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Role and Regulation of *Bacillus subtilis* Glutamate Dehydrogenase Genes

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The complete *Bacillus subtilis* genome contains two genes with the potential to encode glutamate dehydrogenase (GlutDH) enzymes. Mutations in these genes were constructed and characterized. The *rocG* gene proved to encode a major GlutDH whose synthesis was induced in media containing arginine or ornithine or, to a lesser degree, proline and was repressed by glucose. A *rocG* null mutant was impaired in utilization of arginine, ornithine, and proline as nitrogen or carbon sources. The *gudB* gene was expressed under all growth conditions tested but codes for a GlutDH that seemed to be intrinsically inactive. Spontaneous mutations in *gudB* that removed a 9-bp direct repeat within the wild-type *gudB* sequence activated the GudB protein and allowed more-efficient utilization of amino acids of the glutamate family.

Interconversions of 2-ketoglutarate and glutamate are a major link between carbon and nitrogen metabolism in all living organisms. These reactions are catalyzed by several enzymes: glutamate synthase [2-ketoglutarate + glutamine + NAD(P)H $\rightarrow$ 2 $\times$ glutamate + NAD(P)], glutamate dehydrogenase (GlutDH) [2-ketoglutarate + NH_3 + NAD(P)H $\leftrightarrow$ glutamate + NAD(P)], and glutamate aminotransferases (transaminases) [2-ketoglutarate + amino acid $\leftrightarrow$ glutamate + keto acid] (Fig. 1).

Synthesis of *Bacillus subtilis* glutamate synthase, encoded by the *gltAB* operon, is positively regulated by GltC, a member of the LysR family of transcriptional regulators (5, 9), and negatively regulated by TnrA (4), a protein that also regulates other genes involved in nitrogen metabolism (47). Recently, we demonstrated that GltC activity is modulated by the activity of cellular GlutDH(s) and could be related to the rate of 2-ketoglutarate production (4). In order to understand GltC regulation, we sought to characterize mutations in genes coding for *B. subtilis* GlutDHs. The very existence of GlutDH activity in *B. subtilis* was a matter of some controversy (24, 27, 40), but the complete *B. subtilis* genome sequence (28) contains two genes highly similar to GlutDH-encoding genes of other organisms. The putative products of *B. subtilis* genes *yweB* (*ipa-75d*) (19), located at 331.0° on the chromosomal map (28) and here called *rocG*, and *ypcA* (41), located at 205.2° (28) and here called *gudB*, consist of 424 and 426 amino acids (aa), respectively, and are 74% identical to each other, 51 to 54% identical to GlutDHs from another gram-positive bacterium, *Clostridium difficile* (29), and an archaeon, *Pyrococcus furiosus* (14), and similar to many other hexameric GlutDHs from different organisms. The functions of the two *B. subtilis* genes were not known. In this work we analyzed involvement of these two genes in utilization of amino acids of the glutamate family. The *rocG* gene proved to encode the major catabolic GlutDH, while *gudB* seemed to encode an intrinsically inactive GlutDH. Spontaneous mutations in *gudB* generated active catabolic GlutDH.

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MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains were grown at 37°C in DS nutrient broth medium or in TSS minimal medium (16) with 0.5% glucose or 0.4% succinate as a carbon source and a 0.2% nitrogen source. The same media with the addition of agar were used for growth of bacteria on plates. L broth or L agar (31) was used for growth of *Escherichia coli* strains. The following antibiotics were used when appropriate: chloramphenicol (2.5 μg/ml), neomycin (2.5 to 5 μg/ml), phleomycin (0.25 μg/ml), tetracycline (15 μg/ml), or the combination of erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml) for *B. subtilis* strains and ampicillin (50 to 100 μg/ml), kanamycin (25 μg/ml), or tetracycline (10 μg/ml) for *E. coli* strains.

DNA manipulations and transformation. The methods for plasmid isolation, agarose gel electrophoresis, use of restriction and DNA modification enzymes, DNA ligation, PCR, and electroporation of *E. coli* cells were as described by Sambrook et al. (34). *B. subtilis* chromosomal DNA was isolated by modification of a published procedure (16). Transformation of *B. subtilis* by chromosomal or plasmid DNA was performed by modification of the method of Anagnostopoulos and Spizizen (2).

Cloning of the *rocG* gene. A PCR product corresponding to the 5′ end of *rocA*, the gene immediately downstream of *rocG* (Fig. 2), was synthesized by using *rocA*-specific oligonucleotides. The 0.52-kb *Cla*I-*Xho*I fragment derived from this product was cloned in pBB544, a derivative of pBluescript SK(−) (Stratagene, Inc.), containing a neomycin resistance marker (7). The resulting plasmid, pBB901, was integrated into the chromosome of *B. subtilis* SMY, creating strain BB1252. To clone DNA adjacent to the site of integration of pBB901, the chromosomal DNA of strain BB1252 was digested with *Pst*I, self ligated, and introduced by electroporation into *E. coli* cells. The isolated plasmid, pBB907, had a 2.75-kb insert of chromosomal DNA carrying the entire coding regions of *rocG* and the upstream gene *yweA*.

Cloning of the *gudB* gene. A PCR product corresponding to an internal part of the *ypdA* gene (Fig. 3) was synthesized by using *ypdA*-specific oligonucleotides. The 0.41-kb *Eco*RI-*Kpn*I fragment derived from this product was cloned in pBB544 (7). The resulting plasmid, pBB902, was integrated into the chromosome of *B. subtilis* SMY. The chromosomal DNA of the resulting strain, BB1253, was digested with *Bam*HI and *Bgl*II, self ligated, and introduced by electroporation into *E. coli* cells. The isolated plasmid, pBB908, had a 2.33-kb insert of chromosomal DNA carrying the entire coding region of *gudB*.

