induced by GABA. Furthermore, the results confirm that expression of *gabTDP* is strictly required for growth with GABA.

2.6.4 GabR is a octamer

To get further insights into the regulatory mechanism, we purified GabR for interaction studies with the promoter P_{gabTDP} by electrophoretic mobility shift assays (EMSAs). GabR was produced in *E. coli* BL21(DE3) and purified by means of an N-terminal decahistidine tag and Ni-NTA affinity chromatography. 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. Based on the calibration curve (K_{av} versus log M_r) derived from the standard proteins, 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 is an octamer.

2.6.5 Determination of the GabR binding site(s) in the *gabTDP* promoter region

In the first EMSA experiment, binding of GabR to a 500 bp fragment covering the entire *gabTDP* promoter region was compared with a DNA fragment of similar size of the *ldhA* gene of *C. glutamicum* as negative control. The *gabR* promoter fragment was shifted partially with 80 nM GabR (monomer) and fully with 240 nM GabR, whereas no shift was observed for the negative control fragment up to 320 nM GabR. In the following, we reduced the size of the DNA fragments step by step to localize the binding site(s) of GabR between positions -87 and -23 with respect to the TSS of *gabT*.

Regulator binding sites are often conserved among closely related species, which is especially 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* WT*, Corynebacterium deserti*, and *Corynebacterium callunae* using MEME (Bailey et al., 2009). The search revealed a 21 bp repeat with the consensus sequence ANTTMKCCAANTTGGMKAANT that was present 1-2 times in each input sequence. An alignment of the input sequences 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. Further EMSA studies suggested that each of the two binding sites can be bound separately by GabR, but binding is much stronger when both are present.

2.6.6 Investigation of reasons for the inhibitory effect of ammonium on growth with GABA

In our growth experiments, *C. glutamicum* grew much worse with GABA when ammonium sulfate and/or urea were present as additional nitrogen sources. This inhibitory effect was unexpected and triggered studies with the aim to elucidate the molecular basis of the inhibition. The promoter activity tests with the reporter plasmid pJC1-P_{gabT}-eYFP revealed that $(NH_4)_2SO_4$ and urea reduce expression of *gabTDP* with GABA as carbon source in comparison to the cultures with GABA as sole carbon and nitrogen source. In the growth experiments, the strain with pAN6-*gabTDP* grew faster than the control strain with pAN6 with GABA and $(NH_4)_2SO_4$, showing that the growth inhibition by $(NH_4)_2SO_4$ is in part due to reduced expression of *gabTDP*. However, growth of the strain with pAN6-*gabTDP* with GABA and $(NH_4)_2SO_4$ was still worse than growth of the same strain with GABA alone indicating that increased expression of *gabTDP* cannot completely overcome the growth inhibition by $(NH_4)_2SO_4$.

Besides the negative influence of $(NH_4)_2SO_4$ on *gabTDP* expression, it might also inhibit GABA aminotransferase activity. Besides GabT (Cg0566), the protein encoded by *bioA* (cg2885) has also been reported to exhibit GABA aminotransferase activity (Shi et al., 2017). We overproduced and purified both enzymes as described in a

previous study (Marienhagen et al., 2005) and tested the effect of $(NH_4)_2SO_4$ on their activity. The specific activity of GabT without ammonium was 9.69 ± 0.12 µmol min^{-1} mg^{-1}, which was only marginally higher than the specific activity in the presence of 30 mM $(NH_4)_2SO_4$ (9.07 ± 0.33 µmol min^{-1} mg^{-1}). In this assay, no activity was detected for BioA. According to these measurements, ammonium has only a slightly negative effect on GabT activity at the tested concentration.

2.7 Conclusions and outlook

We found that *C. glutamicum* can use GABA alone as sole carbon and nitrogen source and that the transcription of the GABA catabolic genes, *gabTDP*, is induced by GABA in the growth medium. Transcription of *gabTDP* is activated by the divergently encoded regulator GabR, which is strictly required for GABA metabolism. The presence of additional glucose or ammonium besides GABA inhibited *gabTDP* transcription to some extent. Using EMSAs, we showed that GabR binds specifically to the *gabTDP* promoter region, presumably to a tandem inverted repeat. GabR is the first characterized member of PucR family without a GGDEF-like domin. In this thesis, the reporter plasmid pJC1-P_{gabT}-eYFP was constructed. In the future, this could give a hint to construct a GABA biosensor and it could help to increase the production of GABA. A GABA biosensor could be constructed containing the *gabR* gene, the promoter of *gabTDP* and the eYFP gene. Due to the fluorescence produced in precence of GABA, the best GABA producing strain can be found by high throughput screening after evolution or mutation. Till now, no such biosensor was used for improving the GABA production.

Transcriptional activators for catabolic pathways often require the corresponding substrate or one of its degradation products as co-activator. But GABA has no influence on the DNA affinity of purified GabR. There are two hypotheses why GABA is not needed for the DNA-binding of GabR, but is essential for activation of *gabTDP* transcription: a) GABA may cause conformational changes in GabR, which is necessary to activate *gabTDP* transcription; b) GABA binding may change GabR−DNA interaction, which is essential for *gabTDP* transcription. Further studies are required to identify the co-activator of GabR and the mechanism of transcriptional activation.

Furthermore, the GabR binding sites in the *gabTDP* promoter region were identified. But both binding sites block the *gabR* transcription indicating that GabR is a repressor of *gabR*. In the microarray experiments of *C. glutamicum* comparing cells cultivated in minimal medium with GABA and urea to minimal medium with glucose and urea, *gabR* was up-regulated with a ratio of 4.3, even though the increase was much lower than for *gabT* (86.9). So GabR apparently not always functions as a repressor of *gabR* transcription. So how GabR influences *gabR* transcription is still not completely clear and requiresne further studies.

2.8 References

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3 Search for new carbon sources of *Corynebacterium glutamicum*

3.1 Substrates known to be used as carbon sources by *C. glutamicum*

For industrial amino acid fermentations, molasses or starch hydrolysates, which contain high concentrations of sugars, are utilized as feedstocks (Hermann, 2003). But variations of complex raw materials negatively influence process performances, so defined carbon sources such as glucose and sucrose are often used in amino acid productions (Eggeling and Bott, 2005). Feedstock costs became a large extent of the overall fermentation costs. To broaden the spectrum of possible substrates, several compounds have been tested whether they can be used as carbon source by *C. glutamicum*. Till now, *C. glutamicum* is reported to be able to use a variety of other carbon sources, which are listed in Table 1. The metabolism of most of the substrates has been studied extensively or at least to some extent (Fig. 3). For some other substrates, the genes involved in their metabolism have been predicted, but need to be experimentally verified. For example, in order to determine the effect of *myo*-inositol (MI) utilization on global gene expression, whole-genome DNA microarrays of *C. glutamicum* were performed (Krings et al., 2006). Twenty-one genes showed an up to 18-fold increase of the mRNA level, indicative of a possible function within *myo*-inositol catabolism. By comparing the amino acid sequences encoded by the genes involved in MI catabolism between *C. glutamicum* and *B. subtilis*, the amino acid sequences in *C. glutamicum* were found to be similar to the corresponding genes in *B. subtilis*. However, most of the enzymes have not been studied biochemically.

Table 1 Substrates that can be metabolized by *C. glutamicum* wild type or as sole carbon source.

Carbon substrates	Metabolic pathway	References
	Carbohydrates	
Glucose	Known	(Kiefer et al., 2004)
Maltose	Known	(Seibold et al., 2009)
Ribose	Known	(Nentwich et al., 2009)
Mannose	Known	(Sasaki et al., 2011)

Fructose	Known	(Kiefer et al., 2004)
Sucrose	Known	(Wittmann et al., 2004)
Gluconate	Known	(Lee et al., 1998)
β-Glucosides	Known	(Tanaka et al., 2009)
Organic acids		
Pyruvate	Known	(Cocaign-Bousquet and Lindley, 1995)
Lactate	Known	(Cocaign-Bousquet and Lindley, 1995)
Acetate	Known	(Gerstmeir et al., 2003)
Propionate	Known	(Claes et al., 2002)
Citrate	Known	(Netzer et al., 2004a)
Glutamate	Known	(Kronemeyer et al., 1995)
L-Malate	Spontaneous mutant	(Youn et al., 2009)
Fumarate	Spontaneous mutant	(Youn et al., 2009)
Succinate	Spontaneous mutant	(Youn et al., 2009)
Glutamine	Known	(Rehm et al., 2010)
Aromatic compounds		
Benzoate	Known	(Shen et al., 2005a)
Phenol	Known	(Liu and Liu, 2004)
3-Hydrobenzoate	Known	(Shen et al., 2005b)
Gentisate	Known	(Shen et al., 2005b)
Quinate	Known	(Shen et al., 2012)
Resorcinol	Known	(Huang et al., 2006)
Caffeate	Known	(Qi et al., 2007)
Vanillate	Known	(Merkens et al., 2005)
Cinnamate	Known	(Brinkrolf et al., 2006)
Naphthalene	Genes need to be verified	(Shen et al., 2012)
Protocatechuate	Known	(Shen and Liu, 2005)
Other compounds		
Arabitol	Known	(Laslo, 2013)
Glucosamine	Spontaneous mutant	(Uhde et al., 2013)
Uridine	Known	(Brinkrolf et al., 2008)
Mannitol	Known	(Peng et al., 2011)
Ethanol	Known	(Arndt et al., 2008; Witthoff et al., 2013)

myo-Inositol	Genes need to be verified	(Krings et al., 2006)
Benzyl alcohol	Genes need to be verified	(Shen et al., 2012)

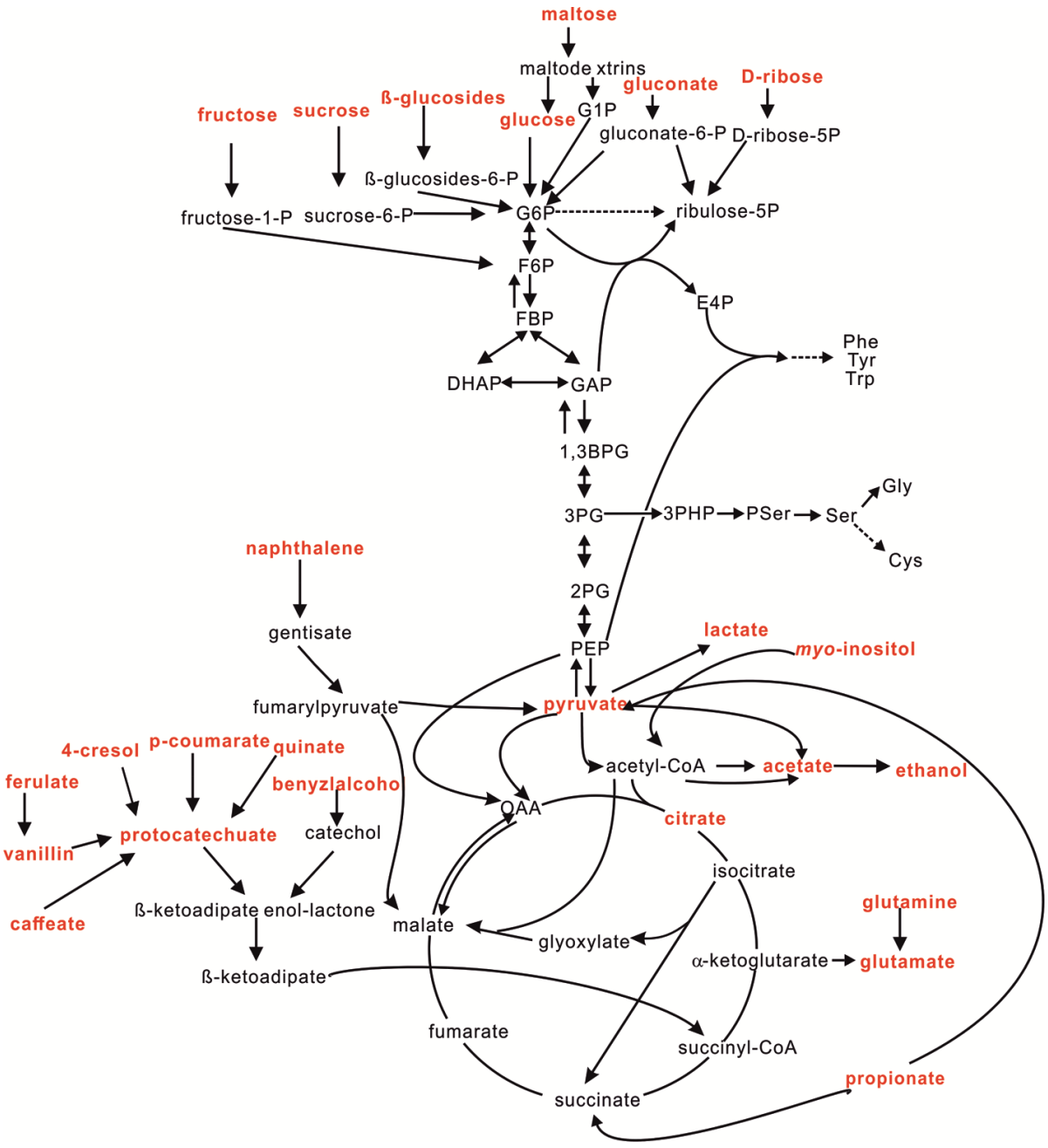


Fig. 3 Catabolic pathways of substrates which can be used as sole carbon sources by *C. glutamicum*. The substrates with red color mean that they can be used as sole carbon sources and the pathways have been studied.

3.2 Alternative carbon sources used by engineered *C. glutamicum* strains

Several cheaply available and therefore interesting carbon sources such as glycerol or xylose cannot be utilized by *C. glutamicum* WT. Therefore, recombinant strains were constructed enabling the use of such carbon sources (Table 2).

Table 2 Substrates that can be used by engineered *C. glutamicum* as sole carbon source.

Carbon substrates	Specific growth rate	References
Galactose	0.19 h^{-1}	(Barrett et al., 2004)
Glycerol	0.29 h^{-1}	(Rittmann et al., 2008)
Lactose	0.13 h^{-1}	(Barrett et al., 2004)
Arabinose	0.27 h^{-1}	(Kawaguchi et al., 2008)
Xylose	0.07 h^{-1}/0.20 h^{-1}	(Radek et al., 2014; Kawaguchi et al., 2006)
Cellobiose	0.23 h^{-1}	(Kotrba et al., 2003)

The metabolic pathways of these substrates are shown in Fig. 4 (taken from Zahoor et al., 2012). For example, overproduction of aldose-1-epimerase, galactokinase, UDP-glucose-1-P-uridylyltransferase, and UDP-galactose-4-epimerase of *Lactococcus lactis* allowed *C. glutamicum* to use galactose as sole carbon source (Barrett et al., 2004). *C. glutamicum* already possesses a gene encoding a xylulokinase, thus the expression of the xylose isomerase gene (*xylA*) of *E. coli* was sufficient to enable growth with xylose as sole carbon source (Kawaguchi et al., 2006). Heterologous expression of the *E. coli* aerobic glycerol utilization genes encoding for a glycerol facilitator, glycerol kinase, and glycerol-3-phosphate dehydrogenase enabled *C. glutamicum* grow with glycerol as sole carbon source (Rittmann et al., 2008).

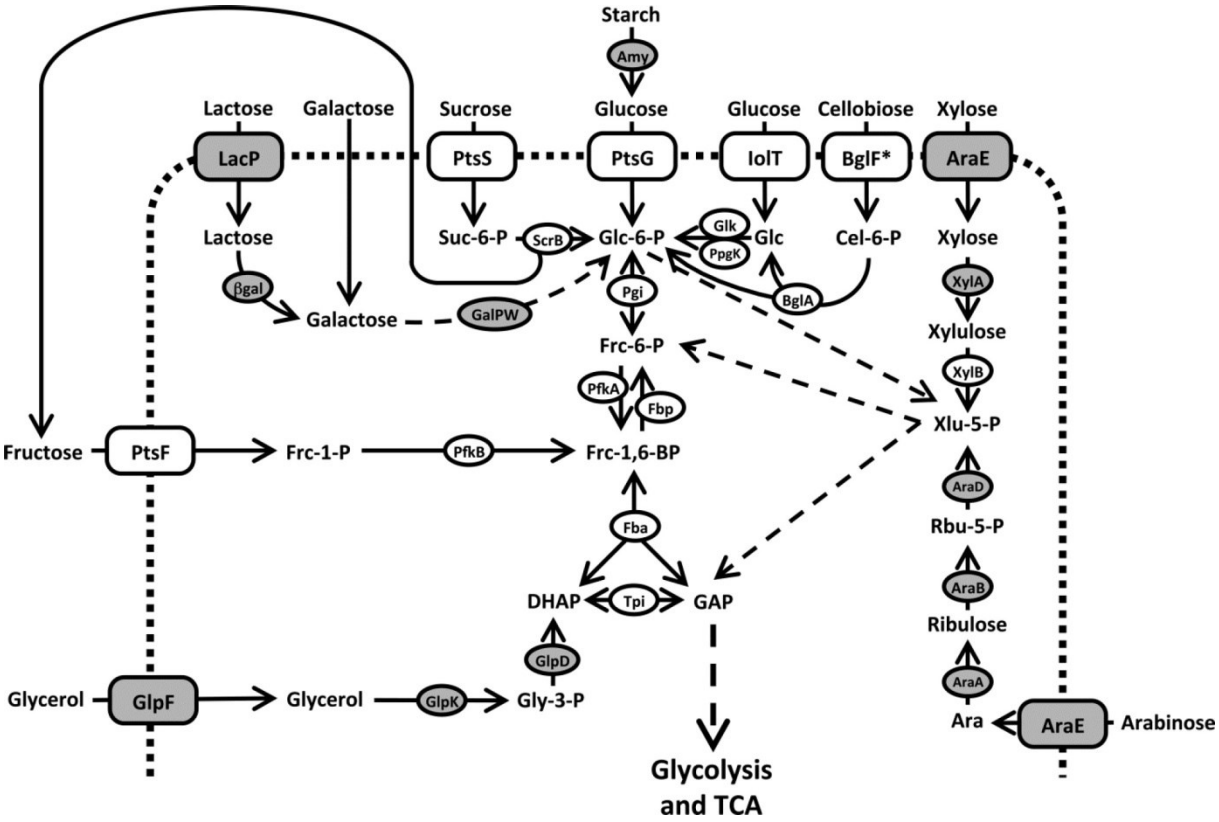


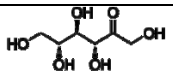
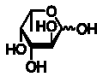
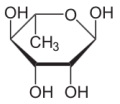
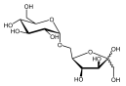
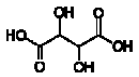
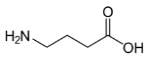
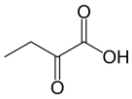
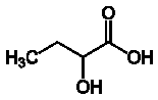
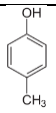
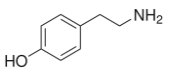
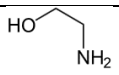
Fig. 4 Carbon source utilization in *C. glutamicum* (taken from Zahoor et al., 2012). Open boxes: *C. glutamicum* enzymes, gray shaded boxes: enzymes from other organisms produced in *C. glutamicum* by heterologous expression. Abbreviations: Ara, arabinose; AraA, arabinose isomerase; AraB, ribulokinase; AraD, ribulose 5-phosphate 4-epimerase; AraE, arabinose transporter; βgal, β-galactosidase; BglA, phospho-β-glucosidase; BglF*, cellobiose specific PTS; Cel-6-P, cellobiose-6-phosphate; DHAP, dihydroxyacetonephosphate; Fba, fructose-bisphosphate aldolase; Fbp, fructose-bisphosphatase; Frc-1,6-BP, fructose-1,6-bisphosphate; Frc-6-P, fructose-6-phosphate; Frc-1-P, fructose-1-phosphate; GalPW, galactose pathway; GAP, glyceraldehyde-3-phosphate; Glc-6-P, glucose-6-phosphate; Glc, glucose; Glk, ATP dependent glucokinase; GlpD, glycerol-3-phosphate dehydrogenase; GlpF, glycerol facilitator; GlpK, glycerol kinase; Gly-3-P, glycerol-3-phosphate; IolT, inositol transporter, also accepting glucose; LacP, lactose permease; PfkA, 6-phosphofructokinase; PfkB, 1-phosphofructokinase; PpgK, polyphosphate dependent glucokinase; PtsF, fructose specific PTS; PtsG, glucose specific PTS; PtsS, sucrose specific PTS; Rbu-5-P, ribulose-5-phosphate; ScrB, sucrose-6-phosphate hydrolase; Suc-6-P, sucrose-6-phosphate; Tpi, triosephosphate isomerase; Xlu-5-P, xylulose-5-phosphate; XylA, xylose isomerase; XylB, xylulokinase.

