Enhancement of Glutamine Utilization in *Bacillus subtilis* through the GlnK-GlnL Two-Component Regulatory System

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During DNA microarray analysis, we discovered that the GlnK-GlnL (formerly YcbA-YcbB) two-component system positively regulates the expression of the *glsA-glnT* (formerly *ybgJ-ybgH*) operon in response to glutamine in the culture medium on Northern analysis. As a result of gel retardation and DNase I footprinting analyses, we found that the GlnL protein interacts with a region (bases –13 to –56; +1 is the transcription initiation base determined on primer extension analysis of *glsA-glnT*) in which a direct repeat, TTTTGTN4T TTTGT, is present. Furthermore, the *glsA* and *glnT* genes were biochemically verified to encode glutaminase and glutamine transporter, respectively.

Various organisms have developed sophisticated signaling systems for eliciting a variety of adaptive responses to their environment. The two-component regulatory system is one such signaling system in prokaryotes, lower eukaryotes, and plants, and it consists of at least two signal transduction proteins (the sensor kinase and the response regulator). The sensor kinase senses environmental or intercellular signals and then transmits them to the response regulator through phosphoryl group transfer. The response regulator often binds to the promoter regions of target genes and regulates their transcription through activation or repression (28).

Genome sequencing of *Bacillus subtilis* has revealed the presence of 36 sensor kinases and 35 response regulators, each of 30 kinase-regulator pairs residing in an operon on the genome (20). According to the classification of sensor kinases as to the sequence around the phosphorylated histidine, CitS, DctS, MalK, and GlnK (formerly YcbA) belong to the same group of kinases (group IV), which are paired with CitT, DctT, MalR, and GlnL (formerly YcbB) on the genome, respectively (9). Out of the four kinase-regulator pairs, three (the exception being GlnKL) have been reported to sense the presence of the respective tricarboxylic acid cycle intermediates in the culture medium and to positively regulate each system of their utilization. The CitST system positively regulates the expression of *citM*, which encodes the Mg-citrate transporter (36), whereas DctST enhances the transcription of the *ydbEFGH* operon, which is involved in the utilization of fumarate and succinate (3). Recently, the MalKR system was reported to trigger the transcription of *ywkA*, which encodes a malic enzyme (8), and that of *maeN* and *yflS* (30), which encodes malate transporters, indicating that this system is involved in the transport and utilization of malate. Thus, only the function of the fourth

system (GlnKL) belonging to this group remained to be characterized.

Glutamine, the best nitrogen source for *B. subtilis*, serves as an amino acid for protein synthesis and a nitrogen donor for the synthesis of various nitrogenous molecules in the cell (10). It also functions as a gauge of the nitrogen supply level in the cell, being a feedback inhibitor of glutamine synthetase. Glutamine synthetase interacting with glutamine is able to trap and inhibit TnrA, which functions as a global regulator under nitrogen-limited conditions (34). Therefore, uptake of glutamine from the medium and its de novo synthesis by glutamine synthetase, as well as glutamine utilization, should be rigorously and highly regulated in the cell.

In this communication, we report that the GlnK-GlnL two-component system positively regulates the expression of the *glsA-glnT* (formerly *ybgJ-ybgH*) operon in response to glutamine in the culture medium. It was verified that the *glsA* and *glnT* genes encode the glutaminase and glutamine transporter, respectively, indicating that the GlnKL system is involved in glutamine utilization.

MATERIALS AND METHODS

Bacterial strains and cultivation media. The *B. subtilis* strains used in this study are listed in Table 1. *Escherichia coli* BL21(DE3) (Novagen, Inc.) was used for overproduction of His-tagged GlnL and GlsA. *E. coli* BL21(DE3) was cultured in Luria-Bertani (LB) medium (26). *B. subtilis* cells were precultured at 30°C overnight on tryptose blood agar base (Difco) plates supplemented with 0.18% glucose (TBABG) and then cultivated in a liquid minimal medium (MM) plus tryptophan (50 µg/ml) (38) containing 13.6 mM glutamine or 13.6 mM Na glutamate plus 20 mM NH₄Cl as the nitrogen source at 37°C, with shaking. If necessary, ampicillin (50 µg/ml), erythromycin (0.3 µg/ml), or chloramphenicol (5.0 µg/ml) was added to the medium.