Construction of a *rocG* null mutant. A deletion-insertion mutation within the *rocG* gene was created by replacing the 0.64-kb *Eco*RI-*Bst*BI fragment of pBB907 (Fig. 2) with a 1.6-kb *Eco*RI-*Acc*I *ble* cassette, excised from pJPM136 (25). (pJPM136 was constructed by cloning the 1.4-kb *Hin*cII-*Ssp*I fragment of pMK3 derivative [44]), carrying the *ble* gene of pUB110 [38] that determines resistance to phleomycin (Phl^r), into the *Sma*I site of pJPM1 [33]. The orientation of the *ble* gene in this construction coincides with that of the *lacZ* gene.) The orientation of the *ble* gene in the resulting Δ*rocG*::*ble* plasmid, pBB918 (Fig. 2), is opposite that of the *rocG* gene. pBB918 was introduced into *B. subtilis* SMY, and Phl^r Neo^s transformants, arising from double-crossover homologous recombination events, were selected. The replacement of the chromosomal *rocG* gene by the Δ*rocG*::*ble* allele in strain BB1267 was confirmed by comparing sizes of the PCR fragments from the wild-type and mutant *rocG* chromosomal loci.

Construction of a *gudB* null mutant. A deletion-insertion mutation within the *gudB* gene was created by replacing the 1.16-kb *Eco*RV-*Hin*dIII fragment of

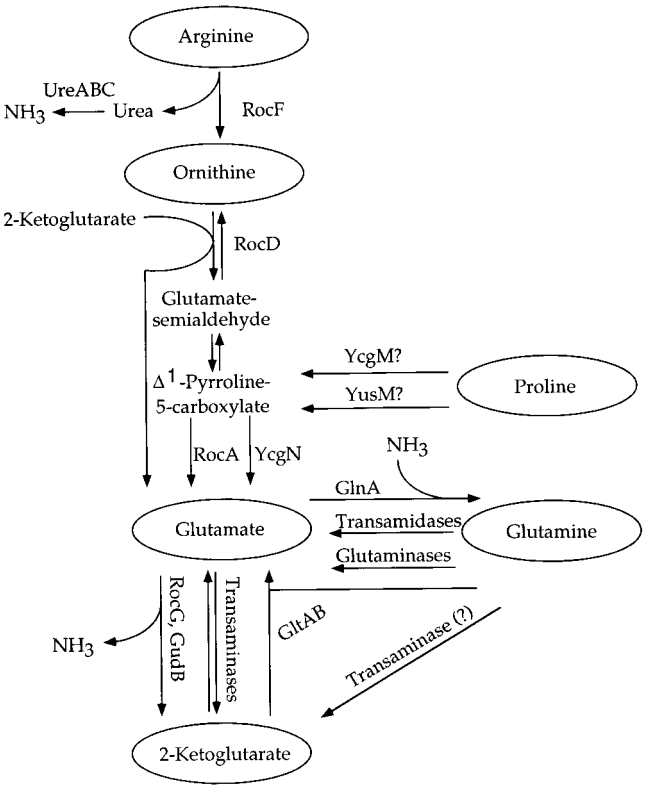

FIG. 1. Pathways of utilization of amino acids of the glutamate family. Some enzymes are indicated by the names of their corresponding genes as follows UreABC, urease; RocF, arginase; RocD, ornithine aminotransferase; RocA and YcgN, Δ^1-pyrroline-5-carboxylate dehydrogenases; YcgM and YusM, proline oxidases (proline dehydrogenases); RocG and GudB, glutamate dehydrogenases; GltAB, glutamate synthase; and GlnA, glutamine synthetase. Pathways of arginine and proline biosynthesis from glutamate are not shown.

pBB908 (Fig. 3) with a 1.9-kb *Bam*HI-*Hin*dIII *tet* cassette, excised from pBEST307 (23). The orientation of the *tet* gene in the resulting Δ*gudB*::*tet* plasmid, pBB920 (Fig. 3), is opposite that of the *gudB* gene. pBB920 was introduced into *B. subtilis* SMY, and Tet^r Neo^s transformants, arising from double-crossover homologous recombination events, were selected. The replacement of the chromosomal *gudB* gene by the Δ*gudB*::*tet* allele in strain BB1268 was confirmed by comparing sizes of the PCR fragments from the wild-type and mutant *gudB* chromosomal loci.

TABLE 1. Bacterial strains used in this study

Organism and strain	Genotype	Source or reference
E. coli JM107	*endA1 gyrA96 thi hsdR17* (r_k^- m_k^+) *supE44 relA1* λ$^-$ Δ(*lac-proAB*) e14$^-$/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15	48
B. subtilis		
SMY	Wild type	P. Schaeffer
BB1252	*rocA*::pBB901 (*neo*)	SMY × pBB901
BB1253	*ypdA*::pBB902 (*neo*)	SMY × pBB902
BB1267	Δ*rocG*::*ble*	SMY × pBB918
BB1268	Δ*gudB*::*tet*	SMY × pBB920
BB1271	Δ*rocG*::*ble* Δ*gudB*::*tet*	BB1267 × DNA of BB1268
BB1283	Δ*rocG*::*ble gudB1*	Spontaneous mutant of BB1267
BB1284	Δ*rocG*::*ble gudB1 ypdA*::pBB902 (*neo*)	BB1283 × pBB902
BB1302	*gudB1 ypdA*::pBB902 (*neo*)	SMY × DNA of BB1284
BB1401	Δ*amyE*::(*gudB′-lacZerm*)	SMY × pBB933