3.3 Potential new carbon sources for *C. glutamicum*

For the further definition of the catabolic potential of *C. glutamicum*, potential new carbon sources were chosen and tested whether they can be metabolized by *C.*

glutamicum. Substrates that have not been reported to be usable as sole carbon sources by *C. glutamicum* or with unknown metabolic pathways were chosen for investigation. The physical properties of these substrates are listed in Table 3.

Table 3 Substrates that were chosen for tests whether they can be metabolized by *C. glutamicum*.

Substrate	CAS No.	Chemical formula	Structure	Molar mass
Carbohydrates				
Psicose	23140-52-5	$C_6H_{12}O_6$		180.16
Fucose	2438-80-4	$C_6H_{12}O_5$		164.16
Rhamnose	3615-41-6	$C_6H_{12}O_5$		164.16
Palatinose	13718-94-0	$C_{12}H_{22}O_{11}$		342.30
Organic acids				
L-Tartaric acid	87-69-4	$C_4H_6O_6$		150.087
GABA	56-12-2	$C_4H_9NO_2$		103.120
2-Keto-butyric acid	600-18-0	$C_4H_6O_3$		102.089
2-Hydroxy butyric acid	565-70-8	$C_4H_8O_3$		126.09
Aromatic compounds				
4-Cresol	106-44-5	C_7H_8O		108.14
Tyramine	51-67-2	$C_8H_{11}NO$		137.179
Alcohols				
Aminoethanol	141-43-5	C2H7NO		61.08

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 and Company, New Jersey, USA) with 90 g

L^{-1} sorbitol were 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 KH_2PO_4/Na_2HPO_4 pH 7.0). Growth experiments were performed in a Biolector microcultivation system (m2p-labs, Baesweiler, Germany) using 48-well FlowerPlates (m2p-labs) containing 750 µL CGXII minimal medium supplemented with 30 mg L^{-1} 3,4-dihydroxybenzoate as iron chelator and with the different carbon sources. In the growth experiments, glucose was used as positive control, and growth without any carbon source was used as negative control. The initial OD_{600} values and the concentrations of the carbon sources are shown in Table 4. The final OD_{600} values were measured after 4 days of growth. The results are shown in Table 4. The standard deviations were calculated based on three biological replicates. As shown in Table 4, most of the selected substrates could not be used as sole carbon source by *C. glutamicum*. Good growth was only observed with GABA. With 10 mM 4-cresol, a 60% increase of the initial OD_{600} was observed after 4 days, suggesting minor growth. With 36 mM 4-cresol, the initial OD_{600} decreased after 4 days, indicating an inhibitory effect of the compound.

Table 4 The final OD_{600} values of *C. glutamicum* after 4 days growth in the Biolector system in 48-well FlowerPlates. BHIS medium was used for precultivation of *C. glutamicum*. The cells of these precultures were harvested by centrifugation and washed with phosphate buffer. Growth experiments were performed at 30°C and 1,200 rpm in a Biolector system in 48-well FlowerPlates containing 750 µL CGXII minimal medium supplemented with different carbon sources. The underlined substrates led to growth in the growth experiment.

Substrate	Concentration	Start OD_{600}	Final OD_{600}
Water		0.40 ± 0.03	0.08 ± 0.02
Glucose	84 mM	0.68 ± 0.02	27.63 ± 1.52
Rhamnose	10 mM	0.40 ± 0.01	0.09 ± 0.02
Rhamnose	42 mM	0.40 ± 0.02	0.08 ± 0.01
Palatinose	10 mM	0.40 ± 0.02	0.05 ± 0.004
Palatinose	21 mM	0.41 ± 0.04	0.03 ± 0.01
Fucose	18 mM	0.49 ± 0.01	0.14 ± 0.01
Psicose	16 mM	0.50 ± 0.01	0.16 ± 0.01
GABA	10 mM	0.39 ± 0.02	0.96 ± 0.20
GABA	63 mM	0.42 ± 0.03	9.95 ± 0.92

Tartaric acid	42 mM	0.50 ± 0.03	0.15 ± 0.02
Tartaric acid	63 mM	0.41 ± 0.02	0 ± 0
Keto-butyrate	10 mM	0.45 ± 0.08	0.05 ± 0.02
Keto-butyrate	63 mM	0.54 ± 0.04	0.03 ± 0.01
Hydroxybutyrate	10 mM	0.52 ± 0.03	0.33 ± 0.05
Hydroxybutyrate	63 mM	0.56 ± 0.05	0.16 ± 0.01
4-Cresol	10 mM	0.51 ± 0.002	0.81 ± 0.05
4-Cresol	36 mM	0.46 ± 0.04	0.04 ± 0.004
Tyramine	10 mM	0.47 ± 0.03	0.11 ± 0.002
Tyramine	31 mM	0.41 ± 0.01	0.04 ± 0.01
Aminoethanol	10 mM	0.39 ± 0.06	0.09 ± 0.01
Aminoethanol	125 mM	0.47 ± 0.03	0.11 ± 0.01

Besides these substrates mentioned above, there are some other substrates that cannot be metabolized as sole carbon source by *C. glutamicum*. For example, *C. glutamicum* can utilize uridine as a sole source of carbon for growth, but other nucleosides such as adenosine, inosine, guanosine, cytidine, thymidine, deoxyuridine, deoxyadenosine, and deoxyguanosine cannot serve as carbon source for growth of the bacterium (Grishchenkov et al., 1978). Creatinine cannot be used as carbon source, but as nitrogen source (Bendt et al., 2004).

Table 5 Substrates that cannot be metabolized as sole carbon source by *C. glutamicum.*

Substrate	References
Thymidine	(Grishchenkov et al., 1978)
Inosine	(Grishchenkov et al., 1978)
Guanosine	(Grishchenkov et al., 1978)
Deoxyguanosine	(Grishchenkov et al., 1978)
Cytidine	(Grishchenkov et al., 1978)
Deoxyuridine	(Grishchenkov et al., 1978)
Deoxyadenosine	(Grishchenkov et al., 1978)
Adenosine,	(Grishchenkov et al., 1978)
Glycolate	(Zahoor et al., 2014)
Creatinine	(Bendt et al., 2004)
Putrescine	(Schneider and Wendisch, 2010)
L-Phenylalanine	(De Boer and Dijkhuizen, 1990)

L-Tyrosine	(Kallscheuer and Marienhagen, 2018)
L-Tryptophan	(Kallscheuer and Marienhagen, 2018)
L-Serine	(Netzer et al., 2004b)
Methanol	(Witthoff et al., 2013)

The genes involved in 4-cresol catabolism were partially identified in *C. glutamicum* (taken from Du et al., 2016). The pathway initiates with the phosphorylation of the hydroxyl group of 4-cresol, which is catalyzed by a novel 4-methylbenzyl phosphate synthase, CreHI. Next, a unique class I P450 system, CreJEF, specifically recognizes phosphorylated intermediates and successively oxidizes the aromatic methyl group into carboxylic acid functionality via alcohol and aldehyde intermediates. Moreover, CreD (phosphohydrolase), CreC (alcohol dehydrogenase), and CreG (aldehyde dehydrogenase) were found to be required for efficient oxidative transformations in this pathway. Subsequently, 4-hydroxybenzoate undergoes variant modifications to form protocatechuic acid (i.e. 3,4-dihydroxybenzoate) or benzoyl-CoA, both of which can be diverted into the central metabolism (i.e. TCA) (Fig. 5) (Du et al., 2016). Although the *cre* pathway was genetically identified in part, the catalytic functions of some enzymes were not determined. For example, *creR* is a putative regulatory gene and the function is still not known. And CreG and CreC appear to be unnecessary in the major route from 4-cresol to 4-hydroxybenzoate. But a previous publication showed that the *creG* knock-out strain was unable to grow using 4-cresol as the sole carbon source (Li et al., 2014). So the function of CreG in 4-cresol catabolism is still unknown.

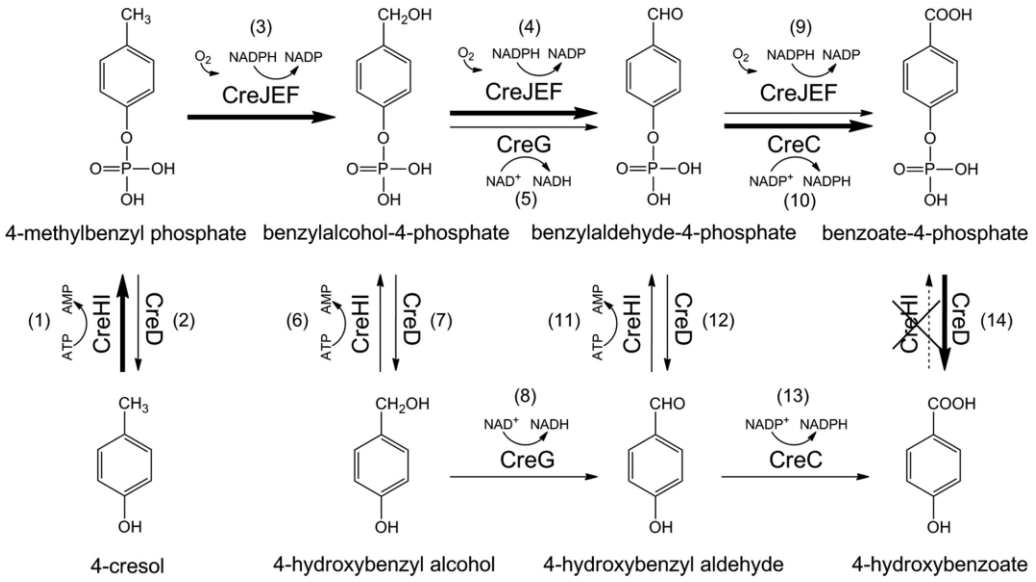


Fig. 5 The catabolic pathway for 4-cresol from *C. glutamicum* (taken from Du et al., 2016). Bold arrows indicate the major phosphorylated pathway comprised of enzymatic steps with greater k_{cat}/K_m values. Each number in brackets denotes an individual enzymatic step.

From the tested new substrates, GABA turned out to be a good carbon source for *C. glutamicum*. GABA degradation by *C. glutamicum* has partially been analyzed in previous studies, but many features, such as the underlying regulatory mechanisms were just proposed and not fully experimentally proven (Jorge et al., 2016; Ni et al., 2015; Shi et al., 2017). Therefore, further studies are required to investigate GABA metabolism and elucidate its regulation in *C. glutamicum*.

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4 γ-Aminobutyrate (GABA) utilization and its regulation by GabR in *Corynebacterium glutamicum*

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MBo, MBa, and LZ designed the study. LZ performed all experiments, wrote the initial version of the manuscript, and prepared the figures. TP processed and uploaded the microarray data to GEO. MBa revised the initial version of the manuscript. MBo revised the manuscript and prepared the final published version.

γ-Aminobutyrate (GABA) utilization and its regulation by GabR in *Corynebacterium glutamicum*

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For the microarray data, the following secure token has been created to allow review of GEO record GSE138829 while it remains in private status: ahupaoiipdeprol

https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138829

ABSTRACT

γ-Aminobutyric acid (GABA) is a non-proteinogenic amino acid formed by decarboxylation of L-glutamate and is widespread in nature from microorganisms to plants and animals. In this study, we analyzed GABA metabolism and its regulation in the Gram-positive soil bacterium *Corynebacterium glutamicum*. This actinobacterial species showed good growth with GABA as sole carbon and nitrogen source. Ammonia and to a lesser extent urea inhibited growth on GABA, whereas glutamine stimulated it. Possible reasons for these effects were analyzed. 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 strictly dependent on GABA and the 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 could be restored by plasmid-based expression of either *gabR* or of *gabTDP*. Glucose caused reduced expression of *gabTDP* presumably via the cAMP-dependent global regulator GlxR. GabR belongs to the PucR family of transcriptional regulators. Purified GabR eluted as an octamer with an apparent mass of 420 kDa in size-exclusion chromatography and bound specifically to two binding sites in the *gabR-gabT* intergenic region extending from -36 to -56 and from -67 to -87 upstream of the *gabT* transcriptional start site. The results uncover new features of actinobacterial GABA utilization.

IMPORTANCE

The non-proteinogenic amino acid γ-aminobutyric acid (GABA) is widespread in nature and a valuable carbon and nitrogen source for many microorganisms. In this study, we provide a detailed analysis of GABA utilization by *Corynebacterium glutamicum*, an intensively studied representative of the *Actinobacteria*. We elucidated the regulation of the *gabTDP* genes required for GABA metabolism by the transcriptional regulator GabR. GabR belongs to the PucR family and is the first characterized member of this family without a GGDEF-like domain. We also disclose new aspects relevant for the utilization of GABA as sole carbon and nitrogen source. Many of the results obtained here will probably also be relevant for other *Actinobacteria* capable of utilizing GABA as carbon and nitrogen source.

INTRODUCTION

Corynebacterium glutamicum is a Gram-positive soil bacterium widely used in the industrial production of amino acids such as L-glutamate (1, 2) and L-lysine (3-5). It is able to use a variety of carbohydrates (6, 7), organic acids (8-13), or alcohols (14) as single sources of carbon and energy for growth. The elucidation of metabolism and its regulation in *C. glutamicum* has made essential contributions to the development of production strains and production processes, but is still far from complete.

γ-Aminobutyric acid (GABA) is a non-proteinogenic amino acid, which is widespread in nature from microorganisms to plants and animals. Recent studies revealed that GABA plays a role in the human gut microbiome (15). In the brain of mammals, GABA is an important neurotransmitter (16, 17). In plants, GABA plays a role in stress responses and at the interface of carbon and nitrogen metabolism (18). GABA has also gained much attention as a building block for the synthesis of 2-pyrrolidone and the biodegradable polyamide nylon 4 (19). In recent years, microbial synthesis of GABA has been studied (20, 21). The major pathway of GABA biosynthesis is the irreversible α-decarboxylation of L-glutamate, which is catalyzed by glutamate decarboxylase and is typically a part of the acid stress response (22). As *C. glutamicum* is an excellent glutamate producer, it is also a promising candidate for the large scale production of GABA, although it does not possess an endogenous glutamate decarboxylase. Besides GABA synthesis by glutamate decarboxylation, an alternative synthetic pathway via ornithine, putrescine, and γ-aminobutyraldehyde was recently established (23).

GABA degradation in bacteria occurs via transamination to succinate semialdehyde with concomitant conversion of 2-oxoglutarate to glutamate followed by oxidation of succinate semialdehyde to succinate, an intermediate of central metabolism (24-26). Several aspects related to GABA degradation by *C. glutamicum* have been analyzed in previous studies (23, 27, 28) (Fig. 1). GabP (Cg0568) is a GABA-specific importer and essential for the use of GABA as carbon or as nitrogen source (27, 29). The gene *gabT* (cg0566) encodes γ-aminobutyrate aminotransferase, which catalyzes the conversion of GABA and 2-oxoglutarate to succinate semialdehyde and L-glutamate. GabT has recently been crystallized (30). A *gabT* deletion strain was still able to degrade GABA, which suggested the

presence of at least one additional enzyme able to catalyze this reaction (27). The protein encoded by *bioA* (cg2885) was recently reported to possess low GABA transaminase activity (28). For succinate semialdehyde dehydrogenase, the situation is even more complex, as three genes encoding this enzyme have been annotated in the *C. glutamicum* genome, *gabD* (cg0566), cg0067, and cg3004 (Fig. 1).

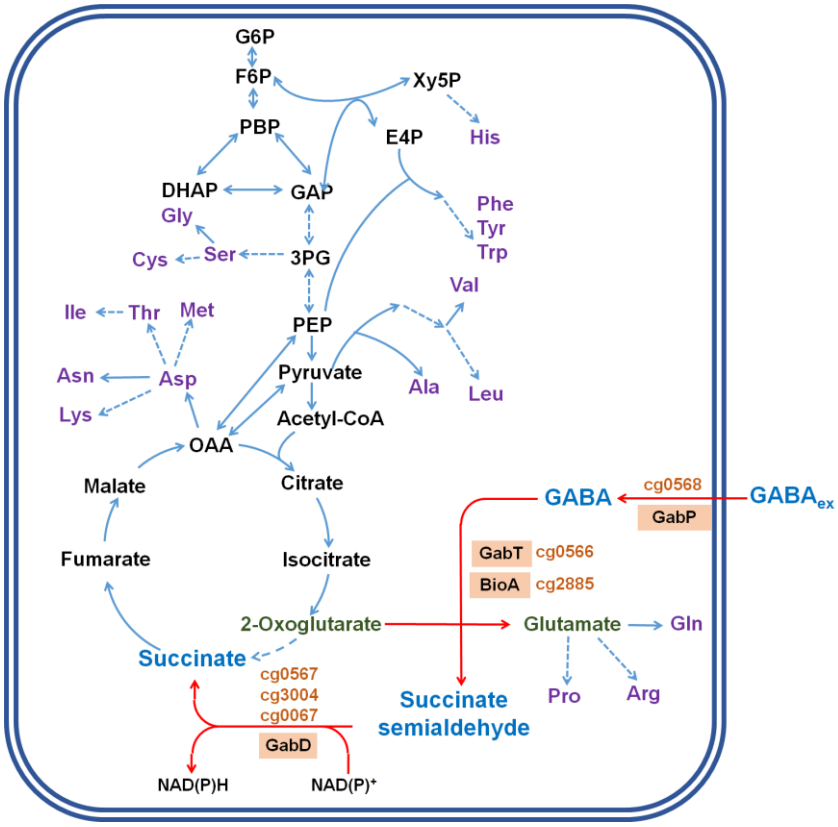

Fig. 1. Model of GABA metabolism in *C. glutamicum* and integration into central metabolism. The metabolites and reactions specifically involved in GABA metabolism are indicated by blue letters and red arrows, respectively. The proteins involved in GABA transport and degradation and the locus tags encoding proteins with the corresponding activity are indicated in orange. GabP is a sodium ion-coupled secondary transporter for GABA uptake, GabT is a γ-aminobutyrate aminotransferase encoded by cg0566. BioA (cg2885) was reported to also possess some GABA aminotransferase activity. GabD (cg0567) is a succinate semialdehyde dehydrogenase. Two alternative proteins with this enzymatic activity are encoded by cg0067 and cg3004. $GABA_{ex}$ represents extracellular GABA. Metabolites involved in central metabolism are shown in black letters, amino acids in purple letters. Solid blue arrows indicate conversions catalyzed by a single enzyme, dashed lines represents conversions composed of several enzymatic steps. Abbreviations: DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; GAP, glyceraldehyde 3-

phosphate; G6P, glucose 6-phosphate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; Xy5P, xylulose 5-phosphate.