***B. subtilis* mutant construction.** Strain FU707 carrying a *glnQ* deletion (Δ *glnQ::cat*) was constructed as follows. The PCR fragment in which the regions flanking the *glnQ* gene sandwiched the *cat* gene was prepared by means of long-flanking homology PCR (32). The two regions upstream and downstream of *glnQ* were amplified by PCR using primer pairs (glnQ-F1 and glnQ-F2 and glnQ-R1 and glnQ-R2, respectively) (Table 2) and DNA of strain 168 as a template. In addition, the chloramphenicol acetyltransferase gene (*cat*) cassette was amplified by PCR using a primer pair (cat-M1 and cat-M2) (Table 2) and DNA of plasmid pCBB31, a derivative of plasmid pUC118 carrying *cat* from plasmid pC194 (17). Joint PCR (32) involving the above three PCR products and

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TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source
168	<i>trpC2</i>	Wild type (laboratory stock)
YCBbd	<i>glnL::pMUTIN trpC2</i>	JAFAN ^a
YBGJd	<i>glsA::pMUTIN trpC2</i>	JAFAN
YBGHd	<i>glnT::pMUTIN trpC2</i>	JAFAN
1A71	<i>gltA1 trpC2</i>	BGSC ^b
FU707	<i>ΔglnQ::cat trpC2</i>	This study
FU708	<i>ΔglnQ::cat glnT::pMUTIN trpC2</i>	This study
FU801	<i>gltA1 glsA::pMUTIN trpC2</i>	This study

^a JAFAN, Japan Function Analysis Network for *B. subtilis*.^b BGSC, *Bacillus* Genetic Stock Center (Ohio University).

a primer pair—a nested primer pair (*glnQ*-F11 and *glnQ*-R22) (Table 2)—resulted in a product, which was used for transformation of strain 168 to chloramphenicol resistance (5 μg/ml). Correct deletion of the *glnQ* gene from strain 168 was confirmed by means of PCR and sequencing.

Strain 708 (*ΔglnQ::cat ybgH::pMUTIN*) was constructed by transformation of strain FU707 (*ΔglnQ::cat*) with DNA of strain YBGHd (*glnT::pMUTIN*) to erythromycin resistance (0.3 μg/ml). Strain FU801 (*gltA1 glsA::pMUTIN*) was constructed by transformation of strain 1A71 with PCR products amplified by the use of primer pairs (*glsA*-M1/*glsA*-M2) (Table 2) to erythromycin resistance (0.3 μg/ml).

β-Galactosidase (β-Gal) assay. *B. subtilis* cells carrying *lacZ* fusions were grown overnight at 30°C on TBAG containing erythromycin (0.3 μg/ml). The cells were inoculated into 50 ml of MM medium containing glutamine or glutamate plus ammonium as the nitrogen source and then incubated at 37°C. During growth, 1-ml aliquots of the culture were withdrawn, and β-Gal activity was determined as described previously (38).

Northern analysis. *B. subtilis* cells were grown in MM medium containing glutamine or glutamate plus ammonium as the nitrogen source at 37°C to optical density at 600 nm (OD₆₀₀) of 0.6 (mid-logarithmic phase). Total RNA was extracted and purified from cell pellets as described previously (39). For Northern analysis, RNA was electrophoresed in a glyoxal gel and then transferred to a Hybond-N membrane (Amersham Biosciences) (26). To prepare probes for the detection of transcripts carrying *glsA*, *glnT*, and *ybgG*, the respective products amplified by PCR using primer pairs (*glsA*-N1-*glsA*-N2, *glnT*-N1-*glnT*-N2, and *ybgG*-N1/*ybgG*-N2) (Table 2) and chromosomal DNA of strain 168 as a template were labeled with a BcaBEST labeling kit (Takara Shuzo Co., Ltd., Kyoto, Japan) and [α -³²P]dCTP (Amersham Biosciences). Hybridization and transcript detection were carried out as described previously (26).