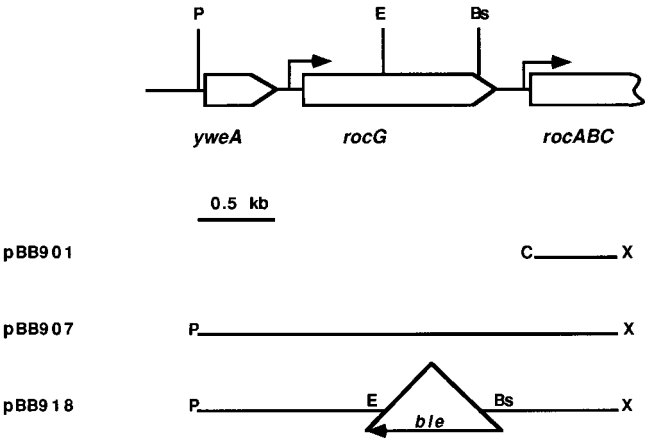

FIG. 2. Genetic map of the *rocG* region (19) and plasmids carrying different parts of this region. The restriction sites are abbreviated as follows: Bs, *Bst*BI; C, *Cla*I; E, *Eco*RI; P, *Pst*I; and X, *Xho*I. The *Cla*I and *Xho*I sites of the insert in pBB901 and derivative plasmids were constructed by PCR. All plasmids are derivatives of pBB544 (7). Transcription initiation sites for *rocG* (6) and *rocABC* (11) are shown by the arrows. The 1.4-kb *ble* cassette is not drawn to scale.

Mapping and cloning of the *gudB1* mutation. pBB902 (Fig. 3) was integrated into the chromosome of *B. subtilis* BB1283 (*rocG gudB1*), creating strain BB1284. The genetic linkage between the integrated plasmid and the *gudB1* mutation was scored by the disappearance of the pale colony phenotype characteristic of *rocG* mutants (see Results) during subsequent transformation of BB1267 (*rocG*) cells to neomycin resistance by using BB1284 chromosomal DNA. The *gudB1* allele of BB1284 was cloned as described above for the wild-type *gudB* allele, resulting in pBB928 (Fig. 3). The *gudB* mutations from other strains were cloned in a similar way.

Construction of a *gudB-lacZ* fusion. The 0.89-kb *Bst*YI fragment of pBB908 (Fig. 3) containing the 5′ part of *gudB* and 305 bp upstream of the *gudB* initiation codon was cloned at the *Bam*HI site of pJPM82 (7). The resulting plasmid, pBB933 (Fig. 3), contained the *gudB* fragment fused to the promoterless *E. coli lacZ* gene in the proper orientation. Strain BB1401 (Table 1), carrying a *gudB-lacZ* transcriptional fusion integrated at the *amyE* locus, was isolated after transformation of strain SMY with pBB933, selecting for resistance to erythromycin and screening for loss of α-amylase production, which indicated a double-crossover homologous recombination event. The Amy phenotype was assayed with colonies grown overnight on tryptose blood agar base (Difco)–0.2% starch plates (16).

RNA isolation and primer extension. Cells of *B. subtilis* were grown in TSS-glucose medium to late exponential phase. Pelleted cells from an 8-ml culture were resuspended in 0.1 ml of 20% sucrose–150 mM NaCl–1 mM EDTA and treated with lysozyme (0.4 mg/ml) for 20 min at 37°C. One milliliter of Trizol reagent (Gibco BRL, Life Technologies) was added, and RNA was extracted according to the manufacturer's instructions. Primer extension experiments were performed by a modification of a previously described protocol (34) with the use of Superscript II reverse transcriptase (Gibco BRL, Life Technologies) and as primers, oligonucleotide oBB57 (5′-CTTATGTATTACGGTTTGGGTTG) or oBB60 (5′-CAATTCGTATACCTCTTCGGG), corresponding to positions +81 to +58 and +123 to +103, respectively, relative to the *gudB* initiation codon.

DNA sequencing. Relevant parts of the *gudB* gene were sequenced by the dideoxy chain termination method of Sanger et al. (35) by using vector- or *gudB*-specific oligonucleotides as primers and a Sequenase reagent kit (Amersham Life Science) as recommended by the manufacturer. Plasmid double-stranded DNA to be sequenced was purified by a modification of an alkaline-lysis procedure (34) or by using a QIAprep Spin Miniprep Kit (Qiagen). DNA and protein sequences were analyzed by using DNA Strider and the BLAST program (1).

Enzyme assays. β-Galactosidase activity was determined as described previously (8). The specific activity was expressed in Miller units (31).

To determine GlutDH activity, 30-ml cell cultures in the mid-exponential stage of growth were harvested, washed, and subjected to sonication in 3 ml of 50 mM Tris-Cl (pH 7.5)–20% glycerol–100 mM NaCl–1 mM EDTA–1 mM phenylmethylsulfonyl fluoride. Cell extracts were clarified by low-speed centrifugation, and 10- to 100-μl samples were assayed at room temperature in 1 ml of 55 mM Tris-Cl (pH 7.5)–2% glycerol–10 mM NaCl–100 mM NH_4Cl–10 mM 2-ketoglutarate–0.2 mM NADH. Oxidation of NADH was monitored as the decrease in absorption at 340 nm. Nonspecific oxidation of NADH was determined from reactions lacking ammonia. One unit of GlutDH activity was defined as the amount needed to convert 1 nmol of NADH per mg of protein per min to NAD. The protein concentration was determined by the Bio-Rad Protein Assay with bovine serum albumin as a standard.