Despite the fact that many bacteria possess genes involved in GABA metabolism, regulation of these genes has so far only been studied in a few species. 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 (31, 32). $GabR_{Bs}$ 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 pyridoxal 5'-phosphate (PLP) (33). Surprisingly, in the close relative *Bacillus thuringiensis* the regulation of GABA metabolism is different. Here the regulator $GabR_{Bt}$ is composed of a Per-ARNT-Sim (PAS) domain, a σ^{54} interaction domain, and a C-terminal HTH domain and activates its target genes *gabT* and *gabRD* in a GABA-dependent manner (34, 35). In *E. coli*, the genes *gabTDPC* are organized in an operon and activated under nitrogen limitation (36). $GabC_{Ec}$ belongs to the FadR subfamily of the GntR family of transcriptional regulators and seems to repress this operon, but the exact physiological function is unclear up to now . The isozymes of GabT and GabD, PuuE and YneI, are induced by putrescine and repressed by succinate (37). The transcriptional regulator PuuR seems to be involved in the regulation of *puuE* but not of *yneI* (37). In summary, regulation of genes involved in GABA metabolism appears to be rather diverse in bacteria.

In this study, our aim was to investigate GABA metabolism in *C. glutamicum* in more detail and elucidate its regulation. We show that *C. glutamicum* is able to grow in a minimal medium containing GABA as sole carbon and nitrogen source, that ammonia and urea inhibit growth with GABA while glutamine stimulates it, and that GabR (GabR without subscript refers to the protein encoded by *gabR* of *C. glutamicum*), which belongs to the PucR family of transcriptional regulators, activates the *gabTDP* gene cluster in the presence of GABA. GabR is shown to be an unusual regulator that forms octamers and binds to two 21 bp inverted repeats in the *gabTDP* promoter.

RESULTS

Growth of *C. glutamicum* with GABA and other nitrogen sources. In initial growth studies, *C. glutamicum* WT was cultivated in CGXII minimal medium containing either glucose (41.7 mM corresponding to 250 mM carbon) or GABA (62.5 mM corresponding to 250 mM carbon). In the GABA-containing medium, growth was much slower than in the glucose-containing medium and the cultures reached a lower final backscatter (parameter representing cell density) (Fig. 2a). Surprisingly, growth with GABA was much better and cultures reached similar final backscatter values as on glucose, when the standard nitrogen sources of CGXII medium, ammonium sulfate (151 mM) and urea (83 mM), were excluded from the medium and GABA served as sole carbon and nitrogen source (Fig. 2a). Measurement of the final optical density at 600 nm (OD_{600}) in a spectrophotometer after 50 h revealed no significant difference between the cultures with glucose, $(NH_4)_2SO_4$ and urea on the one hand (OD_{600} = 12.17 ± 0.54) and the cultures with GABA without additional N-sources on the other hand (OD_{600} = 11.81 ± 0.96). Further growth experiments revealed that $(NH_4)_2SO_4$ alone was sufficient to exert the same inhibitory effect on growth with GABA as in combination with urea (Fig. 2b). Urea alone was also inhibitory, but to a lesser extent. Urea is converted by urease to ammonium and carbon dioxide (38), but the ammonia levels formed in this way are probably much lower than in media containing $(NH_4)_2SO_4$, which could explain the weaker inhibitory effect. When similar experiments were performed with glucose as carbon source, no inhibitory effect of $(NH_4)_2SO_4$ or urea was observed (Fig. 2c). In summary, *C. glutamicum* grew rather well in minimal medium with GABA as sole carbon and nitrogen source and the additional presence of $(NH_4)_2SO_4$ was inhibitory.

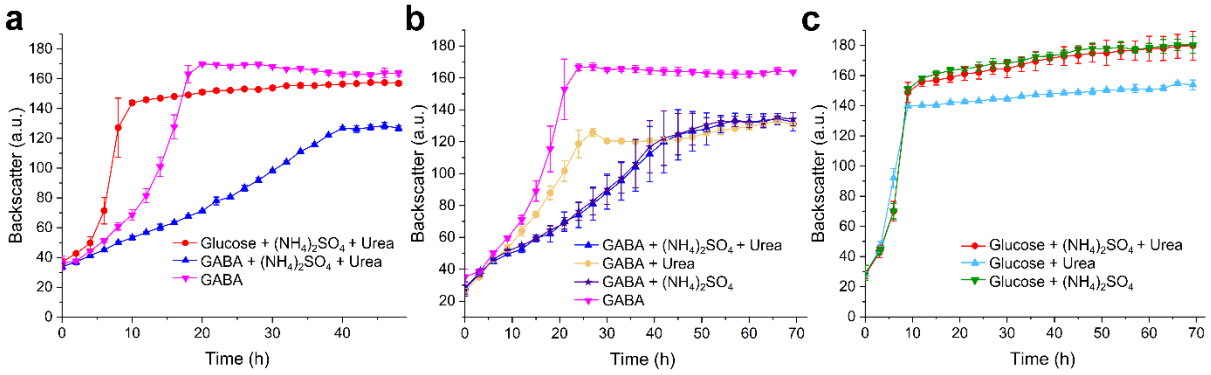

Fig. 2. Growth of *C. glutamicum* WT with different carbon and nitrogen sources. Cells were precultured in BHI medium for 12 hours and subsequently washed with PBS before inoculation of the main cultures to a starting OD_{600} of 1. The main cultivation was performed in a Biolector microcultivation system with 48-well Flower plates each containing 750 µL CGXII minimal medium supplemented with 41.7 mM glucose or 62.5 mM GABA. 151 mM $(NH_4)_2SO_4$ or 83 mM urea were included as indicated. Mean values and standard deviations of three biological replicates are shown.

L-Glutamine synthesis during growth with GABA. In *C. glutamicum*, L-glutamine synthesis is strictly dependent on glutamine synthetase (GlnA) encoded by the *glnA* gene, as cells lacking *glnA* are glutamine-auxotrophic (39). A second putative glutamine synthetase (GlnA2) is encoded in the genome upstream of *glnE*, but *glnA2* cannot complement the glutamine auxotrophy of the Δ*glnA* mutant and the function of the GlnA2 protein is not known yet (40, 41). Glutamine synthetase requires L-glutamate, ATP and NH_4^+ as substrates. When cells grow with GABA as sole carbon and nitrogen source, NH_4^+ is apparently not formed by GABA metabolism and the question arises how L-glutamine is synthesized under these conditions.

To address this question, we analyzed growth with GABA of *C. glutamicum* mutants lacking GlnA (Δ*glnA*), or glutamate dehydrogenase (Δ*gdh*), or glutamate synthase (Δ*gltBD*), or GlnA, GlnA2, and Gdh (Δ*glnA*□*glnA2*□*gdh*). Mutants with deletions of *gdh* or *gltBD* were still able to grow with GABA as sole carbon and nitrogen source and growth was inhibited by ammonium sulfate similar to the situation in the WT (data not shown). This result was expected since GABA transaminase forms glutamate and therefore neither glutamate dehydrogenase nor glutamate synthase are required for this task. The mutant lacking *glnA* showed no

growth at all with GABA as sole carbon and energy source (data not shown), independent of the presence or absence of ammonium sulfate, and the same result was observed for the triple mutant (Δ*glnA*□*glnA2*□*gdh*) (Fig. 3). These data confirmed that GlnA is essential for glutamine synthesis in *C. glutamicum* also during growth with GABA as sole nitrogen source.

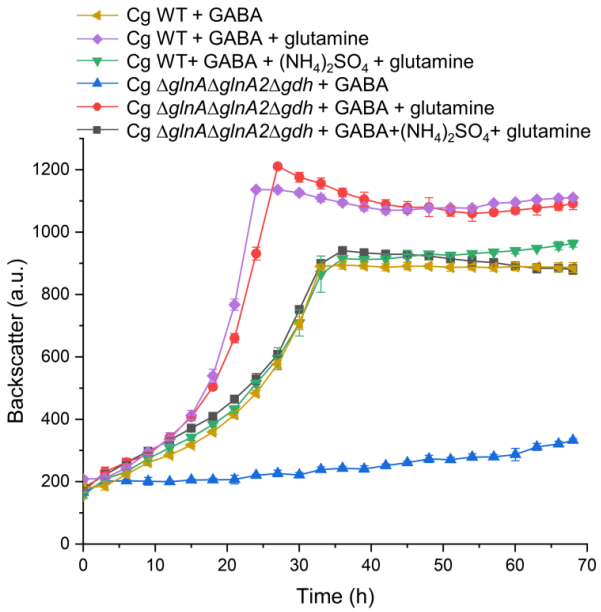

Fig. 3. Influence of L-glutamine supplementation on growth of the indicated *C. glutamicum* strains in CGXII medium with GABA. Cells were precultured in BHI medium for 12 hours and subsequently washed with PBS before inoculation of the main cultures to a starting OD_{600} of 0.5. The main cultivation was performed in a Biolector microcultivation system with 48-well Flower plates each containing 750 µL CGXII minimal medium supplemented with 62.5 mM GABA. 20 mM L-glutamine and 151 mM $(NH_4)_2SO_4$ were included as indicated. Mean values and standard deviations of three biological replicates are shown.

To test for a bottleneck in glutamine synthesis, glutamine was added to the medium containing GABA as sole carbon and nitrogen source. Glutamine significantly improved the growth rate and the final backscatter of the WT compared to growth with GABA alone. Furthermore, glutamine supplementation enabled WT-like growth and GABA utilization by the triple mutant Δ*glnA*□*glnA2*□*gdh*, again confirming the essentiality of GlnA for glutamine synthesis (Fig. 3). When $(NH_4)_2SO_4$ was added to medium with GABA and glutamine, the WT and the triple mutant grew

like the WT with GABA alone (Fig. 3). These results suggest that glutamine synthesis is limiting growth with GABA as sole carbon and nitrogen source, either due to a limiting availability of ammonium as substrate for glutamine synthetase and/or due to a limiting amount of catalytically active glutamine synthetase. This enzyme can be inactivated by adenylylation via GlnE (42).

Genomic location and conservation of the *gabR-gabTDP* gene cluster. Previous studies showed that the *gabTDP* genes are involved in GABA utilization by *C. glutamicum* (23, 27, 29). A gene encoding a transcriptional regulator is located upstream and divergent to *gabTDP*, which might regulate these genes. To check whether this genomic organization is conserved in related species, which is a further 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 Fig. 4, similar gene clusters were found in a number of *Corynebacterium* species and, in a different organization, also in some *Rhodococcus* and *Mycobacterium* species. Interestingly, a second copy of *gabR* and *gabT* is present immediately adjacent to the *gabR-gabTDP* gene cluster in *Corynebacterium aurimucosum*. GabR belongs to the PucR protein family (Pfam PF07905) and has a C-terminal DNA-binding helix-turn-helix motif. The best characterized representative of this family is PucR of *Bacillus subtilis*, which is involved in the regulation of purine catabolism (43, 44).

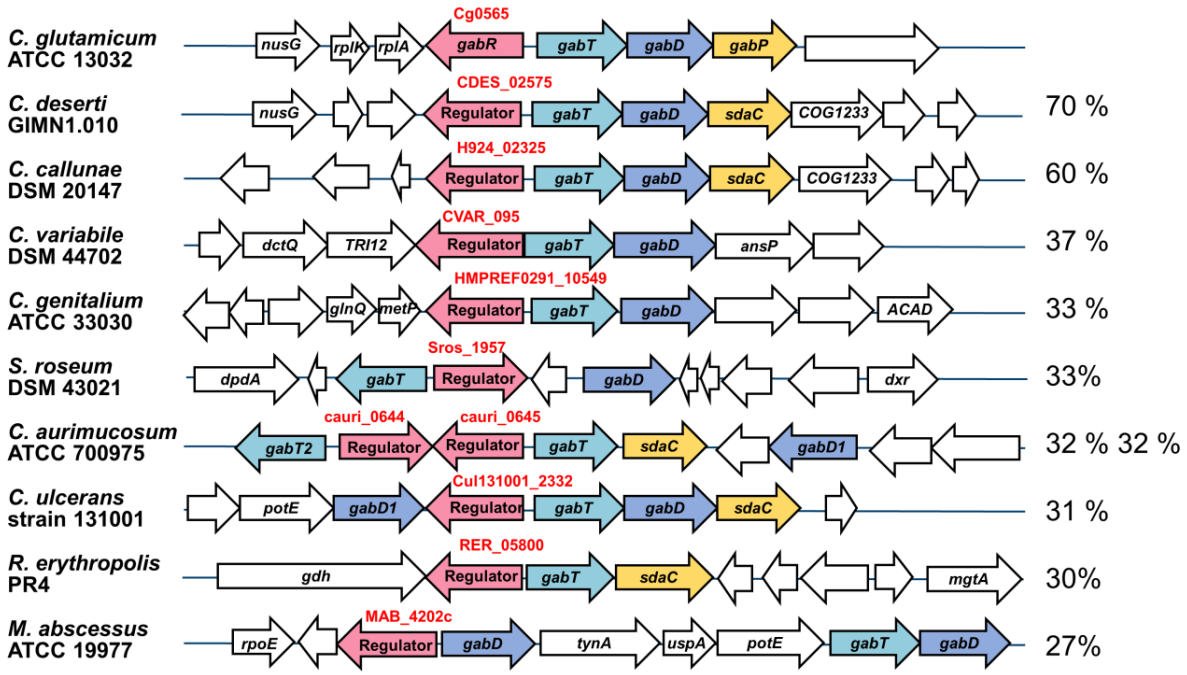

Fig. 4. Genomic organization of the *gabR-gabTDP* gene cluster in different *Corynebacterium* species. Conserved genes are marked with different colors: *gabT* (γ-aminobutyrate aminotransferase, light blue), *gabD* (succinate semialdehyde dehydrogenase, blue), *gabP, sdaC* (GABA-specific importer, yellow), *gabR* (transcriptional regulator, red). Non-conserved genes located next to the *gabR-gabTDP* cluster are shown colorless. The amino acid sequence identity of GabR homologs with GabR of *C. glutamicum*, which was calculated by MultiGeneBlast (91), is given on the right. The sequences were derived from the following genomes: *Corynebacterium deserti* GIMN1.010 (NZ_CP009220), *Corynebacterium callunae* DSM 20147 (NC_020506), *Corynebacterium ulcerans* strain 131001 (NZ_CP010818), *Corynebacterium variabile* DSM 44702 (NC_015859), *Corynebacterium genitalium* ATCC 33030 (NZ_CM000961), and *Corynebacterium aurimucosum* ATCC 700975 (NC_012590). The figure was generated using MultiGeneBlast (91).

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 either with GABA and urea or with glucose and urea (without $(NH_4)_2SO_4$). 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 (Table S1). The genes showing the by far strongest upregulation in GABA-grown cells were *gabTDP* (87-, 78-, and 65-fold, respectively) (Fig. 5 and Table S1). Interestingly, also *gabR* expression was four-fold increased in GABA-grown cells. 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 clearly showed that the *gabTDP* genes are 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.

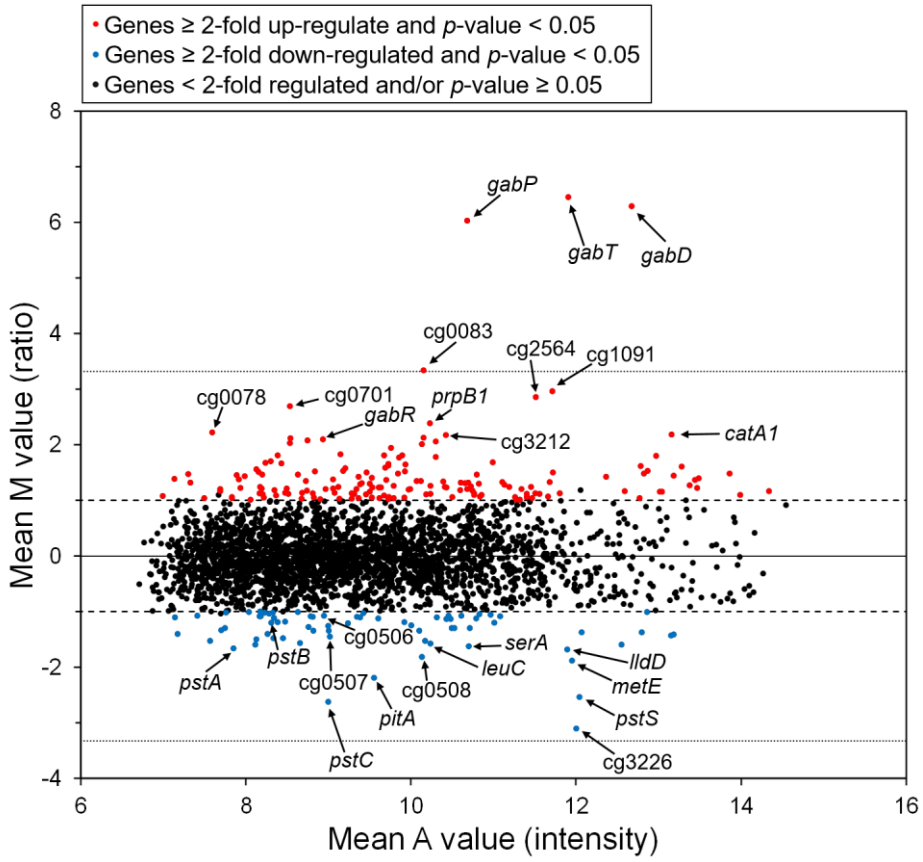

Fig. 5. M/A plot showing differential gene expression in *C. glutamicum* WT cells grown with GABA and urea compared to WT cells grown with glucose and urea. The data shown are based on four two-channel DNA microarray hybridizations starting with cDNA from four independent biological replicates. The dashed lines indicate a 2-fold altered mRNA ratio, the dotted lines a 10-fold altered mRNA ratio.

Relevance of GabR for growth with GABA. To investigate whether GabR is involved in the regulation of GABA metabolism, we generated the deletion mutant *C. glutamicum* Δ*gabR* and compared it with the WT. Both strains grew identically with glucose, but the Δ*gabR* mutant had lost the ability to grow with GABA (Fig. 6a). This suggests that GabR is required for induction of the *gabTDP* genes in the presence of GABA. The growth defect of the Δ*gabR* strain with GABA was abolished after transformation with the expression plasmid pAN6-*gabR* harboring *gabR* under control of a leaky *tac* promoter (Fig. 6b), 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 Δ*gabR* mutant and the WT in comparison to the non-induced cultures (Fig. 6b and Fig. S1). This might be a consequence of too strong expression of the *gabTDP* operon causing e.g. membrane-stress by overexpression of the permease GabP or metabolic disturbances by an excessive GABA metabolism. In summary, these results indicate that GabR is an activator of the *gabTDP* genes and that transcription is induced by GABA.

