Identification and Characterization of γ -Aminobutyric Acid Uptake System GabP_{Cg} (NCgl0464) in *Corynebacterium glutamicum*

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Corynebacterium glutamicum is widely used for industrial production of various amino acids and vitamins, and there is growing interest in engineering this bacterium for more commercial bioproducts such as γ -aminobutyric acid (GABA). In this study, a *C. glutamicum* GABA-specific transporter (GabP_{Cg}) encoded by *ncgl0464* was identified and characterized. GabP_{Cg} plays a major role in GABA uptake and is essential to *C. glutamicum* growing on GABA. GABA uptake by GabP_{Cg} was weakly competed by L-Asn and L-Gln and stimulated by sodium ion (Na⁺). The K_m and V_{max} values were determined to be $41.1 \pm 4.5 \mu\text{M}$ and $36.8 \pm 2.6 \text{ nmol min}^{-1} (\text{mg dry weight [DW]})^{-1}$, respectively, at pH 6.5 and $34.2 \pm 1.1 \mu\text{M}$ and $67.3 \pm 1.0 \text{ nmol min}^{-1} (\text{mg DW})^{-1}$, respectively, at pH 7.5. GabP_{Cg} has 29% amino acid sequence identity to a previously and functionally identified aromatic amino acid transporter (TyrP) of *Escherichia coli* but low identities to the currently known GABA transporters (17% and 15% to *E. coli* GabP and *Bacillus subtilis* GabP, respectively). The mutant RES167 $\Delta\text{ncgl0464/pGXXZ9}$ with the GabP_{Cg} deletion showed 12.5% higher productivity of GABA than RES167/pGXXZ9. It is concluded that GabP_{Cg} represents a new type of GABA transporter and is potentially important for engineering GABA-producing *C. glutamicum* strains.

Transport of metabolic substrates/products across the cellular boundary is vital to microbial adaptation and survival in different environments. The understanding of such transport systems is also very important for the development and improvement of industrial technologies that exploit microbial fermentation or transformation (9, 13, 26). An example is that the manipulation of a tryptophan transport system in *Corynebacterium glutamicum* led to up to a 20% increase of tryptophan production (15).

γ -Aminobutyric acid (GABA) occurs widely in the living world as a nonprotein amino acid. It functions as the chief inhibitory neurotransmitter for the mammalian central nervous system and is involved in many other physiological processes such as induction of hypotension and diuretic effects. Being a potentially important pharmaceutical chemical and food additive, microbial production of GABA has attracted interest from research and industry (22). Many microbes are able to synthesize and metabolize GABA. GABA is generated from glutamic acid (GA) by a GA decarboxylase (GAD; EC 4.1.1.15) as a glutamate-dependent acid stress response and is effective for acid resistance in *Escherichia coli* (25). GABA can be assimilated as a nitrogen and/or carbon source in organisms such as *Saccharomyces cerevisiae* (1) and *E. coli* (2). During microbial assimilation, GABA is initially converted to succinic semialdehyde with a GABA oxoglutarate aminotransferase (GabT), and this product is then oxidized to succinate with succinic semialdehyde dehydrogenase (GabD). Genes encoding those enzymes have been identified in *E. coli* (2) and *Rhizobium leguminosarum* (34). Besides those genes encoding proteins of catalytic function, genes encoding GABA transporters were also identified in *E. coli* (33), *S. cerevisiae* (1), and *Bacillus subtilis* (10). Recently, two ABC-type GABA transport systems were identified in *R. leguminosarum* (12, 47).

Corynebacterium glutamicum has been traditionally and commercially used for production of various amino acids and vitamins (32). Very recently, there has been growing interest in manipulating the highly productive bacterium *C. glutamicum* into robust producing