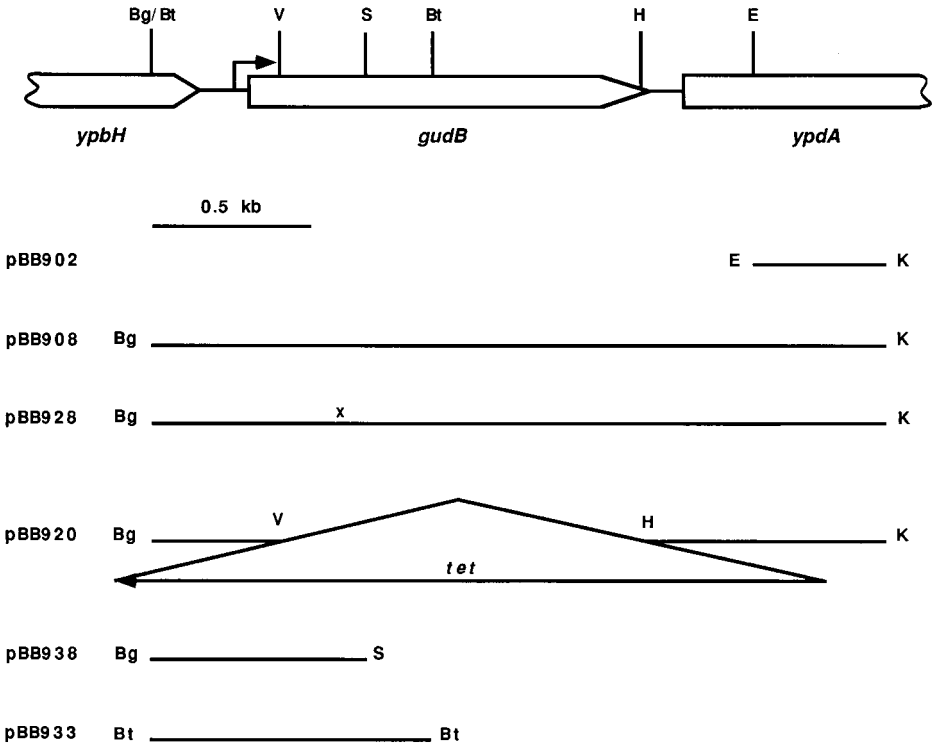

FIG. 3. Genetic map of the *gudB* region (41) and plasmids carrying different parts of this region. The restriction sites are abbreviated as follows: Bg, *Bgl*II; Bt, *Bst*YI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; S, *Sty*I; and V, *Eco*RV. Only relevant *Eco*RV, *Hin*dIII, and *Sty*I sites are shown. The *Kpn*I site of the insert in pBB902 and derivative plasmids was constructed by PCR. Plasmid pBB933 is a derivative of pJPM82 (7); other plasmids are derivatives of pBB544 (7). Construction of some plasmids is described in Materials and Methods; other plasmids were constructed by deleting or subcloning fragments of the *gudB* region. The location of the *gudB* transcription initiation site (see Results) is indicated by the arrow. The 1.9-kb *tet* cassette is not drawn to scale. x denotes the location of the *gudB1* mutation within pBB928.

RESULTS

Characterization of a *rocG* mutant. The *yweB* (*ipa-75d*) gene (19), which we had provisionally named *gudA*, was renamed *rocG* (6) because it belongs to the RocR regulon involved in utilization of arginine and ornithine (11, 17). *rocG* was cloned from the *B. subtilis* chromosome as a 2.8-kb *Pst*I-*Xho*I fragment (Fig. 2), as described in Materials and Methods. A 0.6-kb internal fragment of *rocG* was replaced by the *ble* marker (Fig. 2) and the mutant Δ*rocG*::*ble* gene was substituted for the wild-type *rocG* allele in the chromosome (see Materials and Methods), resulting in strain BB1267 (Table 1).

BB1267 grew more slowly in nutrient broth medium (generation time of 52 min versus 36 min for the wild-type strain) and had a reduced cell yield (final optical density at 600 nm of 1.1 to 1.2 versus 2.3 to 2.5 for a wild-type strain). BB1267 lost the ability to utilize proline, ornithine, or arginine (Fig. 1) as sole carbon source in minimal medium and grew more slowly when proline or ornithine was utilized as sole nitrogen source (Table 2). BB1267 formed heat-resistant spores in nutrient broth medium at a normal frequency, although sporulating cells tended to form clumps and the course of sporulation and spore release was delayed (data not shown). Another insertion in the *ipa-75d* (*rocG*) gene was constructed by P. Glaser (18a) but had not been characterized. We used this mutation in our preliminary experiments and found that the mutant strain had the same phenotype as strain BB1267 (data not shown).

Characterization of a Δ*gudB*::*tet* mutant. The *ypcA* gene (41) was renamed *gudB* because it encodes a second putative GlutDH of *B. subtilis*. *gudB* was cloned from the chromosome as a 2.3-kb *Bgl*II-*Kpn*I fragment (Fig. 3) as described in Materials and Methods. A 1.2-kb internal fragment of *gudB* was replaced by the *tet* marker (Fig. 3) and the mutant Δ*gudB*::*tet* gene was substituted for the wild-type *gudB* allele in the chromosome (see Materials and Methods), resulting in strain BB1268 (Table 1).