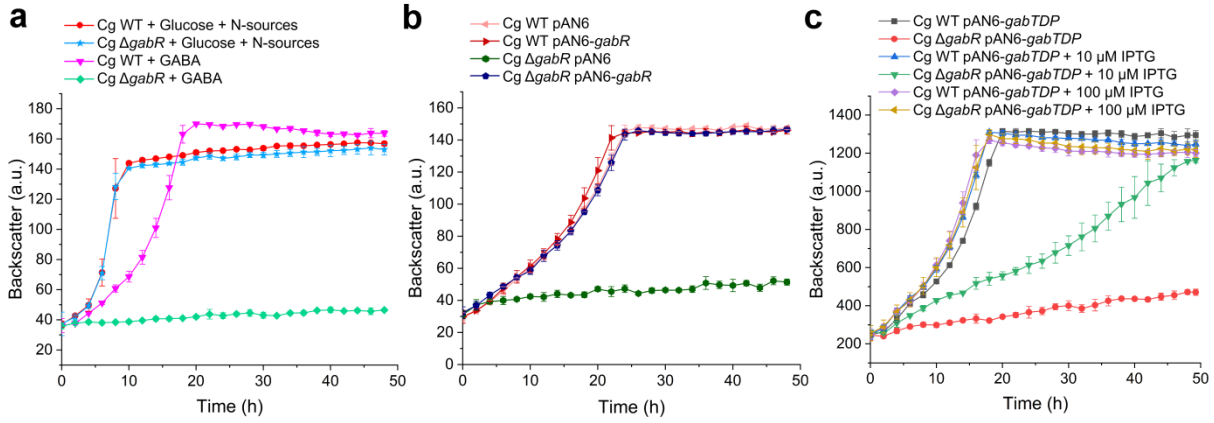

Fig. 6. Growth studies with *C. gutamicum* WT and the Δ*gabR* mutant with or without the plasmids pAN6, pAN6-*gabR*, or pAN6-*gabTDP*. All strains were precultivated in BHI medium and washed with PBS before inoculation of the main culture. (a) Growth of the *C. glutamicum* Δ*gabR* mutant compared to the WT with glucose or GABA as carbon sources. N-sources were 151 mM $(NH_4)_2SO_4$ and 82 mM urea. (b) Influence of plasmids pAN6 and pAN6-*gabR* on growth of *C. glutamicum* WT and the Δ*gabR* mutant with GABA as sole carbon and nitrogen source. (c)

Influence of plasmids pAN6 and pAN6-*gabTDP* on growth of *C. glutamicum* WT and the Δ*gabR* mutant with GABA as sole carbon and nitrogen source. Where indicated, 100 µM IPTG was added to the medium for induction of the P_{tac} promoter controlling expression of *gabTDP*. Since the promoter of pAN6 is known to be slightly leaky, basal transcription of the target genes is independent of IPTG addition. Growth experiments were performed in a Biolector microcultivation system using 48-well Flower plates containing 750 µl CGXII minimal medium with 62.5 mM GABA or 41.7 mM glucose with 151 mM $(NH_4)_2SO_4$ and 83 mM urea. Mean values and standard deviation of three biological replicates are shown.

In a further experiment we tested whether growth of the Δ*gabR* strain on GABA can be restored by plasmid-based expression of *gabTDP. C. glutamicum* WT and the Δ*gabR* mutant were transformed with pAN6-*gabTDP* and cultivated with GABA and different IPTG concentrations (Fig. 6c). Without IPTG addition, the Δ*gabR* 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 confirm that expression of *gabTDP* is strictly required for growth with GABA.

Influence of growth conditions and GabR on the promoter activity of P_{gabTDP}. To analyze the promoter activity of P_{gabTDP} under various conditions, 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 gene. Thereby the promoter activity can easily be monitored by measuring the fluorescence of the culture, e. g. in a Biolector microcultivation device. Cell density (backscatter) and eYFP fluorescence of WT cells with pJC1-P_{gabT}-eYFP cultivated in different media is shown in Fig. 7a,b. As the cells reached different final backscatter values, the specific fluorescence (= ratio fluorescence/backscatter) was calculated for the cultures in the stationary phase at 42 h, which thus represents the activity of P_{gabTDP} over the entire cultivation (Fig. 7c). 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 about ⅓ in

comparison to the cultures with GABA alone, indicating that the additional nitrogen sources inhibited transcription of *gabTDP*. The specific fluorescence of cells cultivated with GABA, glucose, ammonium sulfate and urea (0.90 ± 0.02) was even further reduced, indicating that not only additional nitrogen sources, but also glucose had a negative effect on *gabTDP* transcription. Inhibition by glucose is presumably caused by the regulation of the P_{gabTDP} promoter by GlxR. GlxR is a global cAMP-dependent transcriptional regulator and can function both as activator and repressor, depending on the localization of the binding site with respect to the transcriptional start site (TSS) (45, 46). In P_{gabTDP}, there is a predicted GlxR binding site (5'-TGTATCTCACCTCACA-3') between the TSS (see below) and the translational start site (TLS), which suggests an inhibitory function of GlxR.

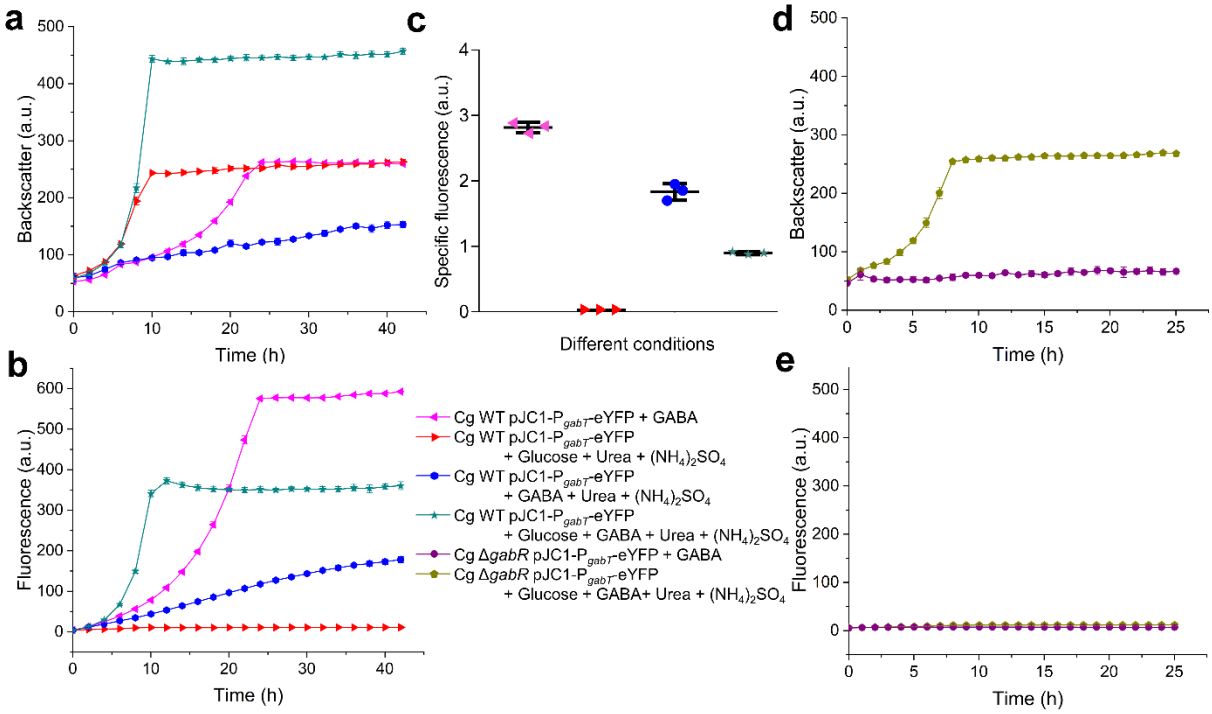

Fig. 7. Relevance of GabR for *gabTDP* transcription. *C. glutamicum* WT or the Δ*gabR* mutant transformed with the reporter plasmid pJC1-P$_{gabT}$-eYFP were precultivated in BHI medium and washed with PBS buffer before inoculation of the main cultures. The cultivations were performed in as described in a Biolector microcultivation system at 30°C and 1200 rpm using 750 µl CGXII minimal medium supplemented with GABA (62.5 mM), glucose (41.7 mM glucose), $(NH_4)_2SO_4$ (151 mM), and urea (83 mM) as indicated. (a, b) Growth and EYFP fluorescence representing the activity of the *gabTDP* promoter of *C. glutamicum* WT with pJC1-P$_{gabT}$-eYFP. (c) Specific fluorescence (ratio fluorescence/backscatter) of the cultures shown

in (a) and (b) after 42 h. (d, e) Growth and fluorescence *C. glutamicum* Δ*gabR* harboring pJC1-P_{gabT}-eYFP. Mean values and standard deviations of three biological replicates are shown.

In further experiments, we analyzed the activity of P_{gabTDP} in the Δ*gabR* mutant (Fig. 7d and 7e). In medium with only GABA 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 observed. This result shows that besides GABA also GabR is essential for expression of the *gabTDP* genes.

Purification of GabR and determination of the native size. The growth experiments and the promoter activity studies indicated 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 electrophoretic mobility shift assay (EMSAs). GabR was overproduced in *E. coli* BL21(DE3) and purified by means of an N-terminal decahistidine tag and Ni-NTA affinity chromatography (Fig. 8a). 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 (Fig. 8b). The peak of GabR appeared at an elution volume of 10.67 mL. 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). Based on the calibration curve (K_{av} versus log M_r) derived from these standard proteins (Fig. 8c) 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.

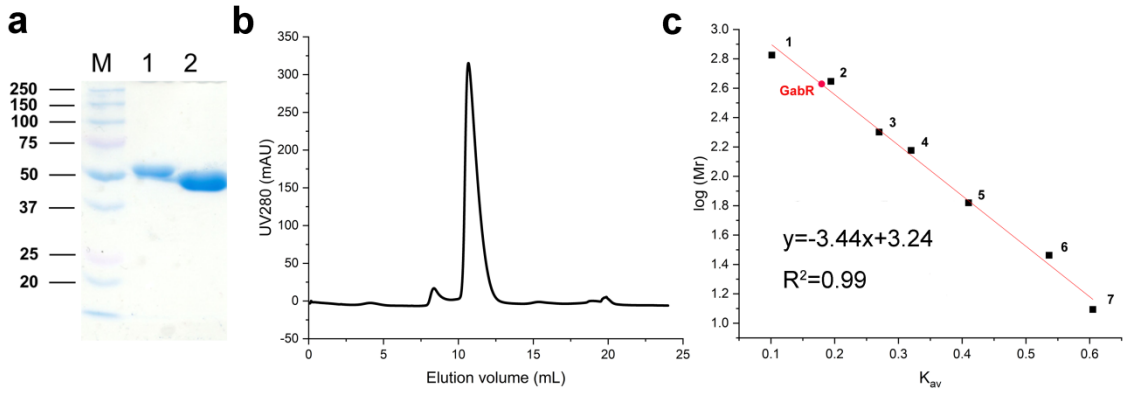

Fig. 8. Purification of GabR and determination of its native molecular mass. (a) Coomassie-stained SDS-polyacrylamide gel showing marker protein (M, molecular mass in kDa) and GabR after Ni-NTA affinity chromatography (lane 1) and after His-tag removal with TEV protease (lane 2). (b) Size exclusion chromatography of tag-free GabR using a Superdex 200 Increase 10-300GL column (GE Healthcare). Protein was detected by absorbance at 280 nm. (c) Calibration curve for the Superdex column obtained with standard proteins: 1, thyroglobulin (669 kDa); 2, apoferritin (443 kDa); 3, amylase (200 kDa); 4, alcohol dehydrogenase (150 kDa); 5, bovine serum albumin (66 kDa); 6, carbonic anhydrase (29 kDa); 7, cytochrome *c* (12.4 kDa). The K_{av} value determined for GabR is marked with a red dot.

Determination of the transcriptional start site of *gabT* and of the GabR binding site(s) in the *gabTDP* promoter region. In a previous RNAseq study, the TSS of P_{gabR} was identified, but not the TSS of P_{gabTDP}, presumably because the genes were not expressed in the absence of GABA (47). Therefore, we determined the TSS of P_{gabTDP} using a 5′/3′-RACE kit and identified a single TSS for *gabT* located 36 bp upstream of the *gabT* start codon (Fig. 9a). The -10 region, the -35 region of P_{gabTDP} and the RBS of *gabT* were predicted according to known consensus sequences (47).

For determination of the GabR binding site, electrophoretic mobility shift assays (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 from the *ldhA* gene of *C. glutamicum* as negative control. The *gabR* promoter fragment was shifted partially with 80 nM GabR (monomer) and fully with 240 nM GabR (Fig. S2), whereas no shift was observed for the control fragment up to 320 nM GabR (Fig. S2). This suggests that GabR binds specifically to the *gabR* 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 Fig. S3, 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 site 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 especially useful to identify a binding motif when only a single target promoter is known (48). We searched for a binding motif in the *gabR-gabT* intergenic regions of *C. glutamicum* WT, *Corynebacterium deserti*, and *Corynebacterium callunae* using MEME (49). The search uncovered a 21 bp repeat that was present 1-2 times in each input sequence (Fig. 9c). An alignment of the input sequences (Fig. S4) 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.

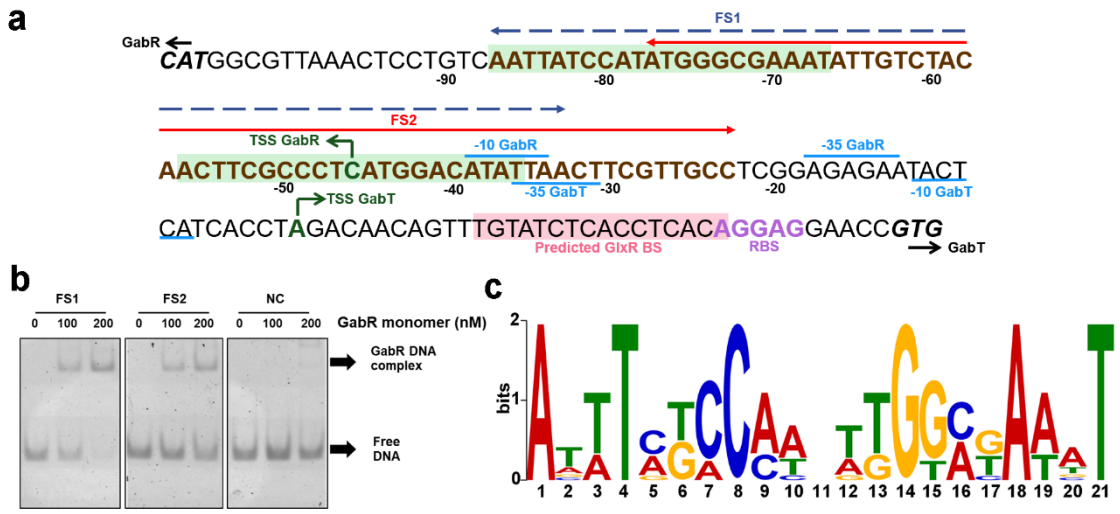

Fig. 9. Determination of the binding site(s) of GabR in the *gabTDP* promoter region. (a) Sequence of the *gabR-gabT* intergenic region. The ATG start codon of *gabR* (cg0565) and the GTG start codon of *gabT* (cg0566) are shown in bold and italic. The transcriptional start sites of *gabR* and *gabT* are indicated with green arrows.The corresponding -10 and -35 regions are labeled in blue. The ribosome binding site of *gabTDP* is labeled in purple, and the predicted GlxR binding site is marked with light red background. The binding region for GabR determined in this

work by EMSAs is marked in bold brown letters (compare Fig. S3). Fragments FS1 (-87 to -33) and FS2 (-77 to -23) are marked in the figure by arrows. Potential binding sites for GabR are marked with light green background. (b) Electrophoretic mobility shift assays with purified GabR and DNA fragments FS1 and FS2 indicated in (a). The fragments were incubated with different GabR concentrations (given in nM of monomers) as indicated. A 31 bp fragment downstream of the predicted binding sites was used as negative control (NC). (c) Proposed GabR consensus binding site identified with MEME software using the *gabR-gabT* intergenic regions of *C. glutamicum* WT*, Corynebacterium deserti* and *Corynebacterium callunae* as input sequences.

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 (Fig. 9a). 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 (Fig. 9b). 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 (Fig. S5). 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.

Attempts to identify the effector of GabR. For understanding of regulator function, not only the target genes and the DNA binding site(s) are important, but also the effector(s) that controls regulator activity. Transcriptional regulators of catabolic pathways are often controlled by the substrate of the respective pathway (13). $GabR_{Bs}$ of *Bacillus subtilis* requires both GABA and PLP for the activation of the

respective catabolic gene cluster (31). Although $GabR_{Bs}$ belongs to a different regulator family than *C. glutamicum* GabR, we nevertheless tested both substances for their influence on binding of GabR to its DNA target. However, both substrates separately and in combination did not change the binding behavior of GabR in EMSAs (Fig. S6). There are several possible explanations for this result: GabR might bind to its target site both in the apo-state and in the ligand-bound state, but changes its conformation upon binding of GABA or another effector metabolite is required to activate transcription of the *gabTDP* genes. Further studies are required to unravel the identity of the GabR effector.

Influence of plasmid-based *gabTDP* expression on growth in the presence of $(NH_4)_2SO_4$. *C. glutamicum* grew much worse with GABA when ammonium sulfate was present as additional nitrogen source (Fig. 2a). The promoter activity tests with the reporter plasmid pJC1-P_{gabT}-eYFP had revealed that $(NH_4)_2SO_4$ reduces expression of *gabTDP* (Fig. 7c). To test whether this effect on transcription is responsible for the growth inhibition by $(NH_4)_2SO_4$, 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 µM IPTG (Fig. 10). With GABA alone, the WT with pAN6-*gabTDP* grew comparable to the control strain with pAN6, indicating that under these conditions chromosomal *gabTDP* expression is 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. However, growth in the presence of $(NH_4)_2SO_4$ with pAN6-*gabTDP* was still worse than growth with GABA alone, even when *gabTDP* expression was induced with 100 µM IPTG (data not shown). These results indicate that increased expression of *gabTDP* can partially but not completely overcome the growth inhibition by $(NH_4)_2SO_4$.

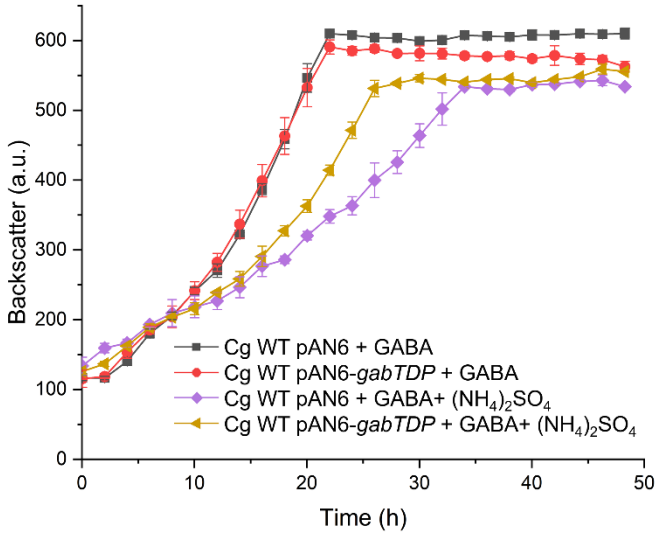

Fig. 10. Growth of *C. glutamicum* WT harboring pAN6-*gabTDP* or pAN6 with different substrates using 10 µM IPTG. Growth experiments were performed in a Biolector microcultivation system with 48-well Flower plates containing 750 µL CGXII minimal medium supplemented with GABA (62.5 mM) and $(NH_4)_2SO_4$ (151 mM) as indicated. BHI medium was used for precultures and the cells were washed with PBS buffer before inoculation of the main culture.

Influence of $(NH_4)_2SO_4$ on global gene expression of WT cells grown with GABA. To elucidate the influence of $(NH_4)_2SO_4$ on global gene expression during growth of WT cells with GABA, the transcriptome of cells cultivated with GABA and $(NH_4)_2SO_4$ was compared with the transcriptome of cells cultivated with GABA and K_2SO_4 (Fig. S7). In total, 160 genes were more than two-fold upregulated and 50 genes showed an at least two-fold lowered mRNA level in the presence of $(NH_4)_2SO_4$ (Table S2). The most strongly upregulated gene was *hmp* (cg3141, 11.5-fold) encoding flavohemoprotein Hmp that is probably involved in the removal of toxic nitrogen species such as nitrite or nitric oxide in *C. glutamicum* (50) followed by *zrf*/*czcD* (cg1447, 8.5-fold) encoding a transporter of the cation diffusion facilitator family that was shown to be involved in alkali and cobalt tolerance in *C. glutamicum* (51). The reasons for increased expression of these genes is unclear. The mRNA levels of *gabTDP* were reduced by 20-33% in the culture with $(NH_4)_2SO_4$, which is in line with the results obtained with the reporter plasmid pJC1-P_{gabT}-eYFP (Fig. 7c). EMSA studies revealed that 50 mM $(NH_4)_2SO_4$ did not change the affinity of GabR to the *gabT* promoter region (Fig. S8), but an effect on transcription initiation cannot be

excluded. Importantly, the transcriptome comparison did not reveal a typical nitrogen starvation response during growth with GABA as sole nitrogen source, which is contrast to a previous study where glutamine as sole nitrogen source was shown to strongly induce the nitrogen starvation response (52).