Primer extension. Primer extension analysis was performed essentially as described previously (37). To map the 5' end of the *glsA-glnT* transcript by primer extension, each of the total RNAs (each, 45 μg) used for Northern analysis was annealed to primer EX-*glsA* (Table 2), corresponding to bases (+76 to +95) that had been labeled at the 5' end with a MEGALABEL kit (Takara Shuzo) and [γ -³²P]ATP (Amersham Biociences). Primer extension was carried out with a thermostable reverse transcriptase (Thermoscript; Invitrogen Corp.) in 40 μl of a reaction mixture comprising 50 mM Tris-acetate (pH 8.4), 75 mM potassium acetate, 8 mM magnesium acetate, 1 mM each deoxyribonucleoside triphosphate, 1 mM dithiothreitol, 40 U RNase OUT (Invitrogen), and 30 U Thermo-riple at 58°C for 1 h. The resultant cDNAs were subjected to urea-polyacrylamide gel electrophoresis as described previously (12). A template for the dideoxy sequencing reactions for ladder preparation starting from the same end-labeled primer was prepared by PCR using a primer pair (*glsA*-F1-EX-*glsA*) (Table 2) and DNA from strain 168 as a template.

Production and purification of GlnL and GlS. To clone and express the *glnL* gene in *E. coli*, the *glnL* region was amplified by PCR using a primer pair (*glnL*-O1-*glnL*-O2) and DNA of strain 168 as a template. The PCR product, which had been digested with BamHI and SphI, was cloned in *E. coli* JM109 into vector pDGHsN2 with selection of an ampicillin-resistant transformant (50 μg/ml), which was a derivative of plasmid pDG148 (a shuttle between *E. coli* and *B. subtilis*) (29). The *glnL* gene cloned in plasmid pDGHsN2 carrying *lacI* was placed under the control of the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *Pspac* promoter, and its product was His tagged at the N terminus. (Construction of plasmid pDGHsN2 by K. Asai remains to be reported.) When *E. coli* BL21(DE3) cells bearing the resultant plasmid pGLNL had grown to an OD₆₀₀ of 0.4 at 37°C in LB medium containing ampicillin (50 μg/ml), 1 mM IPTG was added to induce His-tagged GlnL. After 4 h of incubation, the cells

were harvested, washed twice with 50 mM Tris-Cl buffer (pH 8.0), and then suspended in 50 mM Tris-Cl buffer (pH 8.0) containing 10% (vol/vol) glycerol. The cells were disrupted by sonication, and a supernatant was obtained by centrifugation (10,000 × g; 10 min). His-tagged GlnL was purified to homogeneity by nickel affinity chromatography with the use of a His-binding kit (Novagen).

To produce the GlS protein in *E. coli*, the *glsA* region was amplified by PCR using a primer pair (*glsA*-O1-*glsA*-O2) (Table 2) and DNA of strain 168 as a template; the reverse primer (*glsA*-O2) was designed to eliminate the authentic NdeI site by replacement of A by G. The resulting PCR product was ligated with vector pCR2.1-TOPO, and the ligated DNA was used for transformation of *E. coli* TOP10, according to the instructions for the TA-Cloning kit (Invitrogen) to generate plasmid pTA-GLSA. After correct cloning of the fragment into plasmid pTA-GLSA had been confirmed by nucleotide sequencing, the plasmid DNA was digested with NdeI and NotI. The resulting fragment was ligated with the NdeI-NotI arm of vector pET-22b (Novagen), and the ligated DNA was used for transformation of *E. coli* BL21(DE3) to produce plasmid pGLSA. The GlS protein was overexpressed in *E. coli* BL21(DE3) by the addition of IPTG to the medium, and a crude cell extract was prepared as described above.