No growth defects were detected for strain BB1268 in nutrient broth or in minimal medium (data not shown). The phenotype of a *rocG gudB* double null mutant was very similar to the phenotype of a *rocG* single mutant. We conclude that

TABLE 2. Growth of *B. subtilis* strains in TSS minimal medium

Carbon source	Nitrogen source	Generation time of strain		
		SMY (wild type)	BB1267 (Δ*rocG*::*ble*)	BB1284 (Δ*rocG*::*ble gudB1*)
Glucose	Ammonia	57	56	61
	Glutamine	51	ND[a]	ND
	Glutamate	126	133	57
	Proline	80	110	60
	Ornithine	66	127	57
	Arginine	53	52	57
Proline	Proline	92	NG[b]	90
Ornithine	Ornithine	82	NG	146
Arginine	Arginine	79	NG	138

[a] ND, not done.
[b] NG, no growth under these conditions.

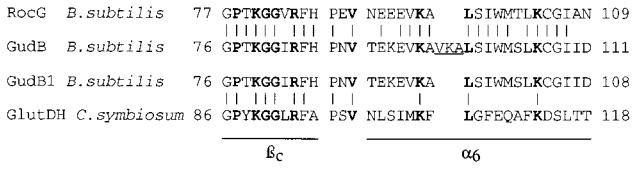

FIG. 4. Partial alignment of the RocG, GudB, GudB1, and *C. symbiosum* GlutDH (45) sequences. Amino acids are numbered with respect to their positions in corresponding proteins. The amino acids comprising β_c and α_6 of *C. symbiosum* GlutDH (3) and the site of a deletion of 3 aa in GudB are underlined. Residues conserved in most GlutDHs (45) are in bold. *C. symbiosum* K89 and K113 are essential lysines involved in glutamate binding (42).

rocG encodes the major GlutDH of *B. subtilis* and that *gudB* contributes little to the total cellular GlutDH activity.

Isolation of *gudB* gain-of-function mutants. Strain BB1267 (*rocG*) forms pale colonies on DS nutrient broth plates, probably due to lower growth yield in this medium. Wild-type-like papillae arising spontaneously at high frequency on the surface of these pale colonies were isolated. These pseudorevertants regained the ability to utilize proline, ornithine, or arginine as sole carbon source and also acquired the ability to utilize glutamate or glutamine as sole carbon source (a wild-type strain cannot grow with glutamate or glutamine as sole carbon source). Spontaneous mutants capable of utilizing glutamate or glutamine as sole carbon source were also directly isolated from a wild-type strain after prolonged incubation in liquid minimal medium containing one of these amino acids as sole carbon and nitrogen sources. The latter mutants formed wild-type-like colonies even when they carried deletions in *rocG*.

Four independent mutations (two from *rocG* pseudorevertants, one from a glutamate-utilizing strain, and one from a glutamine-utilizing strain) were mapped to the vicinity of the *gudB* locus (see Materials and Methods and data not shown). By complementation analysis using an integrative plasmid pBB938 (Fig. 3) and similar plasmids, the mutations were mapped to the 5′ part of the *gudB* gene, upstream of the *Sty*I site (Fig. 3) (data not shown). In accordance with this result, no Gud^+ pseudorevertants could be obtained from a Δ*rocG* Δ*gudB* double mutant. Since these results implied that the mutations were gain-of-function alleles of *gudB*, the chromosomal *gudB* regions of the four *gudB* mutants were cloned (see Materials and Methods) and sequenced. All mutations, called *gudB1*, turned out to be identical deletions of 9 bp in the 5′ coding part of the *gudB* gene. This region of the wild-type *gudB* gene (which was resequenced and found to be identical to the published sequence) contains a direct repeat of 9 nucleotides, GTGAAGGCG, corresponding to a direct repeat of 3 aa at positions 93 to 95 and 96 to 98 of the 426-aa GudB protein. The *gudB1* deletion precisely eliminated one copy of the repeat in both the nucleotide and amino acid sequences (Fig. 4).

Characterization of *gudB1* mutants. Strain BB1284 containing the *gudB1* and Δ*rocG*::*ble* mutations, in addition to its ability to utilize glutamate or glutamine as sole carbon source, also grew faster than a wild-type strain in glucose minimal medium with glutamate, proline, or ornithine as sole nitrogen source (Table 2). In contrast, this strain had a slight growth defect in glucose-ammonia minimal medium that was more pronounced on glucose-ammonia agar plates (Table 2 and data not shown). The growth phenotype of a *gudB1* single mutant (strain BB1302) in glucose medium was very similar to the growth phenotype of strain BB1284 (data not shown); i.e., the activity of RocG did not affect the GudB1 phenotype.

GlutDH activities of wild-type and mutant strains. GlutDH activity was determined in cell extracts in the presence of ammonia and 2-ketoglutarate as substrates (see Materials and Methods). The level of total GlutDH activity in cells grown in nutrient broth medium was low in early exponential phase but reached higher levels in the middle and late stages of exponential growth (Table 3 and data not shown). GlutDH activity was not detected in cells grown in nutrient broth medium in the presence of glucose (Table 3), in agreement with earlier results on total GlutDH activity (27). GlutDH activity was very similar in a wild-type strain and in a Δ*gudB*::*tet* mutant, but very little, if any, activity was detected in a Δ*rocG*::*ble* mutant or in a double *rocG gudB* null mutant (Table 3). Thus, RocG but not GudB contributed to GlutDH activity in wild-type cells grown in nutrient broth medium.