Effects of ammonium on GABA aminotransferase activity. Besides the negative influence of $(NH_4)_2SO_4$ on *gabTDP* expression, it might also inhibit GABA aminotransferase activity. Besides GabT (Cg0566), the protein encoded by *bioA* (cg2885) has also been reported to exhibit GABA aminotransferase activity (28). We overproduced and purified both enzymes as described in a previous study (53) and tested the effects of $(NH_4)_2SO_4$ on their activity. The activity was measured in a discontinuous assay with GABA and 2-oxoglutarate as substrates (see Materials and Methods section). Samples were taken at defined time points after start of the reaction and the glutamate concentration was determined by HPLC. The specific activity of GabT in the presence of 30 mM $(NH_4)_2SO_4$ (9.07 ± 0.33 µmol min^{-1} mg^{-1}) was only 6% lower than without ammonium (9.69 ± 0.12 µmol min^{-1} mg^{-1}). This small effect appears insufficient to explain the strong inhibition of growth on GABA by ammonium. Surprisingly, no GABA transaminase activity was detected in our experiments for BioA.

DISCUSSION

The aim of this study was to elucidate GABA metabolism and its regulation in the actinobacterial model organism *C. glutamicum*. We found that *C. glutamicum* can use GABA alone as sole carbon and nitrogen source and that the transcription of the GABA catabolic genes, *gabTDP*, is induced by GABA in the growth medium. Transcription of *gabTDP* is activated by the divergently encoded regulator GabR, which is strictly required for GABA metabolism. Expression of *gabTDP* was diminished in the presence of glucose, presumably due to repression by the cAMP-dependent regulator GlxR.

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* and, interestingly, about half of the sequences in the database contain a GGDEF-like domain, which suggests that they might possess diguanylate cyclase activity. However, a GGDEF-like domain is absent from *C. glutamicum* GabR. All PucR-type regulators characterized to date contain the GGDEF-like domain. These are *B. subtilis* PucR involved in purine catabolism (43), *B. subtilis* PutR involved in proline utilization (54), *B. subtilis* AdeR involved in alanine catabolism (55), *Streptomyces ambofaciens* Srm22 (SrmR) controlling expression of a polyketide synthase gene for the synthesis of an antibiotic (56, 57), and *Escherichia coli* CdaR (SdaR), which regulates genes involved in the uptake and metabolism of galactarate and glucarate (58). Interestingly, two of these regulators are involved in degradation of an amino acid, similar to GabR. 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 (54, 55). The binding site of PucR is not palindromic (43). The binding sites for Srm22 and CdaR have not been determined yet.

C. glutamicum GabR is the first characterized PucR-like regulator without a GGDEF-like domain and appears to be an octamer. 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 meningitides* forms octameric rings and is proposed to bind to two binding sites in its target promoter with two stacked octameric rings (59). The two CrgA binding sites cover a region of 63 bp,

which is quite similar to the region covered by our two proposed motifs. CrgA also binds to DNA fragments containing just one of the binding sites (59), 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* (60), LrpA of *Pyrococcus furiosus* (61), LrpA of *Mycobacterium tuberculosis* (62, 63), AldR of *M. tuberculosis* (64), or BarR of the archeum *Sulfolobus acidocaldarius* (65).

Transcriptional activators for catabolic pathways often require the corresponding substrate or one of its degradation products as co-activator. Thus, we tested whether GABA has an influence on the DNA affinity of purified GabR, but did not observe any change. *B. subtilis* $GabR_{Bs}$ always binds to its target promoter and causes a conformational change of the promoter region in the presence of GABA and PLP, which triggers transcription of the target genes (31). However, $GabR_{Bs}$ belongs to a different protein family than GabR of *C. glutamicum*. In case of PutR, the presence of proline increased the affinity for its target DNA about 10-fold (54). For AdeR, the addition of L-alanine did not increase the affinity for the target promoter (55). In summary, further studies are required to identify the co-activator of GabR and the mechanism of transcriptional activation.

In our studies, we observed that ammonium inhibits growth with GABA as carbon source. This is caused partially by an influence on *gabTDP* transcription, which was reduced about 1/3 in the presence of ammonium (Fig. 7). 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 (66-68). 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 part 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, but this does not exclude an effect of ammonia on GabR-activated transcription initiation.

The inhibitory effect of ammonium on growth with GABA is surprising, as ammonia is required as substrate for glutamine formation via glutamine synthetase. Supplementation of GABA medium with glutamine strongly increased the growth rate, indicating that glutamine synthesis is limiting when GABA serves as sole nitrogen source. GABA metabolism does not involve a reaction in which ammonia is obviously formed, leading to the question where the ammonia for glutamine synthesis comes from. One possibility is the reaction catalyzed by aspartate ammonia-lyase, which catalyzes the conversion of aspartate to fumarate and ammonia (25). 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. However, also other enzymes forming ammonia, which are not regulated at the transcriptional level, can be involved in providing ammonia for glutamine synthesis during growth with GABA as sole nitrogen source.

Glutamine synthetase was shown to be essential for growth with GABA as sole carbon and nitrogen source. The corresponding *glnA* gene is always expressed to ensure glutamine synthesis, but it is two- to threefold upregulated under nitrogen starvation via derepression by AmtR (40). Derepression is achieved by complex formation of AmtR with the adenylylated form of the P_{II} protein GlnK, which is formed under nitrogen starvation by the adenylyl transferase GlnD (for review see (41)). In the presence of ammonium, GlnK is deadenylylated and degraded (69). Therefore, a lower glutamine synthetase activity in the presence of ammonium due to *glnA* repression by AmtR might contribute to inhibition of growth on GABA by ammonium.

Furthermore, the activity of glutamine synthetase is also directly regulated by covalent modification. Under nitrogen sufficiency, GlnA is at least partially inactivated by adenylylation via GlnE and under nitrogen limitation GlnA is deadenylylated by GlnE. The signal controlling the activity of GlnE is unknown (70). If ammonium addition causes adenylylation and thus inactivation of GlnA, this can contribute to the inhibitory effect of ammonium during growth with GABA.

The use of a single substrate as sole carbon and nitrogen source for *C. glutamicum* has so far only been described for two compounds. *C. glutamicum* was shown to grow with 70 mM L-glutamine as sole carbon and nitrogen source, but growth was quite slow with a growth rate of 0.02 h^{-1} (52). The additional presence of 150 mM ammonium sulfate had no influence on glutamine-dependent growth. In another study, the *C. glutamicum* mutant M4 was isolated, which is able to grow efficiently with glucosamine as sole carbon and nitrogen source (71). In this case, glucosamine 6-phosphate formed during uptake of glucosamine by PTS^{Glc} is converted by glucosamine 6-phosphate deaminase to fructose 6-phosphate + NH_3, thus providing the standard nitrogen source.

In summary, our study has unraveled novel features of GABA metabolism and a new PucR-type regulator without a GGDEF-like domain that controls the GABA catabolic genes. Many of the results obtained here will probably also be relevant for other *Actinobacteria* capable of utilizing GABA as carbon and nitrogen source.

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 were 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 KH_2PO_4/Na_2HPO_4 pH 7.0). Growth experiments were performed in a Biolector microcultivation system (m2p-labs, Baesweiler, Germany) using 48-well FlowerPlates (m2p-labs) containing 750 µL CGXII minimal medium (72) supplemented with 30 mg L^{-1} 3,4-dihydroxybenzoate as iron chelator. *E. coli* was grown at 37 °C in lysogeny broth (73). When required for plasmid maintenance, media were supplemented with kanamycin (50 µg mL^{-1} for *E. coli*, 25 µg mL^{-1} for *C. glutamicum*) or chloramphenicol (20 µg mL^{-1}).

Table 1: Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or Reference
E. coli		
DH5α	F- Φ80*dlac*Δ(*lacZ*)M15 Δ(*lacZYA-argF*) U169 *endA1 recA1 hsdR17* (r_K^- , m_K^+) *deoR thi-1 phoA supE44* λ- *gyrA96 relA1*; strain used for cloning procedures	(83)
BL21(DE3)	F- *ompT hsdS*$_B$ (r_B-, m_B-) *gal dcm* (DE3); host for protein production	(84)
C. glutamicum		
ATCC 13032 (Cg WT)	Biotin-auxotrophic wild type	(85)
Δ*gabR*	WT derivative with an in-frame deletion of *gabR* (cg0565)	This work
Δ*gdh* (LNΔGDH)	ATCC 13032 with deletion of *gdh* (cg2280)	(86)
Δ*glnA* (LNΔGS)	ATCC13032 with deletion of *glnA* (cg2429)	(86)
Δ*gltBD* (LNΔ*gltBD*)	ATCC13032 with deletion of *gltB* (cg0229) and *gltD* (cg0230)	(52)
Δ*glnA*Δ*glnA2*Δ*gdh* (DA-2)	ATCC13032 with deletion of *gdh* (cg2280), *glnA* (cg2429) and *glnA*2 (cg2477)	(87)
Plasmids		
pK19*mobsacB*	Kan^R; plasmid for allelic exchange in *C. glutamicum*; (pK18 *ori*$V_{E.c.}$, *sacB*, *lacZ*α)	(76)
pK19*mobsacB*-Δ*gabR*	Kan^R.; pK19mobsacB derivative containing a PCR product covering the up- and downstream regions of *gabR* (cg0565)	This work
pAN6	Kan^R; *C. glutamicum*/*E. coli* shuttle vector for regulated gene expression using the P_{tac} promoter, derivative of pEKEx2.	(13)
pAN6-*gabR*	Kan^R; pAN6 derivative for expression of *gabR* (cg0565) under control of P_{tac}	This work
pAN6-*gabTDP*	Kan^R; pAN6 derivative for expression of *gabTDP*	This work

	cluster	
pET-TEV	Kan^R; pET28b derivative for overexpression of genes in E. coli, adding an N-terminal decahistdine tag and a TEV protease cleavage site to the target protein (pBR322 oriVE.c., PT7, lacI)	(88)
pET-TEV-*gabR*	Kan^R; pET-TEV derivative for overproduction of GabR (cg0565)	This work
pJC1-P_{tac}-*eyfp*	Kan^R; pJC1 derivative containing the *eyfp* gene under the control of P_{tac}	(89)
pJC1-venus-term	Kan^R; *E. coli-C. glutamicum* shuttle vector, pJC1 derivative carrying the venus coding sequence and additional terminators	(90)
pJC1-P_{gabT}-eYFP	Kan^R; pJC1-venus-term derivative carrying the promoter of *gabT* (cg0566) fused to *eyfp*	This work
pJM0462	Cm^R; pASK-IBA-3C derivative with the coding sequence of *gabT* (cg0566, NCgl0462)	(53)
pJMbioA	Cm^R; pASK-IBA-3C derivative with the coding sequence of *bioA* (cg2885)	(53)

Recombinant DNA work and construction of deletion mutants. Routine methods such as PCR, DNA restriction and ligation were performed using standard protocols (74, 75). All oligonucleotides used in this study are listed in Table S3 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 correct 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* (76) as described before (77). 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 (78) with pK19*mobsacB* which had been digested with BamHI and PstI. 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 NdeI and NheI, 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 corresponding oligonucleotides (gabtdpF and 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 oligonucleotides PgabTF and PgabTR with genomic DNA of *C. glutamicum* WT as template. The *eyfp* 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 (79). Briefly, for the first experiment, *C. glutamicum* WT was cultivated with 62.5 mM GABA and 83 mM urea or with 41.7 mM glucose and 83 mM urea. 20 mM K_2SO_4 was added to these cultures to prevent a possible sulfate limitation. For the second experiment, *C. glutamicum* WT was cultivated with 62.5 mM GABA and 151 mM $(NH_4)_2SO_4$ or with 62.5 mM GABA and 20 mM K_2SO_4. 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 (4000*g*, 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 labelled 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 labelled 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, USA). For background correction of spot intensities, ratio calculation and ratio normalization, data were processed using the BioConductor R-packages limma and marray (http://www.bioconductor.org). The full microarray data sets of this study have been deposited in the NCBI Gene Expression Omnibus and can be found under the GEO accession number GSE138829.

Determination of the transcriptional start site (TSS) of *gabTDP*. The TSS of *gabTDP* was 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 primer GABTTS1R (Table S3). 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 (Table S3). The PCR products were sequenced to determine the TSS. The TSS determination was performed independently for three biological replicates.

Protein overproduction and purification. For GabR overproduction and purification, *E. coli* BL21(DE3) harboring pET-TEV-*gabR* was first cultivated in 5 mL LB medium with 50 µg mL^{-1} kanamycin in 20 mL glas 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 (4,000*g*, 10 min, 4 °C). The harvested cells were resuspended in binding buffer (20 mM Tris-HCl, 500 mM sodium chloride, 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 12,000*g* for 20 min to remove cell debris and the supernatant was subjected to Ni^{2+} affinity chromatography using Ni-NTA Superflow (Qiagen, Hilden, Germany). His-tagged GabR protein was eluted with elution buffer (20 mM Tris-HCl, 500 mM sodium chloride, 1 mM DTT and 300 mM imidazole, pH 7.8). Subsequently, the His-tag was cleaved off with tobacco etch virus (TEV) protease (80) by incubation of 12 mg His-tagged GabR with 0.24 mg His-tagged TEV protease overnight at 4 °C. Subsequently, GabR was further purified by gel filtration with a Superdex™ 200 increase 10/300 GL column (GE Healthcare, Freiburg, Germany) connected to an Äkta™ 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}.

Overproduction and purification of the transaminases GabT and BioA were performed according to a previous publication (53). *E. coli* BL21(DE3) was transformed with pJM0462 and pJMbioA and was precultivated in 5 mL LB medium with 20 µg 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 µg mL^{-1} chloramphenicol in a 500 mL shake flask, which was cultivated at 37 °C and 120 rpm. Induction of target gene expression was induced 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 then 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, 16,000*g*, 4 °C), protein purification was performed by affinity chromatography on ice using Strep-TactinXT Sepharose (IBA, Göttingen, Germany). 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 (EMSAs). EMSAs were performed as described previously (81). 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% (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× 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).

Assay for GABA transaminase activity. The activity of GABA transaminase was measured as described (53). 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. 30 mM $(NH_4)_2SO_4$ was added to test if ammonium affects GABA transaminase activity. 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 µg mL^{-1}. Six 500 µl samples were collected over a period of 26 minutes 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, 16,000*g*). Subsequently, the glutamate concentration in the sample was measured by HPLC (Agilent 1260 series) equipped with an Agilent EclipseXDB-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 $Na_2B_4O_2$ (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*-phthaldialdehyde (82). 25-1000 µM sodium glutamate was used as standard.

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5 Supplementary data

Supplemental material to the manuscript "γ-Aminobutyrate (GABA) utilization and its regulation by GabR in *Corynebacterium glutamicum*"

Supplemental tables

Table S1. Genes with at least 2-fold increased or decreased mRNA levels (p value ≤0.05) **in WT cells grown in CGXII medium with GABA and urea compared to WT cells grown in CGXII medium with glucose and urea.**

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg0052		iron-siderophore ABC transporter, permease	2.05	0.040
cg0053		iron-siderophore ABC transporter, ATPase	2.59	0.022
cg0078		put. membrane protein	4.65	0.000
cg0083		put. nicotinamide mononucleotide uptake permease, PnuC-family	10.08	0.000
cg0105		hypothetical protein	2.05	0.004
cg0111		hypothetical protein	3.82	0.001
cg0120		put. esterase/lipase/thioesterase-family protein, hydrolase	2.38	0.000
cg0365		put. membrane protein	2.36	0.005
cg0387	*adhE*	mycothiol-dependent formaldehyde dehydrogenase	2.05	0.000
cg0518	*hemL*	glutamate-1-semialdehyde 2,1-aminomutase	2.15	0.008
cg0519		protein of histidine phosphatase superfamily, phosphoglycerate mutase-like protein	2.17	0.003
cg0520	*ccsX*	periplasmic thioredoxin	2.08	0.002
cg0522	*ccsA*	transmembrane disulfide interchange protein involved in cytochrome *c* biogenesis, DsbD family	2.38	0.001
cg0523		transmembrane cytochrome *c* biogenesis, ResB-family	2.34	0.002
cg0524	*ccsB*	transmembrane cytochrome *c* assembly protein, CcsA family	2.78	0.001
cg0565	*gabR*	put. transcriptional regulator, similar to PurR of *Bacillus subtilis* that regulates purine catabolism	4.28	0.004
cg0566	*gabT*	4-aminobutyrate aminotransferase, AT class II	86.90	0.001
cg0567	*gabD*	succinate semialdehyde dehydrogenase	77.87	0.001
cg0568	*gabP*	GABA-specific uptake transporter	64.83	0.001
cg0569		put. Cd^{2+}/cation-transporting P-type ATPase	2.35	0.008
cg0575		put. secreted protein	2.30	0.001
cg0637	*creC*	put. NAD^{+}-dependent 4-hydroxybenzaldehyd dehydrogenase subunit (EC 1.2.1.64)	2.74	0.009
cg0638	*creD*	put. *p*-cresol methylhydroxylase subunit	3.39	0.000
cg0639	*creE*	ferredoxin reductase	2.84	0.004
cg0640	*creF*	ferredoxin	2.88	0.003
cg0641	*creG*	put. 4-hydroxybenzyl-alcohol dehydrogenase	2.74	0.019

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg0642	*creH*	put. PEP-utilizing enzyme	2.69	0.017
cg0644	*creI*	put. pyruvate phosphate dikinase	3.19	0.040
cg0701		put. drug/metabolite transporter, DMT superfamily	6.43	0.003
cg0776		put. ABC-type siderophore transporter, secreted substrate-binding lipoprotein	3.54	0.000
cg0796	*prpD1*	2-methylcitrate dehydratase	2.74	0.001
cg0797	*prpB1*	2-methylisocitrate lyase	5.22	0.000
cg0798	*prpC1*	2-methylcitrate synthase	3.48	0.000
cg0932		put. membrane protein	2.52	0.000
cg0968		put. ATP-dependent helicase	2.07	0.008
cg0997	*cgtS2*	two component histidine kinase	2.07	0.005
cg0998	*htrA*	secreted serine protease	2.53	0.001
cg1045		hypothetical protein, conserved	2.08	0.001
cg1069	*gapB*	glyceraldehyde 3-phosphate dehydrogenase involved in gluconeogenesis	2.09	0.000
cg1082		put. membrane protein	2.36	0.003
cg1083	*cgtS10*	two component histidine kinase	2.15	0.001
cg1084	*cgtR10*	two component response regulator	2.29	0.000
cg1087		put. membrane protein	2.26	0.002
cg1091		hypothetical protein	7.78	0.000
cg1095		hypothetical protein	2.02	0.011
cg1106		hypothetical protein, conserved	2.03	0.015
cg1145	*fumC*	fumarase	2.21	0.000
cg1179		put. sensory box/GGDEF-family membrane protein	2.34	0.002
cg1180		put. glycosyltransferase	2.16	0.001
cg1182		put. membrane protein	2.15	0.002
cg1201		hypothetical protein	2.08	0.006
cg1202		hypothetical protein, conserved	2.25	0.003
cg1271	*sigE*	ECF-type sigma factor	2.41	0.003
cg1292		put. flavin-containing monooxygenase	3.12	0.001
cg1293		put. secreted protein	2.03	0.000
cg1300	*cydB*	cytochrome *bd* oxidase, subunit II	2.04	0.001
cg1392		put. transcriptional regulator, CRO/CI-family, HTH_3-family	2.08	0.013
cg1410	*rbsR*	transcriptional repressor, LacI family	3.15	0.002
cg1411	*rbsA*	ribose/xylose ABC transporter, ATPase	3.01	0.005
cg1412	*rbsC*	ribose/xylose ABC transporter, permease	2.68	0.003
cg1413	*rbsB*	ribose/xylose ABC transporter, secreted sugar-binding protein	2.86	0.002
cg1447	*zrf*	zinc exporter, cation diffusion facilitator	2.21	0.001
cg1471		hypothetical protein	2.29	0.043
cg1476	*thiC*	phosphomethylpyrimidine synthase	2.01	0.003
cg1498		put. RecG-like helicase	2.34	0.003
cg1513	*tnp23a*	transposase, put. pseudogene of CGP1 region	2.05	0.000
cg1514		put. secreted protein, CGP1 region	2.78	0.019
cg1515	*tnp24a*	transposase, put. pseudogene of CGP1 region	2.18	0.000
cg1516		hypothetical protein, CGP1 region	2.18	0.003
cg1517		put. secreted protein, CGP1 region	2.05	0.005