strains for cadaverine (30), putrescine (38), polyhydroxy acids (27), isobutanol (5), succinate (23), as well as GABA (41). Transporters of C₄-dicarboxylates like succinate, fumarate, L-malate, and glutamate (20, 44, 49) in *C. glutamicum* have been characterized, but how GABA is transported and metabolized in *C. glutamicum* remains unknown. According to genome annotation, the *C. glutamicum* ATCC 13032 genome carries putative *gabD* (*ncgl0462/cg0567*) and *gabT* (*ncgl0463/cg0566*) genes (14, 17), indicating that *C. glutamicum* was possibly metabolizing GABA. Two additional genes (*ncgl1062* and *ncgl1108*) were annotated as GABA permeases (http://www.ncbi.nlm.nih.gov/nuccore/NC_003450). However, *C. glutamicum* NCgl1062 and NCgl1108 were experimentally identified to be a general aromatic amino acid transporter (AroP_{Cg}) (45) and an L-Phe-specific transporter (PheP_{Cg}) (50), respectively. In this study, we identified and experimentally verified that *ncgl0464* encodes the major and active *C. glutamicum* GABA transporter (GabP_{Cg}), and disruption of this gene is potentially important for construction of highly GABA-producing *C. glutamicum* strains.

MATERIALS AND METHODS

Bacterial strains and plasmids, cultural media, and growth conditions.

All the bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strains were grown aerobically in Luria-Bertani (LB) broth on a rotary shaker (200 rpm) at 37°C (36) or on LB plates with 1.2% (wt/vol) agar. *C. glutamicum* strains were routinely grown at 30°C on a rotary shaker (200 rpm) in LB broth. Minimal MMI medium (19) was applied to

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacY169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
<i>C. glutamicum</i>		
RES167	Restriction-deficient mutant of ATCC 13032; Δ <i>cglIIM</i> Δ <i>cglIIR</i> Δ <i>cglIIR</i>	University of Bielefeld
RES167 Δ <i>ncgl0464</i>	<i>ncgl0464</i> truncated, amino acids 113 to 222 deleted	This study
RES167 Δ <i>ncgl0464</i> /pGXKZ7	RES167 Δ <i>ncgl0464</i> containing pGXKZ7	This study
RES167/pGXKZ9	RES167 Δ <i>ncgl0464</i> containing pGXKZ9	This study
RES167 Δ <i>ncgl0464</i> /pGXKZ9	RES167 Δ <i>ncgl0464</i> containing pGXKZ9	This study
Plasmids		
pMD19-T	PCR cloning vector	Takara
pXMJ19	<i>E. coli</i> - <i>C. glutamicum</i> shuttle vector, Cam ^r <i>Ptac lacI^q</i> pBL1 <i>oriV_{Cg}</i> pK18 <i>oriV_{Ec}</i>	16
pK18 <i>mobsacB</i>	Mobilizable vector, allows selection of double crossover in <i>C. glutamicum</i>	37
pGXKZ7	pXMJ19 carrying PCR-amplified <i>ncgl0464</i> to generate <i>ncgl0464</i> expression or complementation for Δ <i>ncgl0464</i>	This study
pGXKZ8	pK18 <i>mobsacB</i> carrying Δ <i>ncgl0464</i> ; refer to RES167 Δ <i>ncgl0464</i>	This study
pGXKZ9	pXMJ19 carrying PCR-amplified <i>LVIS1847</i> from <i>Lactobacillus brevis</i> ATCC 367 to generate <i>LVIS1847</i> expression	This study

cultivate wild type and mutants of *C. glutamicum* when GABA, tryptophan, tyrosine, or phenylalanine was used as the sole carbon or the sole nitrogen source.

Genetic cloning, disruption, and complementation. The total genomic DNA of *C. glutamicum* was isolated according to the method of Tauch et al. (42). The entire *ncgl0464* gene of *C. glutamicum* was PCR amplified with primers 464F and 464R (see Table S1 in the supplemental material) and was subsequently ligated onto pXMJ19. The resulting plasmid, pGXKZ7, was transformed into *E. coli* cells (36) or electroporated into *C. glutamicum* (43).

A larger fragment (1,961 bp) overlapping the locus *ncgl0464* was PCR amplified using *Pfu* DNA polymerase (Takara, Japan) and the primers 464FK and 464RK (see Table S1 in the supplemental material). The PCR products were purified by using an agarose gel DNA fragment recovery kit (Sangon, China) and cloned into the pMD19-T simple vector (Takara, Japan), resulting in pMD19-*ncgl0464*. *In vitro* disruption of *ncgl0464* was performed by *HincII* restriction of pMD19-*ncgl0464*, thus removing the region from positions 814 to 1,150 bp (positions referring to *ncgl0464*) and resulting in pMD19- Δ *ncgl0464* carrying a truncated *ncgl0464*. The disrupted *ncgl0464* was PCR amplified with primers 464FK and 464RK and was ligated into pK18*mobsacB*, resulting in plasmid pGXKZ8. pGXKZ8 was transformed into *C. glutamicum* RES167 by electroporation (43). Screening for the first and second recombination events, as well as confirmation of the chromosomal deletion, was performed as described previously (37). The deletion of the target gene in pGXKZ8 and in *C. glutamicum* mutants was verified by PCR amplification and DNA sequencing. The resulting strain was designated *C. glutamicum* RES167 Δ *ncgl0464*.