Gel retardation and DNase I footprinting experiments. Gel retardation and DNase I footprinting experiments were performed as described previously (40). The purified His-tagged GlnL protein was prepared as described above. This protein was treated at 37°C for 1 h in 50 μl of a reaction mixture containing 50 mM Tris-Cl (pH 7.5), 5 mM MgCl₂, 50 mM acetylphosphate (Sigma), and 1 mM dithiothreitol, essentially as described previously (25). For gel retardation analysis, the probe DNA containing the *glsA-glnT* promoter region (bases -205 and +25) was a PCR product amplified from DNA of strain 168 using a primer pair (*glsA*-F1-*glsA*-F2). The probe was labeled with a BcaBEST labeling kit and [α -³²P]dCTP (Amersham Biosciences). For DNase I footprinting, the same probe DNA was prepared by PCR amplification using the above primers (*glsA*-F1-*glsA*-F2), either of which were labeled at the 5' terminus with a MEGA-LABEL kit and [γ -³²P]ATP (Amersham Biosciences).

Paper chromatography to identify the enzyme reaction product of GlS. The reaction mixture (50 μl) comprising 50 mM potassium phosphate buffer (pH 7.2) and 20 mM L-glutamine was incubated overnight at 30°C after the addition of a crude cell protein extract (10 μg), which had been prepared from *E. coli* BL21(DE3) cells carrying either plasmid pGLSA or pET-22b, as described above. An aliquot (5 μl) of the reaction mixture was subjected to paper chromatography together with authentic L-glutamate and L-glutamine solutions, the developing solvent being *n*-butanol:acetic acid:H₂O at a ratio of 4:1:1. Spraying with 0.7% ninhydrin localized the spots of the reaction product and two other authentic amino acids on the chromatogram.

TABLE 2. Primers used in this study

Primer	Sequence
<i>glnT</i> -N1	TGCAATCTTCTGGATGTGGA
<i>glnT</i> -N2	TTGAAAAGATTCCCGCTCTG
<i>glsA</i> -N1	GAGCCTGATGGAACGATGAT
<i>glsA</i> -N2	TCGTGTGCAATCGGATGATA
<i>ybgG</i> -N1	AACGCAAGGGCTGTAATTTG
<i>ybgG</i> -N2	TTTGGCTTCAGTCAGACACG
Ex- <i>glsA</i>	CGAATTCCTCCTCACTGAT
<i>glsA</i> -F1	AGGGCCAGTATATATCCGTC
<i>glsA</i> -F2	AACGCTTTCATAATAGCGGA
<i>glsA</i> -O1	GAGGAATCATATGAAAGAATTGATAAAAGA ^a
<i>glsA</i> -O2	TTAAAAATGCTGAGCTCCCATTCCTCGCCATGTG ^b
<i>glnL</i> -O1	GGAGGATCCATGCGTTTTTTTATTGCAGAT ^a
<i>glnL</i> -O2	CTCCTCGAGCATGCTTATATCCTTCTTGTTCATC G ^a
<i>glsA</i> -M1	CTTCATGAATCTCTCCGCCAGTCTCAGGA
<i>glsA</i> -M2	GGCATCCGAAACAGAAATGATAAAAGCGGT
<i>glnQ</i> -F1	CAACAAGAACCCTTGACT
<i>glnQ</i> -F11	TCGCAATTAACAACGAGGAACAGCTCCGA
<i>glnQ</i> -F2	GTATCCGCTCACATTCGCAATCCTCCTATCAGAA
<i>glnQ</i> -R1	CGTCGTGACTGGGAAACATAAACAGAAATGGGGGAGC
<i>glnQ</i> -R2	ACTGCCGGCCAGCGCGATA
<i>glnQ</i> -R22	ACACTTGGCAGCAAGGTATGGTAAAAACCG
cat-M1	GAATTGTGAGCGGATAAC
cat-M2	GTTTTCACGTCACGACG

^a Restriction enzyme sites are underlined.^b The boldface G was introduced in place of A to eliminate the NdeI site.