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg1543	*uriH*	inosine-uridine preferring nucleoside hydrolase	2.74	0.000
cg1545	*uriT*	permease of the major facilitator superfamily	2.59	0.003
cg1546	*rbsK1*	put. ribokinase protein	4.36	0.000
cg1547	*uriR*	transcriptional regulator of uridine utilization and ribose transport, LacI family	4.08	0.000
cg1617		put. GTP-binding protein EngA	2.29	0.001
cg1662		put. secreted protein	2.29	0.001
cg1673	*ppmN*	polyprenol-phosphate-mannose synthase domain 2	2.16	0.024
cg1683		put. superfamily II DNA and RNA helicase	2.93	0.009
cg1697	*aspA*	aspartate ammonia-lyase	2.50	0.000
cg1734	*hemH*	ferrochelatase	2.49	0.001
cg1759		put. Fe-S cluster assembly protein	2.17	0.004
cg1760	*sufU*	cysteine desulfhydrase	2.24	0.003
cg1761	*sufS*	Fe-S cluster assembly protein	2.08	0.008
cg1762	*sufC*	Fe-S cluster assembly ATPase	2.22	0.001
cg1763	*sufD*	Fe-S cluster assembly membrane protein	2.63	0.000
cg1764	*sufB*	Fe-S cluster assembly protein	2.57	0.004
cg1765	*sufR*	transcriptional repressor of the *suf* operon, ArsR-family	3.03	0.000
cg1895		put. secreted protein, CGP3 region	3.14	0.000
cg1901		hypothetical protein, CGP3 region	2.17	0.004
cg1902		put. secreted protein, CGP3 region	2.30	0.001
cg1903		put. ABC-type multidrug transport system, ATPase	2.35	0.000
cg1904		put. membrane protein, CGP3 region	2.48	0.001
cg1905		hypothetical protein, CGP3 region	2.78	0.001
cg1906		hypothetical protein, CGP3 region	3.06	0.000
cg1907		put. phosphopantothenoylcysteine synthetase/decarboxylase, CGP3 region	3.47	0.000
cg1908		hypothetical protein, CGP3 region	3.20	0.000
cg1942		put. secreted protein, CGP3 region	2.30	0.025
cg1943		hypothetical protein, CGP3 region	2.16	0.007
cg1955		put. secreted protein, CGP3 region	2.11	0.000
cg1956	*recJ*	single-stranded-DNA-specific exonuclease	2.33	0.042
cg1961		hypothetical protein, CGP3 region	2.31	0.035
cg1963		put. superfamily II DNA/RNA helicase, CGP3 region	2.01	0.000
cg2004		protein similar to 232 protein-lactobacillus bacteriophage g1e	3.24	0.028
cg2046		hypothetical protein, CGP3 region	2.04	0.001
cg2051		hypothetical protein, CGP3 region	2.06	0.008
cg2052		put. secreted protein, CGP3 region	2.83	0.002
cg2068		hypothetical protein, CGP3 region	2.48	0.049
cg2069	*psp1*	put. secreted protein, CGP3 region	2.18	0.002
cg2094		hypothetical protein	2.04	0.001
cg2111	*hrpA*	put. ATP-dependent RNA helicase	2.31	0.000
cg2157	*terC*	transmembrane tellurium resistance protein	3.48	0.000
cg2176	*infB*	translation initiation factor IF-2, GTPase	2.08	0.008
cg2265	*smc*	chromosome segregation ATPase	2.17	0.001
cg2270		hypothetical protein, conserved	2.60	0.000

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg2271		put. secondary $Co^{2+}/Zn^{2+}/Cd^{2+}$ efflux transporter, cation diffusion facilitator (CDF) family	2.24	0.003
cg2340		put. ABC-type amino acid transport system, secreted component	2.07	0.003
cg2422	*lipB*	lipoyltransferase	2.45	0.000
cg2430		hypothetical protein	4.01	0.000
cg2477		hypothetical protein, conserved	3.49	0.003
cg2555		hypothetical protein	2.86	0.003
cg2564		hypothetical protein	7.22	0.001
cg2572		hypothetical protein, conserved	3.43	0.000
cg2623	*pcaI*	β-ketoadipate succinyl-CoA transferase subunit	2.27	0.001
cg2624	*pcaR*	transcriptional repressor, IclR-family	4.32	0.000
cg2625	*pcaF*	β-ketoadipyl-CoA thiolase	2.98	0.003
cg2626	*pcaD*	β-ketoadipate enol-lactone hydrolase	2.43	0.002
cg2634	*catC*	muconolactone isomerase	2.82	0.000
cg2635	*catB*	chloromuconate cycloisomerase	2.48	0.001
cg2636	*catA1*	catechol 1,2-dioxygenase	4.52	0.001
cg2637	*benA*	benzoate 1,2-dioxygenase α subunit aromatic ring hydroxylation dioxygenase A	4.20	0.003
cg2638	*benB*	benzoate dioxygenase small subunit	4.14	0.001
cg2639	*benC*	benzoate 1,2-dioxygenase ferredoxin reductase subunit	2.20	0.001
cg2640	*benD*	*cis*-diol dehydrogenase	2.18	0.004
cg2641	*benR*	transcriptional regulator, LuxR-family	2.65	0.006
cg2685		put. short chain dehydrogenase/reductase	2.50	0.002
cg2823		put. dehydrogenase or related protein	2.77	0.000
cg2828		put. membrane protein	2.13	0.003
cg2837	*sucC*	succinyl-CoA synthetase, β subunit, ADP-forming	2.05	0.005
cg2875		hypothetical protein, mycoloylated	2.32	0.040
cg2888	*phoR*	two component response regulator	2.12	0.001
cg2958	*butA*	L-2,3-butanediol dehydrogenase/acetoin reductase	2.40	0.001
cg2962		put. enzyme involved in biosynthesis of extracellular polysaccharides	2.57	0.005
cg3001		put. transcriptional regulator, MarR-family	2.34	0.001
cg3003	*cps*	non-ribosomal peptide synthetase	3.07	0.001
cg3045	*glnH*	put. secreted glutamine-binding lipoprotein	2.53	0.001
cg3046	*pknG*	serine/threonine protein kinase	2.36	0.007
cg3077		put. membrane protein	2.16	0.007
cg3114	*cysN*	sulfate adenyltransferase, subunit 1	2.67	0.001
cg3115	*cysD*	sulfate adenylyltransferase, subunit 2	2.88	0.002
cg3116	*cysH*	phosphoadenosine-phosphosulfate reductase	2.38	0.001
cg3117	*cysX*	ferredoxin-like protein	2.24	0.005
cg3161		put. membrane protein	2.25	0.005
cg3212		put. carboxymuconolactone decarboxylase subunit	4.50	0.000
cg3237	*sodA*	manganese superoxide dismutase	2.45	0.004
cg3280		put. secreted protein, horizontally transferred gene	2.54	0.003
cg3281	*copB*	Cu^{2+}/cation-transporting ATPase transmembrane protein	2.01	0.003
cg3282		put. Cu^{2+}/heavy metal binding transport protein,	2.28	0.012
cg3283		hypothetical protein	2.16	0.014

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg3327	*dps*	DNA-binding protein from starved cells, stores Fe^{3+}	2.71	0.006
cg3332	*qor3*	put. NADPH:quinone oxidoreductase	2.13	0.001
cg3350	*nagK*	fumarylpyruvate hydrolase	2.13	0.001
cg3367		put. ABC-type multidrug transport system, ATPase	2.12	0.004
cg3368		put. ABC-transporter, permease	2.47	0.003
cg0133	*abgT*	p-aminobenzoyl-glutamate transporter	0.39	0.008
cg0134	*abgB*	metal-dependent amidase/aminoacylase/ carboxypeptidase, AbgB homolog	0.46	0.008
cg0158		put. transport protein, MFS family	0.49	0.008
cg0197	*iolC*	carbohydrate kinase, myo-inositol catabolism	0.42	0.002
cg0212		put. phosphate isomerase/epimerase, conserved	0.50	0.000
cg0277	*dccT*	dicarboxylate uptake system for succinate, fumarate or L-malate, DASS family	0.34	0.001
cg0286		put. membrane protein, conserved	0.36	0.005
cg0304		put. membrane protein	0.47	0.001
cg0404		put. protein of nitroreductase family, conserved	0.39	0.000
cg0411		put. membrane protein	0.49	0.002
cg0414	*wzz*	cell surface polysaccharide biosynthesis/chain length determinant protein	0.40	0.002
cg0506		ABC transporter for spermidine/putrescine/iron(Gibson et al.), ATPase	0.47	0.003
cg0507		ABC-transporter for spermidine/putrescine/iron(Gibson et al.), permease	0.37	0.004
cg0508		ABC-transporter for spermidine/putrescine/iron(Gibson et al.), secreted substrate-binding lipoprotein	0.28	0.001
cg0527	*glyR*	transcriptional regulator, ArsR family	0.50	0.050
cg0544		put. membrane protein	0.38	0.044
cg0545	*pitA*	low-affinity phosphate transport protein	0.22	0.008
cg0623	*cbrV*	put. cobalamin ECF transporter, transmembrane component	0.43	0.000
cg0624		put. secreted oxidoreductase	0.41	0.005
cg0665		put. serine protease	0.46	0.000
cg0772		put. sugar efflux permease, MFS type	0.49	0.005
cg0924		putative siderophore ABC transporter, secreted substrate-binding lipoprotein	0.46	0.002
cg0952	*mctB*	put. integral membrane protein	0.46	0.014
cg0953	*mctC*	monocarboxylic acid transporter	0.38	0.004
cg1001	*mscL*	large conductance mechanosensitive channel, MscL family	0.47	0.003
cg1214	*nadS*	cysteine desulfurase-like protein involved in Fe-S cluster assembly, required for maturation of NadA	0.46	0.001
cg1218	*ndnR*	transcriptional repressor, NrtR family	0.48	0.007
cg1290	*metE*	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	0.27	0.003
cg1347		put. secreted phospholipid phosphatase	0.40	0.000
cg1409	*pfkA*	6-phosphofructokinase	0.47	0.001
cg1451	*serA*	phosphoglycerate dehydrogenase	0.32	0.006
cg1479	*malP*	maltodextrin phosphorylase	0.38	0.003
cg1482		put. Zn-dependent hydrolase, including glyoxylases	0.44	0.002
cg1487	*leuC*	isopropylmalate isomerase large subunit	0.33	0.001

Locus tag	Gene name	Annotated function	mRNA ratio GABA/ Glucose	p value
cg1488	*leuD*	isopropylmalate isomerase small subunit	0.47	0.014
cg1492	*gpsA*	NADPH-dependent glycerol-3-phosphate dehydrogenase	0.33	0.023
cg1537	*ptsG*	glucose-specific EIIABC component of PEP-dependent phosphotransferase system (PTS)	0.41	0.000
cg1580	*argC*	N-acetyl-γ-glutamyl-phosphate reductase	0.47	0.012
cg1710	*uppP*	undecaprenyl pyrophosphate phosphatase	0.47	0.001
cg2181	*oppA*	peptide ABC-transporter, secreted component	0.37	0.007
cg2182	*oppB*	peptide ABC-transporter, permease	0.39	0.002
cg2221	*tsf*	elongation factor Ts	0.44	0.001
cg2312	*gip*	put. hydroxypyruvate isomerase	0.35	0.000
cg2391	*aroG*	phospho-2-dehydro-3-deoxyheptonate aldolase	0.47	0.013
cg2429	*glnA*	glutamine synthetase I	0.46	0.003
cg2470		put. branched-chain amino acid ABC transporter, secreted substrate-binding lipoprotein	0.48	0.001
cg2539	*ectP*	ectoine/proline/glycine betaine carrier, BCCT family	0.46	0.010
cg2610		put. dipeptide/oligopeptide/nickel ABC-transporter, secreted component	0.49	0.000
cg2777		put. membrane protein, conserved	0.47	0.002
cg2843	*pstB*	phosphate ABC-transporter, ATPase	0.43	0.011
cg2844	*pstA*	phosphate ABC-transporter, permease	0.32	0.000
cg2845	*pstC*	phosphate ABC-transporter, permease	0.16	0.006
cg2846	*pstS*	phosphate ABC-transporter, secreted phosphate-binding lipoprotein	0.17	0.003
cg2870	*dctA*	secondary H^+/Na^+:C_4-dicarboxylate symporter, dicarboxylate/amino acid:cation symporter (DAACS) family	0.41	0.029
cg2922		put. transcriptional regulator, IclR-family	0.47	0.000
cg2925	*ptsS*	sucrose-specific EIIABC component of PTS	0.41	0.000
cg2938	*siaF*	sialic acid ABC-transporter, permease	0.44	0.000
cg2939	*siaG*	sialic acid ABC-transporter, fused permease and ATPase components	0.35	0.001
cg2940	*siaI*	sialic acid ABC-transporter, duplicated ATPase domains	0.38	0.002
cg2964	*guaB1*	inosine-5-monophosphate dehydrogenase	0.43	0.002
cg3011	*groEL*	chaperone GroEL	0.50	0.002
cg3054	*purT*	5-phosphoribosylglycinamide transformylase	0.42	0.005
cg3107	*adhA*	Zn-dependent alcohol dehydrogenase	0.33	0.004
cg3149	*alaT*	alanine aminotransferase	0.35	0.005
cg3219	*ldhA*	NAD-dependent L-lactate dehydrogenase	0.38	0.010
cg3226		L-lactate permease, MFS-type	0.12	0.007
cg3227	*lldD*	menaquinone-dependent L-lactate dehydrogenase	0.31	0.002
cg3277		hypothetical protein	0.50	0.001
cg3374	*cye1*	put. NADH-dependent flavin oxidoreductase, Old Yellow Enzyme family	0.49	0.006
cg3382		put. dipeptide/tripeptide permease	0.46	0.001
cg3404		putative iron(Gibson et al.) dicitrate ABC transporter, substrate-binding lipoprotein	0.36	0.006

Table S2. Genes with at least 2-fold increased or decreased mRNA levels (p value ≤ 0.05) **in WT cells grown in CGXII medium with GABA and $(NH_4)_2SO_4$ compared to WT cells grown in CGXII medium with GABA and K_2SO_4 instead of $(NH_4)_2SO_4$.**

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg0018		conserved membrane protein	2.43	0.006
cg0040		put. secreted protein involved in zinc metabolism	2.92	0.000
cg0041	*znuA2*	ABC-type Mn/Zn import system Znu2, substrate-binding lipoprotein	4.61	0.006
cg0072		conserved membrane protein	2.13	0.013
cg0144	*rbtT*	put. ribitol transporter, MFS-type	2.23	0.003
cg0159		hypothetical protein	2.04	0.005
cg0183		put. LysE-type translocator	2.18	0.010
cg0210		transcriptional regulator, LacI family	3.21	0.000
cg0223	*iolT1*	myo-inositol transporter 1	2.33	0.001
cg0255		hypothetical protein	2.39	0.001
cg0256		put. protein, conserved	2.99	0.002
cg0261	*moeA1*	molybdopterin cofactor synthesis protein A1, MoeA family	2.04	0.000
cg0272		put. transcriptional regulator, LysR-family	2.83	0.001
cg0292	*tnp16a*	transposase	3.66	0.002
cg0332		put. secreted protein	2.36	0.019
cg0365		put. membrane protein	2.16	0.031
cg0384	*rluC1*	ribosomal large subunit pseudouridine synthase C	2.26	0.001
cg0462		hypothetical protein, conserved	2.10	0.003
cg0551	*menC*	O-succinylbenzoate synthase	2.06	0.005
cg0661		hypothetical protein, conserved	2.85	0.011
cg0689		hypothetical protein	2.04	0.002
cg0740		put. membrane protein	2.07	0.000
cg0785		hypothetical protein	2.72	0.006
cg0794		protein with CobW domain, cobalamin synthesis	2.01	0.008
cg0808	*wbpC*	put. lipopolysaccharide biosynthesis acyltransferase, conserved	2.01	0.003
cg0823	*ntaA*	nitrilotriacetate monooxygenase component A	3.12	0.000
cg0824	*tnp5a*	transposase	2.89	0.002
cg0932		put. membrane protein	3.49	0.007
cg1019		put. metal-dependent hydrolase, conserved	2.10	0.011

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg1020		put. double-stranded β-helix domain	2.82	0.003
cg1023	*tnp6b*	transposase fragment	2.21	0.002
cg1024	*tnp7a*	transposase	3.02	0.000
cg1025		hypothetical protein	2.21	0.021
cg1059		hypothetical protein, conserved	3.14	0.002
cg1065	*urtD*	ABC-type urea uptake system	2.32	0.005
cg1081		put. ABC-type multidrug transport system, ATPase component	2.66	0.000
cg1082		put. membrane protein	2.63	0.001
cg1179		put. sensory box/GGDEF family membrane protein	2.16	0.005
cg1187	*tnp10a*	transposase fragment, put. pseudogene	2.97	0.007
cg1255		put. HNH endonuclease, conserved	3.10	0.003
cg1279		put. secreted protein	4.28	0.010
cg1289		put. multidrug efflux permease of the major facilitator superfamily	2.19	0.001
cg1295		put. hydrolase or acyltransferase, α/β hydrolase superfamily	2.15	0.003
cg1296		put. non-ribosomal peptide synthetase module and related proteins, conserved	2.56	0.006
cg1342	*narJ*	dissimilatory nitrate reductase, δ-subunit	2.25	0.002
cg1347		put. secreted phospholipid phosphatase	2.13	0.001
cg1349		put. membrane protein containing CBS domain	2.02	0.011
cg1352	*moaA*	molybdenum cofactor biosynthesis protein A	2.28	0.005
cg1361	*atpI*	protein put. related to Mg^{2+} uptake	2.56	0.013
cg1441		put. ABC-type nitrate/sulfonate/taurine/bicarbonate transporter, secreted component	2.11	0.038
cg1447	*zrf*	zinc exporter, cation diffusion facilitator	8.53	0.000
cg1469		put. secreted protein	2.68	0.027
cg1622		put. ABC-type multidrug/protein/lipid transporter, ATPase	2.16	0.000
cg1716	*tnp16b*	transposase	3.52	0.003
cg1744	*pacL*	cation-transporting ATPase	2.39	0.002
cg1764	*sufB*	Fe-S cluster assembly protein	2.30	0.001
cg1765	*sufR*	transcriptional repressor of the *suf* operon, ArsR family	2.27	0.002
cg1782	*tnp13b*	transposase	2.26	0.009