For genetic complementation and expression, plasmid pGXZ7 was transformed into *C. glutamicum* RES167 Δ *ncgl0464* by electroporation. The expression of *ncgl0464* on pGXZ7 was induced by addition of 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) to culture broth.

Assays for GABA transport. Uptake experiments with unlabeled and ⁴⁻¹⁴C-labeled GABA were conducted according to the procedures described by Zhao et al. (50). The minimal medium used by Zhao et al. (50) was replaced by minimal MMI medium.

(i) **Uptake assay with unlabeled GABA.** *C. glutamicum* cells were grown in LB medium. Cells at exponential phase were harvested and washed with ammonium-free minimal MMI medium. The harvested cells were resuspended (cell density, optical density at 600 nm of 4 to 5) in minimal MMI medium containing 1 mM GABA and then incubated at

30°C. At the indicated intervals, portions of the reaction mixture were withdrawn, filtered, and analyzed with an Agilent 1200 high-pressure liquid chromatograph (HPLC) equipped with a Zorbax Eclipse-AAA column (Agilent).

(ii) **Uptake assay with ¹⁴C-labeled GABA.** *C. glutamicum* strains were cultivated and harvested according to the above-described procedures. Cells were washed twice with 0.1 M Tris phosphate buffer and resuspended in the same buffer. The reaction mixture (1 ml) contained 100 μ M Tris phosphate, 1 μ M MgSO₄, 10 μ M glucose, 100 μ g chloramphenicol, and 0.1 ml of the cell suspension (approximately 0.2 mg dry cells). The reaction was started by the addition of [4-¹⁴C]GABA (ARC, Inc.). At the indicated intervals, 50 μ l of the reaction mixture was withdrawn, vacuum filtered using nitrocellulose filters with a pore size of 0.45 μ m, and immediately washed two times with 2-ml portions of cold 0.1 M LiCl. The filters containing cells were put into 2.0-ml centrifuge tubes filled with scintillation liquid. Radioactivity was determined by a PerkinElmer MicroBeta liquid scintillation counter. The rate of GABA uptake was calculated with the data from ¹⁴C-labeling liquid scintillation counting. The uptake activity was expressed as nanomoles of GABA taken up per mg of cell dry weight (DW) under the conditions applied in this study.

Determination of kinetic constants. *K_m* and *V_{max}* were determined by linear regression using a Lineweaver-Burk reciprocal plot. The initial rates of [4-¹⁴C]GABA uptake at concentrations of 4, 9, 20, 50, 100, 300, and 500 μ M were determined by the amounts of GABA taken up into cells within 20 s. *K_m* and *V_{max}* were calculated according to the plot.

GABA production. To generate GABA-producing strains, the glutamate decarboxylase gene *gadB1* (*LVIS_1847*) of *Lactobacillus brevis* (41) was cloned into pXMJ19, resulting in pGXKZ9. For genetic expression, the plasmid pGXKZ9 was transformed into *C. glutamicum* RES167 and RES167 Δ *ncgl0464*. The expression of *gadB1* was induced by addition of 1 mM IPTG to the culture broth. After 12 h, cells were harvested and washed twice with 0.9% NaCl. Then, the cells were incubated in a 250-ml flask containing MMI medium (per liter: L-glutamic acid, 50 g; glucose, 4 g; Na₂HPO₄ · 12H₂O, 5 g; and NaH₂PO₄ · 2H₂O, 1 g). GABA production was analyzed using an Agilent 1200 HPLC equipped with Zorbax Eclipse-AAA columns (Agilent).

DNA and protein sequences and bioinformatic analyses. The genome sequence of *C. glutamicum* ATCC 13032 (accession no. NC_003450 and NC_006958) was retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/>). Gene and protein sequences were obtained from NCBI (<http://www.ncbi>

.nlm.nih.gov) and KEGG (<http://www.genome.jp/kegg>). Sequence comparisons were carried out using the BLAST service of NCBI (<http://blast.ncbi.nlm.nih.gov>).