In cells grown in minimal medium, RocG activity, tested in strain BB1268 (Δ*gudB*::*tet*), was very low whenever glucose was present, and detectable activity was seen only when proline, ornithine or arginine was added (Table 4). High RocG activity was observed in the presence of ornithine or arginine when succinate replaced glucose or when these amino acids served as sole carbon and nitrogen sources (Table 4). Intermediate activity was detected when proline served as sole carbon and nitrogen sources (Table 4). The mechanism of glucose repression of RocG and the SigL-dependent regulation of RocG by RocR will be detailed elsewhere (6). GlutDH activity in a

TABLE 3. GlutDH activity of a wild-type strain and GlutDH mutants in nutrient broth medium[a]

Medium	GlutDH activity of[a]:				
	SMY (wild type)	BB1268 (Δ*gudB*::*tet*)	BB1267 (Δ*rocG*::*ble*)	BB1271 (Δ*rocG*::*ble* Δ*gudB*::*tet*)	BB1284 (Δ*rocG*::*ble gudB1*)
DS (nutrient broth)	346	440	≤45	≤45	447
DS (nutrient broth) + 1% glucose	≤45	≤45	≤45	≤45	250

[a] Cells of the indicated strains were grown in DS medium to mid-exponential growth phase (optical density at 600 nm of 0.6 to 0.8). GlutDH activity was assayed and expressed in units as described in Materials and Methods. All numbers are averages of at least two experiments, and the mean errors did not exceed 30%.

TABLE 4. GlutDH activity in minimal medium[a]

Carbon source	Nitrogen source	GlutDH activity of:	
		RocG	GudB1
Glucose	Ammonia	≤45	159
Glucose	Glutamate	≤45	178
Succinate	Glutamate	≤45	ND[b]
Glucose	Glutamine	≤45	ND
Succinate	Glutamine	≤45	ND
Glucose	Proline	76	182
Succinate	Proline	67	391
Proline	Proline	246	465
Glucose	Ornithine	74	158
Succinate	Ornithine	792	430
Ornithine	Ornithine	769	633
Glucose	Arginine	97	106
Succinate	Arginine	998	ND
Arginine	Arginine	1,040	662

[a] Cells were grown in TSS medium. GlutDH activity was assayed and expressed in units as described in Materials and Methods. Strains BB1268 (Δ*gudB*::*tet*) and BB1284 (Δ*rocG*::*ble gudB1*) were used for determination of RocG and GudB1 activities, respectively. All numbers are averages of at least two experiments, and the mean errors did not exceed 30%.

[b] ND, not done.

TABLE 5. Expression of a *gudB-lacZ* fusion in minimal medium[a]

Carbon source	Nitrogen source	β-Galactosidase activity
Glucose	Ammonia	50
	Glutamine	102
	Glutamate	172
	Proline	183
	Ornithine	89
	Arginine	84
Proline	Proline	181
Ornithine	Ornithine	162
Arginine	Arginine	144

[a] Cells of strain BB1401 were grown in TSS medium. β-Galactosidase activity was assayed and expressed in Miller units as described in Materials and Methods. All numbers are averages of at least two experiments, and the mean errors did not exceed 20%.

wild-type strain was indistinguishable from activity in a Δ*gudB*::*tet* mutant (data not shown). No GlutDH activity was detected in *rocG* mutant strain BB1267 under any growth conditions (data not shown), reflecting very low activity or lack of activity of the wild-type GudB in minimal medium.

Intermediate to high levels of GudB1-GlutDH activity could be measured in a Δ*rocG*::*ble gudB1* strain, BB1284, both in nutrient broth and in minimal media (Tables 3 and 4); these activities were decreased two- to sixfold when glucose was present but varied little when cells were grown with different nitrogen sources.

In accordance with previous results on total GlutDH activity in *B. subtilis* cells (27), RocG and GudB1 were specific with respect to NADH and were not active with NADPH as substrate. With glutamate as substrate and NAD as cofactor, the catabolic activities of these enzymes were about 10-fold-less efficient than anabolic activities (data not shown). The inactivity of wild-type GudB, the specific requirements for RocG activation in minimal media, and the very low catabolic activity of RocG in vitro probably explain the failure to detect GlutDH activity in *B. subtilis* in several previous studies (reviewed in reference 27).

Expression of the *gudB* gene. A *gudB-lacZ* transcriptional fusion containing the entire intergenic region between *gudB* and the preceding gene, including its putative transcription terminator (Fig. 3), was constructed (see Materials and Methods). This fusion was active in an otherwise wild-type strain, BB1401, in glucose-ammonia medium; expression of the fusion increased 1.7- to 3.7-fold when glutamate or related amino acids served as nitrogen source (Table 5). Changes in expression of the *gudB-lacZ* fusion under different growth conditions did not correlate perfectly with the activity of GudB1 measured under similar growth conditions (Table 4), but only relatively small variations in activity were seen in each case. Increasing the osmolarity of the medium by the addition of 0.5 M NaCl, which should lead to a highly elevated cellular proline pool (46), did not affect *gudB* expression (data not shown). The *gudB-lacZ* fusion was expressed in nutrient broth medium at a level similar to that in minimal medium and was not significantly affected by the presence of glucose (data not shown).

A primer extension experiment identified the putative transcription start point of *gudB* at position G (−46) with respect to the initiation codon (Fig. 5). Sequences similar to the −35 and −10 regions of a σ^A-dependent promoter could be identified upstream of the *gudB* transcription start point (Fig. 5).