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg1830		put. signal peptidase, membrane protein	2.38	0.000
cg1913		hypothetical protein CGP3 region	3.16	0.003
cg1914		hypothetical protein CGP3 region	2.64	0.001
cg1918		put. secreted protein CGP3 region	3.00	0.001
cg1919		put. membrane protein CGP3 region	4.21	0.008
cg1921		hypothetical protein CGP3 region	2.13	0.000
cg1922		hypothetical protein CGP3 region	2.98	0.020
cg1924		hypothetical protein CGP3 region	3.18	0.017
cg1926		hypothetical protein CGP3 region	2.46	0.012
cg1928		hypothetical protein CGP3 region	2.10	0.000
cg1957		hypothetical protein CGP3 region	2.28	0.019
cg1963		put. superfamily II DNA/RNA helicase CGP3 region	2.19	0.002
cg1964		hypothetical protein CGP3 region	2.76	0.013
cg1965		put. protein, similarity to gp57-phage N15 CGP3 region	2.49	0.009
cg1975		hypothetical protein, conserved CGP3 region	3.16	0.001
cg1983		hypothetical protein CGP3 region	2.27	0.002
cg1986		hypothetical protein CGP3 region	4.41	0.002
cg1987		hypothetical protein CGP3 region	2.39	0.013
cg1990		put. NUDIIX hydrolase CGP3 region	2.10	0.003
cg2004		puative protein, similar to 232 protein-lactobacillus bacteriophage g1e, conserved CGP3 region	2.22	0.002
cg2015		hypothetical protein CGP3 region	2.35	0.009
cg2016		hypothetical protein CGP3 region	2.39	0.000
cg2017		hypothetical protein CGP3 region	3.06	0.010
cg2061	*psp3*	put. secreted protein CGP3 region	2.41	0.003
cg2065		put. superfamily II DNA or RNA helicase CGP3 region	2.83	0.014
cg2070	*int2*	put. phage integrase C-terminal fragment, CGP3 region	3.86	0.025
cg2134		put. membrane protein	2.07	0.006
cg2202	*hrtB*	ABC-type heme exporter, permease	3.82	0.004
cg2226		put. nucleotide-binding protein involved in DNA uptake, conserved	2.33	0.023
cg2242		transcriptional repressor, LacI family	2.89	0.002
cg2243		protein of divalent anion:Na^+ symporter (DASS) family	2.75	0.004

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg2298	*impA*	*myo*-inositol-1or 4-monophosphatase family protein	2.04	0.002
cg2351		hypothetical protein	2.44	0.004
cg2356		put. permease of the drug/metabolite transporter (DMT) superfamily	2.18	0.000
cg2379		hypothetical protein	2.21	0.001
cg2381		hypothetical protein, conserved	2.07	0.001
cg2425	*sucE*	succinate exporter	2.20	0.000
cg2454		hypothetical protein	2.07	0.005
cg2504		hypothetical protein, conserved	3.71	0.001
cg2542		put. secondary malonate transporter, auxin efflux carrier, AEC family	2.25	0.000
cg2568	*dctM*	C_4-dicarboxylate transport system, permease large protein, TRAP family	2.31	0.028
cg2578		put. secreted DNA uptake protein or related DNA-binding protein	2.99	0.004
cg2648		transcriptional regulator, ArsR-family	2.11	0.002
cg2675		ABC transporter, ATPase with duplicated ATPase domains	2.14	0.001
cg2676		put. dipeptide/oligopeptide/nickel ABC transporter, permease	2.21	0.001
cg2677		put. dipeptide/oligopeptide/nickel ABC transporter, permease	2.27	0.006
cg2683		hypothetical protein, conserved	2.77	0.012
cg2699	*ctiP*	copper transport and insertion protein	2.48	0.001
cg2711		put. secreted protein	3.47	0.001
cg2713	*dhaS*	NADP-dependent aldehyde dehydrogenase	2.96	0.000
cg2732	*gntV*	put. gluconokinase	2.32	0.016
cg2748		put. membrane protein, conserved	2.12	0.002
cg2756		hypothetical protein, conserved	2.06	0.005
cg2757	*tnp15a*	transposase	2.08	0.000
cg2759	*tnp15b*	transposase	2.32	0.004
cg2807	*tnp11a*	transposase, put. pseudogene	2.32	0.021
cg2810	*cynT*	high affinity cysteine importer	2.03	0.001
cg2868	*nuc*	extracellular nuclease	2.09	0.004
cg2884		put. dipeptide/tripeptide permease	3.47	0.004

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg2898		put. 3-ketosteroid dehydrogenase	2.09	0.005
cg2916	*ksdD*	3-ketosteroid-1-dehydrogenase	2.20	0.009
cg2918		put. dehydrogenase or related protein	2.26	0.035
cg2920		put. shikimate permease of the major facilitator superfamily	2.65	0.026
cg2921		put. permease of the major facilitator superfamily	3.03	0.003
cg2973		hypothetical protein, conserved	2.39	0.007
cg2987	*pbp4b*	serine-type D-Ala-D-Ala carboxypeptidase	2.56	0.006
cg3028	*mrpF2*	subunit 5 of Na^+K^+/H^+ antiporter Mrp2	2.02	0.007
cg3082		transcriptional regulator, ArsR family	3.00	0.004
cg3087		transcriptional regulator, LacI family	2.48	0.008
cg3111		put. secreted protein	2.15	0.009
cg3115	*cysD*	sulfate adenylyltransferase, subunit 2	2.41	0.003
cg3116	*cysH*	phosphoadenosine-phosphosulfate reductase	2.51	0.004
cg3117	*cysX*	ferredoxin-like protein	2.49	0.010
cg3141	*hmp*	flavohemoprotein	11.54	0.000
cg3145		put. pseudogene	2.60	0.024
cg3161		put. membrane protein	2.04	0.001
cg3166		put. cytoplasmic glycosyltransferase involved in corynomycolate transport	2.25	0.005
cg3202	*farR*	transcriptional regulator, GntR family	2.52	0.001
cg3210	*lcpB*	similar to cg0847, might be involved in cell wall biogenesis	2.45	0.002
cg3225		put. calcineurin-like phosphoesterase	2.34	0.012
cg3232		put. secreted phosphohydrolase, ICC family	2.74	0.001
cg3246		put. transcriptional regulator, MarR family	2.16	0.001
cg3255	*uspA3*	universal stress protein	2.43	0.003
cg3266	*tnp5c*	transposase	2.35	0.002
cg3268		put. membrane protein, put. pseudogene	2.20	0.001
cg3274		put. site-specific recombinases, DNA invertase Pin homolog-fragment, put. pseudogene	2.02	0.016
cg3278	*tnp20a*	transposase, put. pseudogene	2.01	0.008
cg3283		hypothetical protein	2.10	0.019
cg3284	*copS*	two component sensor kinase, copper stress,	2.73	0.001

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg3286		secreted protein involved in copper stress	2.94	0.003
cg3287	*copO*	secreted multicopper oxidase	2.13	0.010
cg3288		protein involved in copper stress	3.38	0.003
cg3289	*tlpA*	thioredoxin-like protein	2.59	0.006
cg3291		transcriptional regulator, Crp family	2.42	0.000
cg3292		copper chaperone	2.33	0.002
cg3294		hypothetical protein	2.44	0.002
cg3295		put. Cd^{2+}/cation transport ATPase	2.05	0.005
cg3298	*tnp19a*	transposase fragment, put. pseudogene	2.09	0.014
cg3303		transcriptional regulator, PadR family	2.46	0.002
cg3354	*genH*	put. aromatic-ring hydroxylase flavoprotein monooxygenase or 3-hydroxybenzoate 6-hydroxylase	2.39	0.008
cg3367		put. ABC-type multidrug transport system, ATPase	2.03	0.001
cg3394		put. secreted protein	2.50	0.017
cg0197	*iolC*	carbohydrate kinase, *myo*-inositol catabolism	0.44	0.004
cg0310	*katA*	catalase	0.49	0.007
cg0405		put. iron(Gibson et al.)-dicitrate ABC transporter, secreted siderophore-binding lipoprotein	0.41	0.001
cg0444	*ramB*	transcriptional regulator of acetate metabolism, MerR family	0.50	0.001
cg0701		put. drug/metabolite transporter (DMT) superfamily	0.40	0.004
cg0723	*crtE*	geranylgeranyl-pyrophosphate sythase	0.46	0.000
cg0727		put. nucleoside-diphosphate-sugar epimerase	0.46	0.001
cg0753		put. secreted protein	0.40	0.002
cg0759	*prpD2*	2-methycitrate dehydratase	0.46	0.000
cg0770		put. iron-siderophore ABC transporter, permease	0.41	0.001
cg0771	*irp1*	put. iron-siderophore ABC transporter, secreted siderophore-binding lipoprotein	0.36	0.000
cg0914	*ftsE*	cell division ATP-binding protein, FtsE family	0.50	0.002
cg0921		put. cytoplasmic siderophore-interacting protein	0.48	0.009
cg0922		put. secreted siderophore-binding lipoprotein	0.31	0.004
cg0924		put. siderophore ABC transporter, substrate-binding lipoprotein	0.20	0.000

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg0926		put. siderophore ABC transporter, permease	0.48	0.001
cg0998	*pepD*	put. trypsin-like serine protease	0.44	0.000
cg1055	*rraA*	ribonuclease activity regulator protein or S-adenosylmethionine:2-demethylmenaquinone methyltransferase	0.42	0.001
cg1056		put. membrane protein	0.39	0.001
cg1085		hypothetical protein	0.47	0.000
cg1120	*ripA*	transcriptional regulator of iron proteins, AraC family	0.32	0.000
cg1139		put. allophanate hydrolase subunit 2	0.43	0.001
cg1140		put. allophanate hydrolase subunit 1	0.49	0.001
cg1142		put. Na^+/proline, Na^+/panthothenate symporter	0.47	0.000
cg1336		put. secreted protein	0.46	0.009
cg1434	*yggB*	glutamate exporter, MscS family	0.40	0.001
cg1612		put. acetyltransferase	0.31	0.000
cg1708		hypothetical protein, conserved	0.40	0.024
cg1810	*gmk*	guanylate kinase	0.39	0.000
cg1811	*ihf*	put. integration host factor, conserved	0.42	0.000
cg2096		put. membrane protein	0.46	0.010
cg2104	*galE*	UDP-glucose 4-epimerase	0.48	0.005
cg2155		hypothetical protein, conserved	0.41	0.001
cg2156		hypothetical protein	0.49	0.031
cg2181	*oppA*	peptide ABC transporter, secreted component	0.43	0.001
cg2221	*tsf*	elongation factor Ts	0.46	0.002
cg2439		hypothetical protein	0.44	0.001
cg2445	*hmuO*	heme oxygenase	0.48	0.003
cg2470		put. branched-chain amino acid ABC transporter, substrate-binding protein	0.48	0.000
cg2481		hypothetical protein, conserved	0.47	0.014
cg2623	*pcaI*	β-ketoadipate succinyl-CoA transferase subunit	0.43	0.002
cg2651		put. protein fragment, conserved	0.46	0.001
cg2887	*phoS*	two component sensor kinase, phosphate homeosatasis	0.48	0.000
cg2888	*phoR*	two component response regulator, phosphate homeosatasis	0.44	0.005
cg2904		hypothetical protein	0.44	0.041

Locus tag	Gene name	Annotated function	mRNA ratio GABA + $(NH_4)_2SO_4$/ GABA + K_2SO_4	p value
cg2927	*scrB*	put. sucrose-6-phosphate hydrolase, β-fructofuranosidase	0.39	0.000
cg3156	*htaD*	secreted heme transport-associated protein	0.38	0.004
cg3216	*gntP*	gluconate permease, gluconate:H^+ symporter, GntP family	0.49	0.001
cg3324		put. secreted protein	0.49	0.003
cg3404		put. iron(Gibson et al.)-dicitrate ABC transporter, substrate-binding lipoprotein	0.44	0.001

Table S3. Oligonucleotides used in this study.

Oligonucleotide	Sequence (5' → 3') and properties[a]
Construction of deletion plasmid pK19mobsacB-Δ*gabR* and PCR-analysis of the resulting mutants	
cg0565frontF	**ATGCCTGCAGGTCGACTCTAGAGGATC**TGAGGCTGCTGGCGCTGACTTCGTT
cg0565frontR	**CGCCCATATGGATAATTGACAGGAGTTTAACGCCAAAGACCCCATGCAAAGCATGGGGCC**
cg0565backF	**GGCCCCATGCTTTGCATGGGGTCTTTGGCGTTAAACTCCTGTCAATTATCCATATGGGCG**
cg0565backR	**CACGACGTTGTAAAACGACGGCCA**GTGAATTGTGATCGCCTGGAGTCAAGGCGTTGAG
cg0565checkF	CAAGAACTACGACGCTTCCATCGAC
cg0565checkR	AGTGGTCCGAATCCGGACTTGTATG
Construction of plasmid pAN6-*gabR*	
0565F	GAACATATGGAAACCCCAACCCAAGACAT
0565R	GAAGCTAGCTTATTGCTTTTCGACGTCTC
Construction of plasmid pET-TEV-*gabR*	
0565F	GAACATATGGAAACCCCAACCCAAGACAT
petfhis0565R	CATCCAAGCTTATTGCTTTTCGACGTCTC
Construction of plasmid pJC1-P$_{gabT}$-eYFP	
PgabTF	**GATCAGCGACGCCGCAGGGGGATC**ATTTCACGGGGGACTTCAAAGA
PgabtR	**CAGCTCCTCGCCCTTGCTCACCAT**GGTTCCTCCTGTGAGGTGAGATAC
eYFPF	**ATGGTGAGCAAGGGCGAGGAGCTG**TTCA
eYFP-termR	**GTAAAACGACGGCCAGTACTAGT**TTATCTAGACTTGTACAGCTCGTCCATGCCG
Construction and sequencing of plasmid pAN6-*gabTDP*	
gabtdpF	**CATGCCTGCAGAAGGAGATATACAT**ATGGAAGATCTCTCATACCGCATC
gabtdpR	**GAACTGTGGGTGGGACCAGCTAGC**CTATGCCCAACCCGCAGGCATTAATC
gabtdpS2	CCAGGTCGGAGCCGAAAACCTCGC
gabtdpS3	CAGGTGTTAGTCGCAGCCCTAGAG
gabtdpS4	CTGATCCTCGCTTGGCTAAAGTCA
gabtdpS5	CAATCCGGCTTCGGCAGAGAAGGC
Sequencing primers for plasmid pK19mobsacB	
pK19F	CGCCAGGGTTTTCCCAGTCAC
pK19R	AGCGGATAACAATTTCACACAGGA
Sequencing primers for plasmid pAN6	
pAN6F	GATATGACCATGATTACGCCAAGC
pAN6R	GACCGCTTCTGCGTTCTGATTTAA

Oligonucleotide	Sequence (5' → 3') and properties[a]
Sequencing primers for plasmid pET-TEV	
pET-TEVF	GAAACAAGCGCTCATGAGCCCGAAG
pET-TEVR	GCAGCAGCCAACTCAGCTTCCTTTC
Sequencing primers for plasmid pJC1	
pJC1F	GACAGATTATCTGCAAACGGTGTGT
pJC1R	CAATGCTTAATCAGTGAGGCACCTA
Oligonucleotides to determine the transcriptional start site of *gabTDP*	
GABTTS1R	GTTTTCGGCTCCGACCTGGGATTC
GABTTS2R	GTAAGACATTGGTGCACGGTAGAC
GABTTS3R	GTCCGTCCGTGGTACGCGTTGTC
Oligonucleotides to generate DNA fragments for EMSAs	
P*GabT*	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FA1	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA1R	TAGGTGATGAGTATTCTCTCCGAGGC
FA2	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA2R	GGCAACGAAGTTAATATGTCCATGAG
FA3	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA3R	CATGAGGGCGAAGTTGTAGACAATA
FA4	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA4R	CAATATTTCGCCCATATGGATAATT
FA5	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA5R	TAATTGACAGGAGTTTAACGCCATG
FA6	
P_{GabT}F	ATTTCACGGGGGACTTCAAAGA
GabrbsA6R	CCATGGAAACCCCAACCCAAGACAT
FB1	
GabrbsB1F	ATGTCTTGGGTTGGGGTTTCCATGG
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FB2	
GabrbsB2F	CATGGCGTTAAACTCCTGTCAATTA
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FB3	
GabrbsB3F	AATTATCCATATGGGCGAAATATTG
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FB4	

Oligonucleotide	Sequence (5' → 3') and properties[a]
GabrbsB4F	TATTGTCTACAACTTCGCCCTCATG
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FB5	
GabrbsB5F	CTCATGGACATATTAACTTCGTTGCC
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FB6	
GabrbsB6F	GTTGCCTCGGAGAGAATACTCATCAC
P_{GabT}R	GTATGAGAGATCTTCCACGGTTCCT
FS	
GabrbsB3F	AATTATCCATATGGGCGAAATATTG
GabrbsA2R	GGCAACGAAGTTAATATGTCCATGAG
FS1	
GabrbsB3F	AATTATCCATATGGGCGAAATATTG
GabrbsS1R	ATATGTCCATGAGGGCGAAGT
FS2	
GabrbsS2F	ATGGGCGAAATATTGTCTACAACTTC
GabrbsA2R	GGCAACGAAGTTAATATGTCCATGAG
FS3	
GabrbsS3F	CTGTCAATTATCCATATGGGCGAAATATTGT
GabrbsS3R	ACAATATTTCGCCCATATGGATAATTGACAG
FS4	
GabrbsS4F	CTACAACTTCGCCCTCATGGACATATTAACT
GabrbsS4R	AGTTAATATGTCCATGAGGGCGAAGTTGTAG
NC1	
NCldhF	GTTGGTGGACAAAAACGTCAACGAT
NCldhR	GAGCAGTGCAGAGACTGGGACTG
NC2	
NC55bpF	TAGACAACAGTTTGTATCTCACCTC
NC55bpR	GTATGAGAGATCTTCCACGGTTCCT
NC3	
NC31bpF	CACAGGAGGAACCGTGGAAGATCTCTCATAC
NC31bpR	GTATGAGAGATCTTCCACGGTTCCTCCTGTG

[a]) Overlaps for Gibson assembly are written in bold letters. Restriction sites are underlined.

Supplemental figures

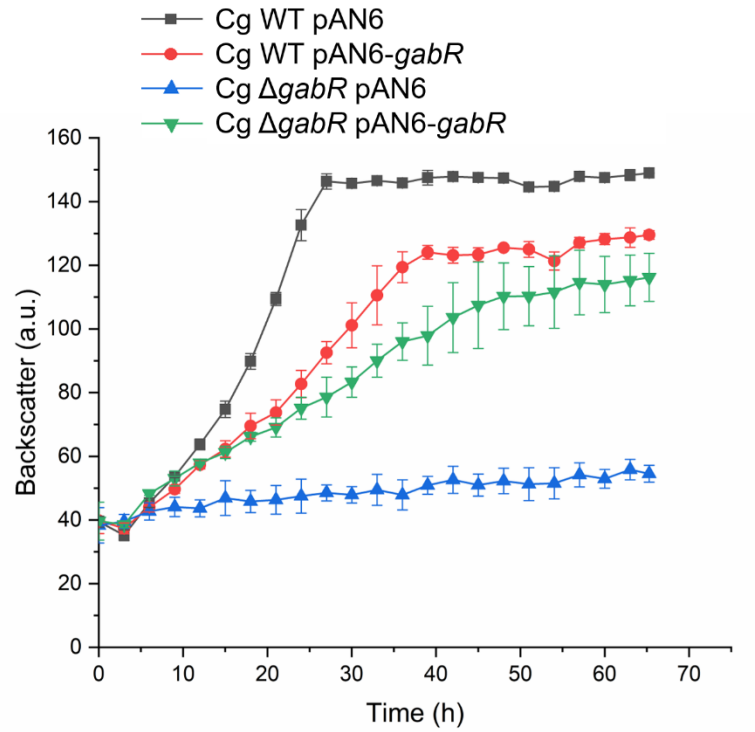

Fig. S1. Influence of *gabR* overexpression on growth of *C. glutamicum* WT and the Δ*gabR* mutant with GABA as sole carbon and nitrogen source. Growth experiments were performed with a Biolector microcultivation system and FlowerPlates containing 750 µl CGXII minimal medium with 62.5 mM GABA and 50 µM IPTG for induction of *gabR* expression. BHI medium was used for precultures and cells were washed with PBS buffer before inoculation of the main cultures. Mean values and standard deviations of three biological replicates are shown.