RESULTS

Genomic data mining of genes relating to GABA metabolism.

The two genome projects (NC_003450 and NC_006958) resulted in high-quality *C. glutamicum* ATCC 13032 DNA sequences and revealed identical genetic clusters that were probably involved in GABA metabolism, namely, *ncgl0462* to *ncgl0464* from NC_003450 and *cg0566* to *cg0568* from NC_006958 (14, 17). *ncgl0462* and *cg0567* encoded a putative succinate-semialdehyde dehydrogenase (GabD_{Cg}). This putative GabD_{Cg} showed amino acid sequence identities to the previously and functionally identified GabD proteins of *E. coli* (41 to 44%), of *B. subtilis* (43%), and of *Mycobacterium tuberculosis* (31%). *ncgl0463* and *cg0566* encoded a putative GABA aminotransferase (GabT_{Cg}). This putative GabT_{Cg} showed 43 to 46% identical amino acids to the previously identified GabT proteins of *E. coli* and *B. subtilis* (see Table S2 in the supplemental material and references 1, 3, 7, 8, 10, 12, 18, 21, 24, 25, 28, 29, 31, 33, and 48).

The third gene, *ncgl0464* or *cg0568*, had been annotated for a putative hydroxyl- or aromatic amino acid transporter (26). BLAST-P searches showed that NCgl0464 had 29% amino acid sequence identity to the L-Tyr-specific permease of *E. coli* (TyrP_{Ec}). Although several GABA transporter systems have been identified, NCgl0464 did not show significant identities (less than 17%) to those proteins involved in the transport systems. Further analysis indicated that *ncgl0464* encodes a theoretical protein of 415 amino acid residues with a calculated molecular mass of 43.4 kDa. This theoretical protein was predicted to be an integral membrane protein with 11 putative transmembrane segments (TMs; TMHMM Server, version 2.0). The above-described analyses suggested that *ncgl0464* was possibly involved in either aromatic amino acid and/or GABA transport.

The mutant RES167 Δ ncgl0464 lost the ability to grow on GABA. In order to investigate its function, *ncgl0464* was disrupted in *C. glutamicum* RES167, resulting in the mutant RES167 Δ ncgl0464. RES167 Δ ncgl0464 and RES167 were cultivated in LB broth and MMI medium with L-Trp, L-Tyr, or L-Phe as the sole nitrogen source. The growth difference between RES167 Δ ncgl0464 and RES167 in LB broth was marginal. There was no observable difference between RES167 Δ ncgl0464 and RES167 in MMI medium with L-Trp, L-Tyr, or L-Phe as the sole nitrogen source. It seems that NCgl0464 does not play a major role for aromatic amino acid uptake in *C. glutamicum*.

RES167 Δ ncgl0464 and RES167 were also cultivated with GABA as the sole nitrogen or carbon source. Results showed that the wild-type strain RES167 was able to grow on GABA. However, the mutant strain RES167 Δ ncgl0464 lost the ability to grow on GABA. The ability to grow on GABA was restored in complemented strain RES167 Δ ncgl0464/pGXXKZ7 (see Fig. S1 in the supplemental material). These results demonstrated that *ncgl0464* was involved in GABA assimilation in *C. glutamicum*.

Mutant RES167 Δ ncgl0464 was impaired in uptake of GABA.

Combining the bioinformatic analysis and the above-described experimental results, it was deduced that *ncgl0464* encoded a GABA transporter. Uptake assays were conducted with unlabeled and 4-¹⁴C-labeled GABA. Figure 1A shows the results obtained with unlabeled GABA. It was observed that the bulk concentrations of GABA in the medium dropped quickly with RES167 and RES167 Δ ncgl0464/pGXXKZ7 but not with the mutant RES167

FIG 1 Uptake of GABA by *C. glutamicum* RES167 (filled squares), RES167 Δ ncgl0464 (empty triangles), and RES167 Δ ncgl0464/pGXXKZ7 (filled triangles) (pH 6.5). (A) Unlabeled GABA, HPLC assay. The initial concentration of GABA was 1 mM, which was calculated to be 100% uptake. (B) ¹⁴C-labeled GABA assay. The initial concentration of [¹⁴C]GABA was 10 μ M. Independent experiments were repeated three times, and the trends were the same.