Attempts to identify the regulator of nitrogen source-dependent *gudB* expression have been unsuccessful so far. Expression of the *gudB-lacZ* fusion was affected less than twofold by mutations in genes known to be involved in nitrogen-source-dependent regulation, including *sigL* (13), *tnrA* (47), *glnR* (37), *ahrC* (30), *gltC* (9), or *codY* (39) (Table 6). In no case was nitrogen-source-dependent regulation eliminated. Mutations in *ccpA* (20), *spo0A* (21), *sigB* (32), or *sigD* (32) also had little or no effect on the regulation of *gudB* expression (Table 6). A mutation in *abrB* (43) reduced *gudB-lacZ* expression threefold in glucose-ammonia medium. *gudB* expression was not affected by Δ*rocG*::*ble* or Δ*gudB*::*tet* mutations; in a *gudB1* strain, *gudB-lacZ* activity in glucose medium was reduced 2.4-fold (Table 6).

DISCUSSION

GlutDH, a ubiquitous metabolic enzyme, catalyzes a reversible reaction that can carry out either the anabolic function of glutamate biosynthesis or the catabolic function of glutamate utilization. In some organisms, both functions are active under different physiological conditions (40). It has been known for many years, however, that *B. subtilis* has little or no anabolic GlutDH, since it possesses only one route of glutamate biosynthesis from ammonia, catalysis by glutamate synthase (36). Although in vitro the anabolic activities of RocG and GudB1 are much higher than their catabolic activities, under no conditions tested could either of these *B. subtilis* GlutDHs compensate in vivo for the defect in glutamate synthesis caused by a glutamate synthase mutation (4). Both RocG and GudB1 utilize NAD(H), rather than NADP(H), as is typical of catabolic enzymes (40). Thus, our data show that both *rocG* and *gudB* (or at least *gudB1*) code for catabolic GlutDHs, involved in utilization of glutamate and other amino acids of the glutamate family.

Our results explain the general inability of *B. subtilis* strains of 168 lineage, derived from strain SMY (10), to utilize glutamate or glutamine as sole carbon source (4, 22), despite the presence of two GlutDH genes. Production of one GlutDH, RocG, requires the presence of arginine, ornithine, or proline; this enzyme is not induced by glutamate or glutamine. The second GlutDH, GudB, while synthesized in the presence of glutamate or glutamine, has either undetectably low enzymatic activity or none at all. Only constitutive expression of RocG, for instance, in strains carrying a *rocR*^{c} mutation (6, 18) or activation of GudB by an unusual gain-of-function mutation, as described herein, allows cells to utilize glutamate or glutamine as sole carbon source. It is likely that at least some reports of growth of *B. subtilis* strains with glutamate as sole carbon source were due to accumulation of *gudB1*-like mutations or mutations causing constitutive expression of *rocG*.

Cells that grow with glutamate, proline, ornithine, or arginine as sole nitrogen source need to produce ammonia in order to synthesize glutamine via the glutamine synthetase reaction (36). Catabolic GlutDHs could be responsible for ammonia production from all these amino acids (Fig. 1). Slow utilization of glutamate as sole nitrogen source (Table 2) again reflects the lack of induction of RocG by glutamate and the inactivity of wild-type GudB. Accordingly, the growth rate in glucose-glutamate medium is not affected by null mutations in *rocG* or *gudB* or both and is highly increased when GudB is made active by the *gudB1* mutation. Aminotransferases, enzymes that transfer the amino group of glutamate to various keto acids, are likely responsible for the low rate of utilization of glutamate as sole nitrogen source in a wild-type strain. Under these conditions ammonia is probably produced by degradation of some of the amino acids generated by transamination.

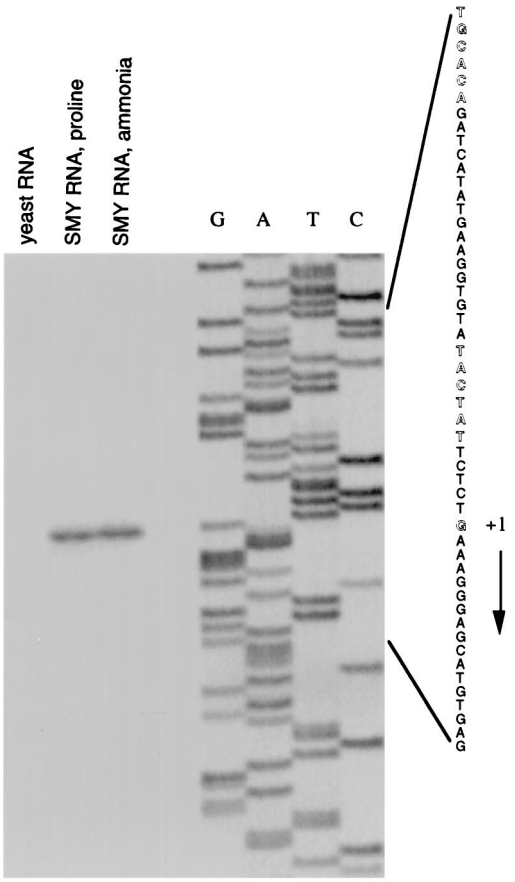

FIG. 5. Primer extension analysis of *gudB* mRNA. Primer oBB60 was extended with reverse transcriptase by using total RNA from *B. subtilis* SMY grown in glucose minimal medium containing ammonia or proline as nitrogen source or from *Saccharomyces cerevisiae* (Sigma Chemical Company) as template. The sequence of the nontemplate strand of plasmid pBB917 deduced from sequencing reactions with oBB60 as primer is shown on the right. The apparent transcription start site of *gudB* and the −10 and the −35 regions of the likely *gudB* promoter are indicated by outlined letters. Primer oBB57 gave the same apparent *gudB* mRNA 5′ end (data not shown). The direction of transcription is shown by the arrow.