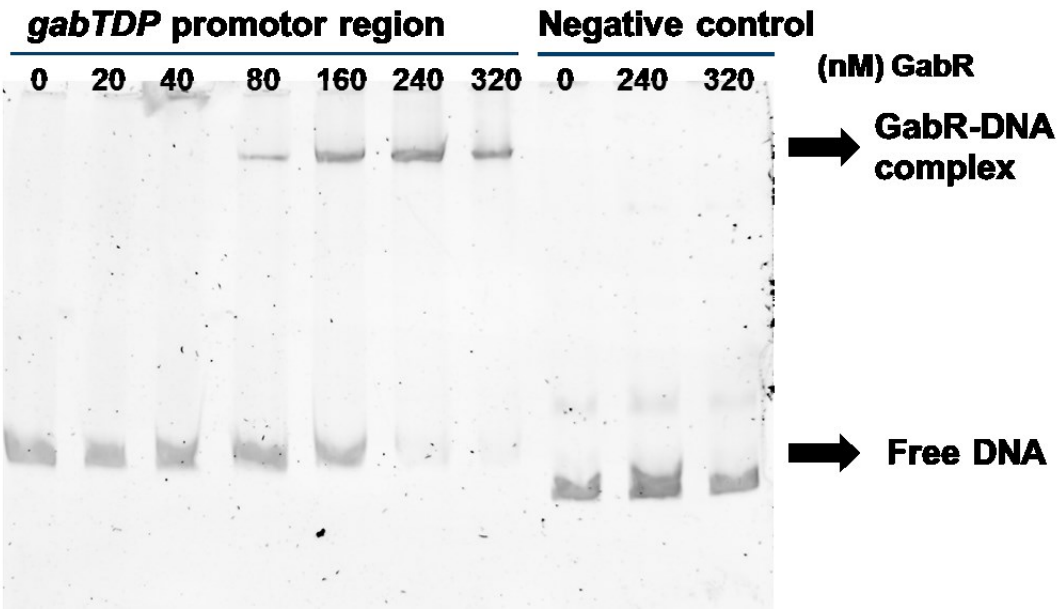

Fig. S2. Electrophoretic mobility shift assays with purified GabR and the *gabTDP* promoter region. A 500 bp fragment covering the *gabT* promoter region (100 ng) was incubated with increasing concentrations (given in nM of monomers) of purified GabR as indicated. A 470 bp fragment of the *ldhA* gene of *C. glutamicum* was used as negative control. Electrophoresis was performed using 6% native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl, 89 mM boric acid, pH 8.2) as running buffer (180 V, 45 minutes). Subsequently, the gel was stained with SYBR Green.

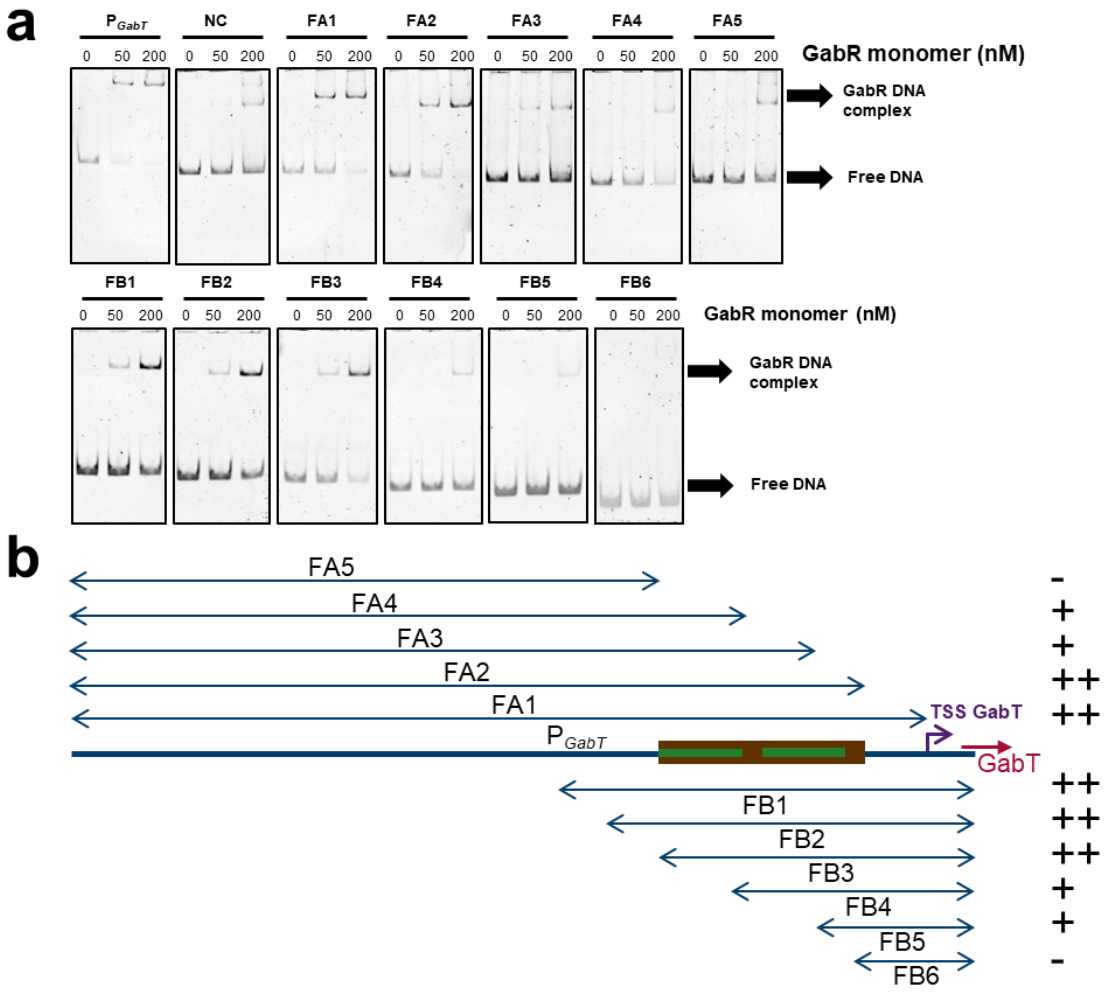

Fig. S3. Localization of the GabR binding region within the *gabT* promoter. (a) EMSAs with different fragments of the *gabT* promoter region (100 ng) incubated with the indicated concentrations of purified GabR (given in nM of monomers). A fragment of the *ldhA* gene of *C. glutamicum* was used as negative control. Electrophoresis was performed using 6% native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl, 89 mM boric acid, pH 8.2) as running buffer (180 V, 40 minutes). (b) Scheme showing the regions covered by the different fragments used in the EMSAs and the qualitative result of GabR binding to these fragments: ++, efficient binding, +, less efficient binding, -, no binding. The binding region derived from this experiment is marked in brown. Potential GabR binding sites are marked in green (compare Fig. 9). The translational start of *gabR* is marked with a red arrow, the TSS of *gabT* is marked with a purple arrow.

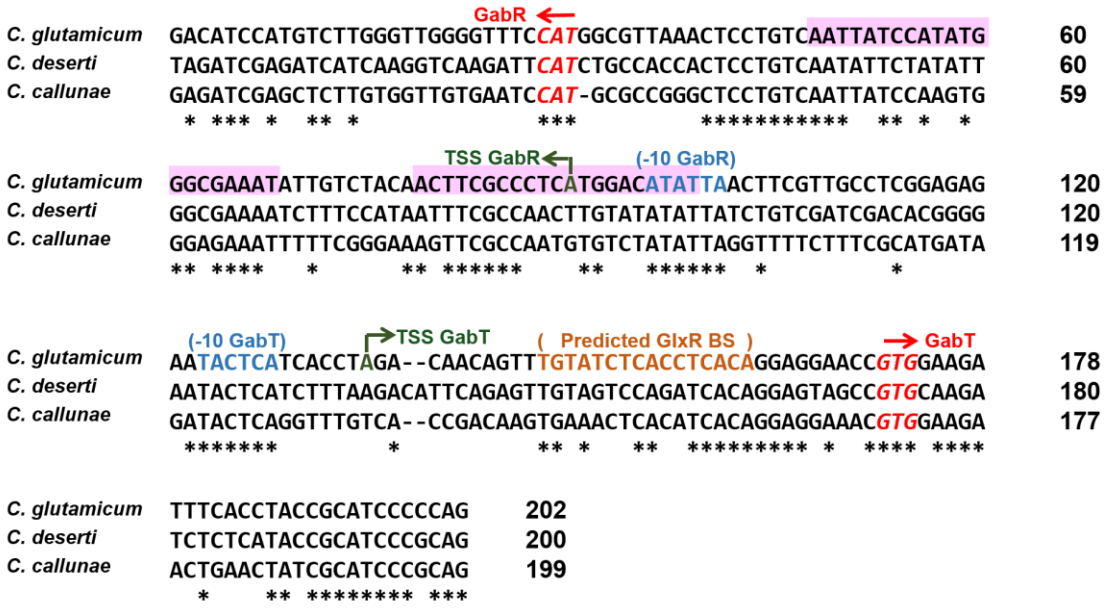

Fig. S4. Alignment of the *gabR-gabT* intergenic regions of the indicated *Corynebacterium* species including 30 bp of each coding region. The start codons of *gabR* and *gabT* are marked in red, the TSSs of GabT and GabR are indicated with green arrows, and the predicted GlxR binding site is marked in brown. The GabR binding sites are marked with pink background.

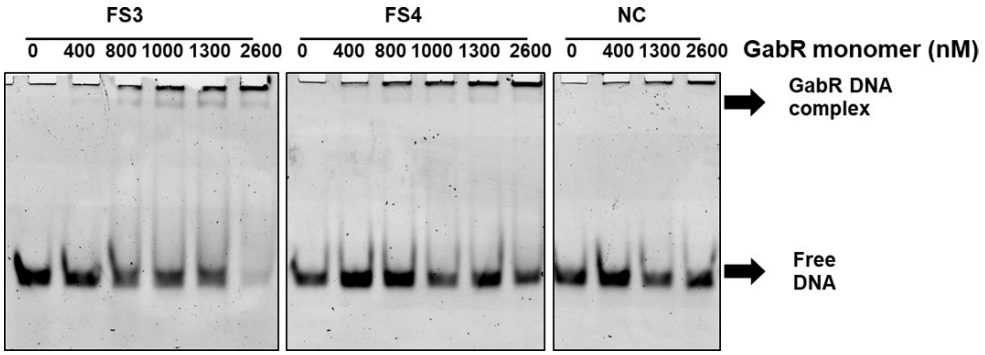

Fig. S5. Electrophoretic mobility shift assays using purified GabR and 31 bp fragments of the *gabTDP* promoter region. Two 31 bp fragments (FS3 and FS4) covering one predicted GabR binding site each were generated by annealing of two complementary oligonucleotides and incubated with different concentrations of GabR (nM of a monomeric form) as indicated. As negative control, a 31 bp fragment of the downstream of the predicted binding sites was used. Electrophoresis was performed using 8% native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl, 89 mM boric acid, pH 8.2) as running buffer (180 V, 40 minutes).

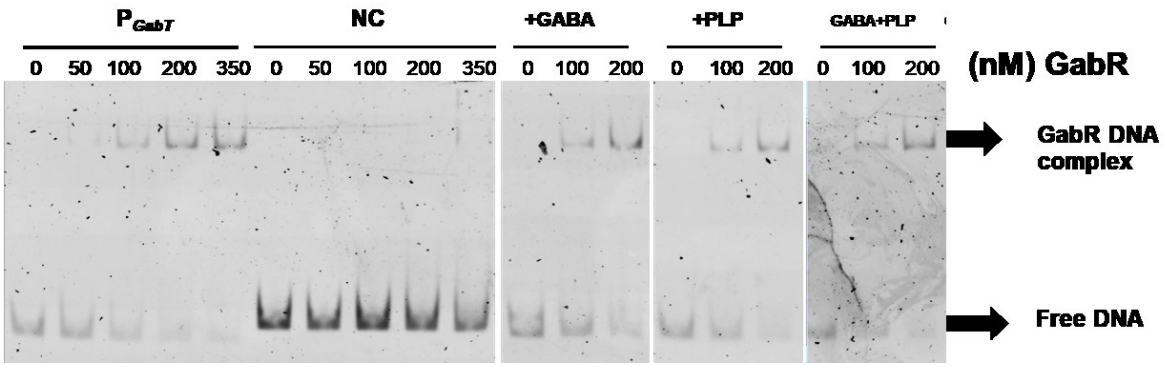

Fig. S6. Influence of GABA and PLP on binding of GabR to the *gabTDP* promoter region. A 65 bp fragment of the *gabT* promoter region (100 ng) was incubated with increasing concentrations of purified GabR (nM of monomeric GabR) as indicated. Electrophoresis was performed using 6% native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl, 89 mM boric acid, pH 8.2) as running buffer (180 V, 40 minutes). GABA (5 mM) and PLP (0.05 mM) were added to the binding buffer.

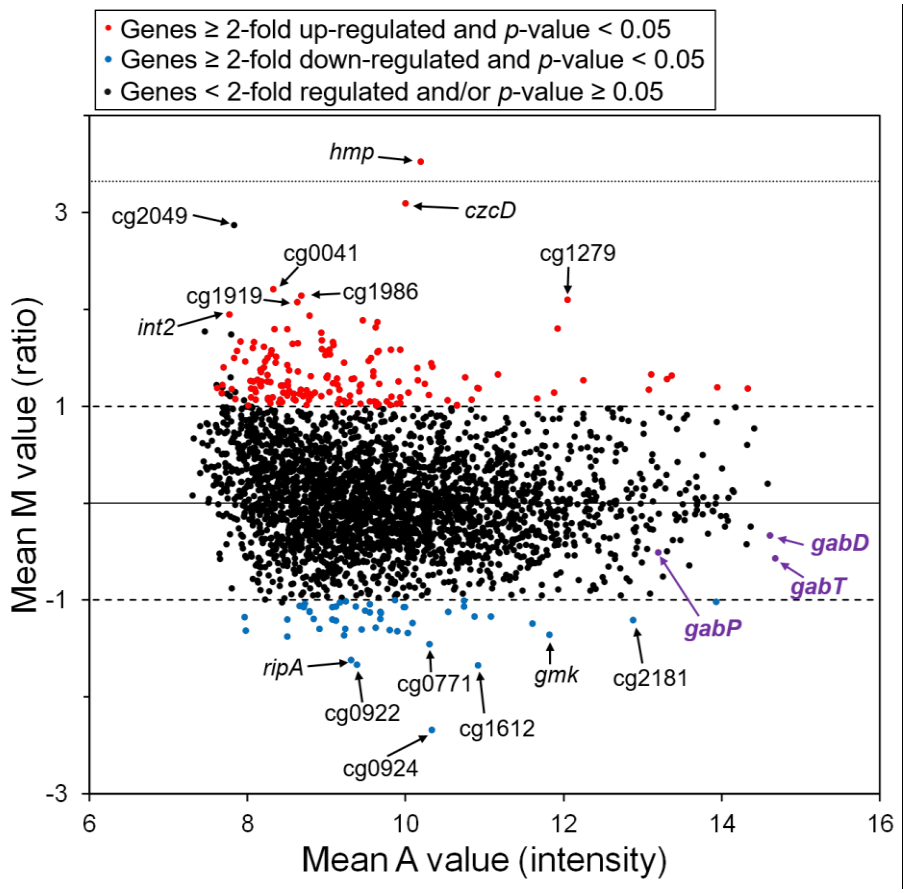

Fig. S7. M/A plot showing differential gene expression in *C. glutamicum* WT cells grown with GABA and $(NH_4)_2SO_4$ compared to WT cells grown with GABA and K_2SO_4 instead of $(NH_4)_2SO_4$. The data shown are based on four two-channel DNA microarray hybridizations starting with cDNA from four independent biological replicates. The dashed lines indicate a 2-fold altered mRNA ratio, the dotted line a 10-fold altered mRNA ratio. The genes *gabTDP* are marked in purple.

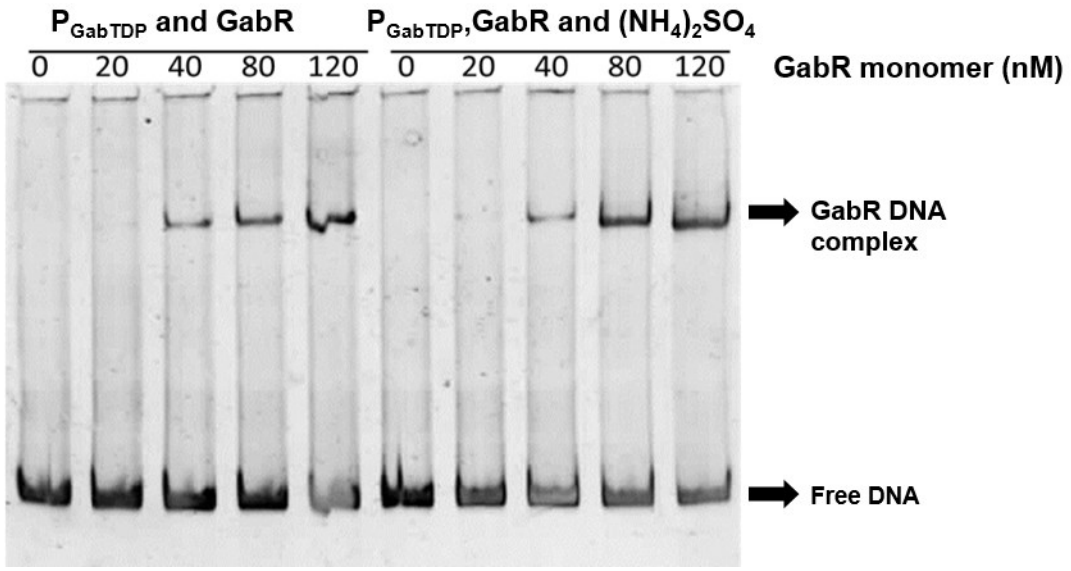

Fig. S8. Influence of 50 mM ammonium sulfate on the binding of GabR to the *gabTDP* promoter region. A 500 bp fragment covering the *gabT* promoter region (300 ng) was incubated with increasing concentrations of purified GabR (nM of monomeric form) as indicated. A 470 bp fragment of the *ldhA* gene of *C. glutamicum* was used as negative control. Electrophoresis was performed using 6% native polyacrylamide gels in an ice bath with TB buffer (89 mM Tris-HCl, 89 mM boric acid, pH 8.2) as running buffer (180 V, 45 minutes).

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The most important heartfelt thanks go to my parents for their unrestricted support.

Erklärung

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

_____

Jülich, den 18.12.2019