Δ ncgl0464. Subsequently, uptake experiments with 4-¹⁴C-labeled GABA were carried out. Results showed that both RES167 and RES167 Δ ncgl0464/pGXXKZ7 imported GABA, and the expression of *ncgl0464* on the multicopy plasmid pGXXKZ7 resulted in an uptake rate in RES167 Δ ncgl0464/pGXXKZ7 even higher than that in RES167. Mutant RES167 Δ ncgl0464 lost the ability to take up any GABA (Fig. 1B). On the basis of these results, it was concluded that *ncgl0464* encoded a GABA transporter and here was named GabP_{Cg}.

GabP_{Cg} is a new transporter specific to GABA. The substrate specificity of GabP_{Cg} was examined with 4-¹⁴C-labeled GABA in the presence of 20-fold of unlabeled amino acids or structurally related compounds (Table 2). Results indicated that L-Gln and L-Asn resulted in 20.0 to 31.6% reductions in GABA uptake, but a 24% increase of 4-¹⁴C-labeled GABA uptake was found with 1,4-diaminobutane. None of the other compounds tested significantly affected the uptake of 4-¹⁴C-labeled GABA by GabP_{Cg}. Thus, it was concluded that GabP_{Cg} represented a new transporter and it was specific to GABA.

GabP_{Cg} activity was dependent on electrochemical ion potentials and subject to pH influence. In order to understand whether the ATP molecule or electrochemical potential was the driving force, uptake of GABA by GabP_{Cg} was examined in the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a molecule that is commonly used for depolarization of the membrane ion gradient. Results (Fig. 2A) showed that the presence of CCCP completely stopped the uptake of GABA by GabP_{Cg}. This result suggested that GabP_{Cg} relied on electrochemical potential for GABA transportation in *C. glutamicum* and did not rely on ATP molecules. We also observed the influence of pH (range, 6.5 to 8.0) on GabP_{Cg} activity. As shown in Fig. 2B, GabP_{Cg} was more active under neutral to slightly alkaline conditions. Further, it was observed that sodium ion (Na⁺) significantly increased the uptake of GABA at pH values of either 6.5 or 7.5 (Fig. 2C).

Uptake kinetics. The rates of uptake by GabP_{Cg} were determined at various concentrations (4, 9, 20, 50, 100, 300, and 500 mM) of [4-¹⁴C]GABA with RES167 Δ ncgl0464/pGXXKZ7. RES167 Δ ncgl0464 was run in parallel, but this mutant basically exhibited no uptake activity on GABA, as demonstrated in the previous experiments. As shown in Fig. 3, uptake rates were determined at pH values of 6.5 and 7.5. The *K_m* and *V_{max}* values were determined

TABLE 2 Effects of various amino acids and structurally related compounds on $[4\text{-}^{14}\text{C}]$ GABA uptake in *C. glutamicum* RES167 $\Delta\text{ncgl0464/pGXKZ7}$ (pH 6.5)

Competitor ^a	Relative rate (%) of $[4\text{-}^{14}\text{C}]$ GABA ^b uptake
Control	100.0 $\pm$ 5.0
GABA	15.4 $\pm$ 0.3
β -Alanine	118.7 $\pm$ 9.3
Succinate	103.6 $\pm$ 0.2
L,4-Diaminobutane	124.0 $\pm$ 7.0
5-Aminovaleric acid	112.7 $\pm$ 8.8
L-Gly	89.4 $\pm$ 0.3
L-Gln	68.4 $\pm$ 2.4
L-Glu	92.3 $\pm$ 2.4
L-Asn	80.0 $\pm$ 4.3
L-Asp	111.2 $\pm$ 4.4
L-Ser	89.5 $\pm$ 2.4
L-Cys	92.8 $\pm$ 7.3
L-Leu	99.8 $\pm$ 2.2
L-Ile	94.3 $\pm$ 2.1
L-Val	104.2 $\pm$ 4.4
L-Ala	100.4 $\pm$ 8.5
L-Trp	89.4 $\pm$ 8.9
L-Phe	100.7 $\pm$ 7.7
L-Tyr	99.7 $\pm$ 3.8
L-Thr	102.2 $\pm$ 4.2
L-Met	105.1 $\pm$ 7.7
L-Arg	104.5 $\pm$ 2.8
L-His	101.6 $\pm$ 5.1
L-Lys	109.6 $\pm$ 1.6
L-Pro	93.0 $\pm$ 4.9

^a The concentration of each competitor was 200 μM .