TABLE 6. Expression of a *gudB-lacZ* fusion in regulatory mutants in minimal medium[a]

Genotype	β-Galactosidase activity in the presence of indicated nitrogen source	
	Ammonia	Proline
Wild type	50	183
tnrA	58	179
glnR	47	179
gltC	NG[b]	209
codY	39	124
ahrC	90	160
abrB	15	150
ccpA	55	119
spo0A	62	150
sigL	51	203
sigB	35	151
sigD	40	140
Δ*gudB*::*tet*	57	197
Δ*rocG*::*ble* Δ*gudB*::*tet*	57	221
gudB1	21	75

[a] Strain BB1401 (*gudB′-lacZ*) and its derivatives were grown in glucose minimal medium. β-Galactosidase activity was assayed and expressed in Miller units as described in Materials and Methods. All numbers are averages of at least two experiments, and the mean errors did not exceed 20%.
[b] NG, no growth under these conditions.

The ability of proline or ornithine to provide intermediate growth rates as sole nitrogen source in glucose medium (Table 2) is probably due to induction of RocG. In accord with this conclusion, a *rocG* null mutant utilized proline or ornithine as sole nitrogen source at the same low rate as it utilized glutamate (Table 2). Less than full induction of RocG-GlutDH activity is sufficient for utilization of ornithine or proline as nitrogen source inasmuch as RocG is poorly expressed in glucose-ornithine or glucose-proline minimal medium (Table 4), but cells grow significantly faster with ornithine or proline than with glutamate as sole nitrogen source (Table 2). More-efficient utilization of ornithine and proline in the presence of glucose was provided by higher levels of activity of GudB1-GlutDH (Tables 2 and 4).

Arginine supports a high growth rate because, in addition to generating glutamate by the three-step *roc* pathway, it liberates urea, which can be degraded to ammonia by the action of urease (Fig. 1), even though the latter is not highly active under these conditions (12). A high growth rate with glutamine as sole nitrogen source (Table 2) is expected, since, in this case, the cells do not need to generate ammonia to synthesize glutamine and glutamate can be synthesized from glutamine by glutamate synthase, by transamidation reactions, or by two putative glutaminases, YbgJ and YlaM (28) (Fig. 1). Because the high growth rates conferred by glutamine or arginine as sole nitrogen source are mostly determined by GlutDH-independent pathways, it is not surprising that growth rates on these substrates were little affected by the lack of GlutDH in a *rocG* mutant (Table 2). In a double *rocG ureC* mutant, lacking urease activity, utilization of arginine as sole nitrogen source was severely impaired (data not shown).

The GlutDH activities of RocG and GudB1 correlated well with the ability of different strains to utilize amino acids of the glutamate family as sole nitrogen sources (Tables 2 and 4). For unknown reasons, little or no correlation was seen when these amino acids were utilized as sole carbon sources (Tables 2 and 4).

Alignment of RocG and GudB sequences showed a single significant gap that coincided with the tandem repeat of 3 aa which is deleted in the *gudB1* mutant (Fig. 4). The same 3-aa gap is seen in pairwise alignments of the wild-type GudB sequence with sequences of other GlutDHs in GenBank (Fig. 4 and data not shown), indicating that the wild-type *gudB* sequence contains an insertion of 3 aa (9 bp) with respect to the common ancestral GlutDH sequence. The mechanism by which this 3-aa (9-bp) insertion occurred is unclear. Elimination of this insertion in the spontaneous *gudB1* mutants could arise by strand slippage mispairing during DNA replication (15). A similar 9-bp deletion within a stretch of DNA containing a near perfect repeat of 9 bp in the *torS* gene of *Escherichia coli* has been described (26). Interestingly, the latter deletion was also a gain-of-function mutation and led to formation of a constitutively active protein (26). Maintenance of the 9-bp insertion in the wild-type *gudB* sequence could be explained by the slight growth defect of *gudB1* mutants in glucose-ammonia medium, presumably because the glutamate-synthesizing activity of glutamate synthase is overwhelmed by the glutamate-degrading activity of GudB1.

The GudB and GudB1 sequences were modelled against the available three-dimensional structures of GlutDH from *Clos-*

tridium symbiosum (3, 42) and *P. furiosus* (49). The 3-aa insertion within GudB was localized to helix α_6 of GlutDH (Fig. 4), and it likely affects both the amino acid composition of this helix and its relative orientation with respect to the upstream strand β_C. Both β_C and α_6 form part of the substrate binding and catalytic pocket of GlutDH and could be involved in intersubunit interactions (3, 42).

In summary, wild-type *B. subtilis* has two genes for GlutDH, one of which (*rocG*) encodes an enzyme that is induced by arginine, ornithine, or proline and contributes to use of these compounds as carbon or nitrogen sources. The second GlutDH gene (*gudB*) encodes a protein with very low or no enzymatic activity. A frequently occurring spontaneous mutation renders this enzyme active, suggesting that natural or laboratory conditions in which *B. subtilis* strains of Marburg/168 lineage have been grown select for emergence and maintenance of the inactive form of GudB.

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