^b The concentration of $[4\text{-}^{14}\text{C}]$ GABA was 10 μM . The $[4\text{-}^{14}\text{C}]$ GABA uptake rate in the absence of competitors was determined to be $8.1 \pm 0.4 \text{ nmol min}^{-1} (\text{mg DW})^{-1}$, and this was calculated to be 100% uptake. Data are averages from three parallel determinations, and the standard deviations are provided.

to be $41.1 \pm 4.5 \mu\text{M}$ and $36.8 \pm 2.6 \text{ nmol min}^{-1} (\text{mg DW})^{-1}$, respectively, at pH 6.5. The K_m and V_{max} values at pH 7.5 were $34.2 \pm 1.1 \mu\text{M}$ and $67.3 \pm 1.0 \text{ nmol min}^{-1} (\text{mg DW})^{-1}$, respectively. GabP_{Cg} exhibited both a high affinity and activity on GABA at pH 7.5 compared to pH 6.5.

GABA production with a mutant with a GabP_{Cg} deletion. In order to generate an efficient GABA producer from *C. glutamicum* and to evaluate the impact of GabP_{Cg} deletion on GABA production, the glutamate decarboxylase gene *gadB1* from *L. brevis* was cloned into pXMJ19, and the resulting plasmid, pGXKZ9, was transformed into RES167 and RES167 $\Delta\text{ncgl0464}$. Results show that the rate of molar conversion of glutamate to GABA by mutant RES167 $\Delta\text{ncgl0464/pGXKZ9}$ was 5.5% higher than that by RES167/pGXKZ9. The productivity of GABA was 2.4 and 2.1 mg of GABA per mg of cell mass by RES167 $\Delta\text{ncgl0464/pGXKZ9}$ and RES167/pGXKZ9 (Table 3), respectively, which correspond to an amount 12.5% higher than that for the GabP_{Cg} mutant. These results clearly demonstrated that disruption of GabP_{Cg} increased the production of GABA by *C. glutamicum*.

DISCUSSION

GABA is currently produced via chemical synthesis. Due to the high demand from industry, biological system-based and environment-friendly processes are desired (41). GABA transport across the cell boundary is a critical step for high GABA produc-

FIG 2 (A) Effect of CCCP on uptake of ^{14}C -labeled GABA by *C. glutamicum* RES167 $\Delta\text{ncgl0464/pGXKZ7}$ at pH 7.5. The initial concentration of $[^{14}\text{C}]$ GABA was 10 μM . Filled squares, control, no addition; filled triangles, addition of CCCP at 0 s; filled diamonds, addition of CCCP at 25 s. (B) Uptake of ^{14}C -labeled GABA by *C. glutamicum* RES167 $\Delta\text{ncgl0464/pGXKZ7}$ at pH 6.0 (empty triangles), pH 6.5 (empty squares), pH 7.2 (filled triangles), pH 7.5 (filled squares), and pH 8.0 (filled diamonds). (C) Effect of Na^+ ion on GABA uptake in *C. glutamicum* RES167 $\Delta\text{ncgl0464/pGXKZ7}$ at pH 6.5 (gray columns) and pH 7.5 (black columns). The final concentration of NaCl, KCl, and NaGLU was 2 mM. Cells were washed twice with deionized H_2O , and then the cells were used in the experiments. Data are averages from 3 independent experiments.

tion by microbial processes. GabP_{Cg} is the first GABA transporter identified in *C. glutamicum*, and deletion of this transporter resulted in a 12.5% increase of productivity compared to that by the parent strain. GabP_{Cg} shows low amino acid sequence identity (less than 17%) to the currently known proteins involved in GABA transport systems and had responses to competitive inhibitors very different from those of the previously functionally characterized GABA transporters. Among the 20 amino acids tested,

FIG 3 Uptake of $[4\text{-}^{14}\text{C}]$ GABA by *C. glutamicum* RES167 $\Delta\text{ncgl0464/pGXKZ7}$ (filled diamonds, pH 6.5; filled squares, pH 7.5) and RES167 $\Delta\text{ncgl0464}$ (empty triangles, pH 7.5). The initial concentration of $[4\text{-}^{14}\text{C}]$ GABA was 1 to 500 μM .

TABLE 3 Effects of genetic deletion of *ncgl0464* in *C. glutamicum* on the extracellular accumulation and productivity of GABA

Strain	GABA production ^a (g · liter ⁻¹)	Molar conversion rate (%)	Productivity (mg · mg ^{-1b})
RES167/pGXXKZ9	23.6 ± 1.2	67.4	2.1
RES167 Δ <i>ncgl0464</i> /pGXXKZ9	25.6 ± 2.3	72.9	2.4

^a The initial concentration of glutamate was 50 g · liter⁻¹, and the incubation time was 1 h. Cell densities were 4.4 to 4.6 mg · ml⁻¹ for all tests.

^b mg of GABA produced per mg of cell mass in 1 h.

L-Asn and L-Gln, each at a concentration of 200 μ M, could compete only by 20.0 to 31.6% with GABA for uptake. Sodium cation (Na⁺) and 1,4-diaminobutane stimulated the uptake of GABA by GabP_{Cg}. In contrast, the uptake of GABA by the GabP of *E. coli* (GabP_{Ec}) was not competed by L-Asn and L-Gln but was competed by L-Asp at 20 mM by approximately 75% (33). The GabP of *B. subtilis* (GabP_{Bs}) showed an equal preference for L-Ala and GABA (6). Rv0522 of *Mycobacterium bovis* took both L-Arg and GABA as substrates (39). In addition, GabP_{Cg} shows low sequence identity to the previously functionally characterized GABA transporters. For example, GabP_{Cg} has only 17% sequence identity to GabP_{Ec} and 15% sequence identity to GabP_{Bs}. Relatively higher sequence identities (40 to 48%) were observed among GabP_{Ec}, GabP_{Bs}, and Rv0522 (12, 39, 45).

Topology prediction and sequence alignments revealed that GabP_{Cg} had 11 putative TMs. The transport of GABA by GabP_{Cg} was more active under neutral to slightly alkaline conditions. In contrast, other experimentally identified hydroxy/aromatic amino acid permease (HAAAP) family members such as TyrP (46), Mtr (11), and SdaC (40) in *E. coli* were symporters of substrate and H⁺ and usually have 12 TMs. Except for the two ABC-type GABA transporters (Bra and Gts) (12, 47), all previously identified microbial GABA transporters, including GabP_{Ec} in *E. coli* (33), GabP_{Bs} in *B. subtilis* (10), and UGA4, GAP1, and PUT4 in *S. cerevisiae* (1, 4), were secondary carriers of the amino acid-polyamine-organocation (APC) superfamily and functioned as solute:cation symporters and solute:solute antiporters (35). GabP_{Cg} is the sole member of the HAAAP family in *C. glutamicum* (26).

GabP_{Cg} plays a major role in GABA transport, and thus, it is essentially involved in GABA assimilation in *C. glutamicum*. It is considered to be the only GABA-specific transport system in *C. glutamicum*, since the GabP_{Cg} mutant (RES167 Δ *ncgl0464*) lost the ability to grow on GABA. A similar phenotype was reported when GabP_{Bs} was inactivated in *B. subtilis* (10). However, more than one GABA transport system has been detected in other microbes or their genomes (see Fig. S2 in the supplemental material). Deletion of *M. bovis* Gab (Gab_{Mb}) resulted in only a 75% decrease in GABA uptake, suggesting that a different and weak GABA transport system might exist (39). Transport of GABA in *S. cerevisiae* by 3 transport systems, a general amino acid permease (GAP1), the proline permease (PUT4), and a specific GABA permease (UGA4), was reported (1). *R. leguminosarum* has two GABA transport systems of the ABC type, the broad-substrate-specificity Bra system for branched-chain amino acids (12) and the GABA-specific Gts system (47). Considering the many physiological roles of GABA in the living world, i.e., as signal molecules in mammals, as shuttle molecules in symbiont *R. leguminosarum*

and its host plant (34), or in nitrogen or carbon nutrition for different microbes, the diverse GABA transport systems identified reflect only a small proportion of the long coevolution and adaptation to different environments.

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