Measurement of L-glutamine uptake. L-Glutamine uptake by strains of *B. subtilis* was measured essentially by a previously described method (13, 40). After cells had been grown at 37°C to an OD₆₀₀ of 1.0 in MM medium containing glutamate plus ammonium as the nitrogen source with or without 5 mM IPTG, 6 ml of the culture was harvested and washed in MM medium plus chloramphenicol (100 µg/ml). The washed cells were suspended in 4 ml of the same medium plus chloramphenicol (100 µg/ml) and then used for measurements. L-Glutamine uptake assays were performed with a final L-glutamine concentration of 500 µM. The assay tubes held 0.7 ml of MM medium plus chloramphenicol (100 µg/ml) containing L-[U-¹⁴C] glutamine (22.2 kBq; 9,250 MBq/mmol) (American Radiolabeled Chemicals, Inc.) and 930 µM L-glutamine and were then kept at 37°C. L-Glutamine uptake was initiated by the addition of 0.6 ml of a cell suspension to each assay tube. After incubation at 37°C for 3 min, the suspension was filtered through a moistened glass microfiber filter (2.4-cm diameter) (GF/C; Whatman). The filter was immediately washed three times with 5 ml of MM medium plus chloramphenicol (100 µg/ml). The filter was dried, and radioactivity was counted in 10 ml of ASCII scintillant (Amersham Biosciences).

RESULTS

Induction of the *glsA-glnT* operon by glutamine, which is positively regulated through the GlnK-GlnL two-component regulatory system. Comprehensive DNA microarray analysis of *B. subtilis* two-component regulatory systems (19) revealed that one of the candidate target genes positively regulated by the GlnKL system is *glnT*, which is adjacent to and located divergently from the *glnKL* genes and appears to constitute an operon with *glsA* (Fig. 1A). The GlS protein exhibited high similarities to glutaminases from various organisms in a protein similarity search involving the BLASTP 2.2.9 program (2; data not shown). A GlS homolog of *Rhizobium etli* has been verified to be a glutaminase (5). So, we performed Northern analysis with specific probes for *glsA*, *glnT*, and *ybgG* to reveal the operon organization of the three genes and to determine if these genes are induced by glutamine and positively regulated by GlnKL (Fig. 1B). *B. subtilis* cells grow almost equally well on glutamine and glutamate plus ammonium as the nitrogen source in MM medium (11). When total RNA samples prepared from cells of strain 168 grown on glutamine (Fig. 1B, lanes 1, 4, and 7) and glutamate plus ammonium (lanes 2, 5, and 8) as nitrogen sources and from cells of strain YCBBd (*glnL::pMUTIN*) grown on glutamine (lanes 3, 6, and 9) were subjected to Northern analysis (Fig. 1B), a transcript (2.5 kb) was only detected in total RNA from strain 168 grown on glutamine with the *glsA* and *glnT* probes (lanes 1 and 4). A 1.1-kb transcript, however, was only detected with the *ybgG* probe in all total RNAs (lanes 7 to 9); the 2.5- and 1.1-kb transcripts were considered to include *glsA* and *glnT*, and *ybgG*, respectively (Fig. 1A). The results indicated that an operon comprising the *glsA* and *glnT* genes (not *ybgG*) was induced by glutamine in the medium; this induction was under positive regulation through the GlnKL system. Moreover, β-Gal synthesis in strain YBGHd (*glnT::pMUTIN*) with a *lacZ* fusion with *glnT* was confirmed to be induced by glutamine in the medium (data not shown). Thus, it is highly possible that GlnK undergoes autophosphorylation when it senses glutamine in the medium and transfers the phosphoryl group to the cognate response regulator of GlnL, which triggers transcription of the *glsA-glnT* operon. Nevertheless, strain YCBBd (*glnL::pMUTIN*) was able to grow well on glutamine as the sole nitrogen source at the same doubling rate as the wild-type strain 168 grew (data not shown).

5' mapping of the *glsA-glnT* transcript. Northern analysis (Fig. 1B) suggested that the *glsA-glnT* operon was likely transcribed from a site upstream of *glsA* to produce the 2.5-kb transcript. To determine the 5' end of this transcript, primer extension analysis was performed with the same RNA samples as those used for Northern analysis. As shown in Fig. 2, a specific band of runoff cDNA was only detected with total RNA prepared from cells of strain 168 grown on glutamine (lane 2), i.e., not with total RNA from ones grown on glutamate plus ammonium (lane 1), which coincided with the results of Northern analysis; the size of the cDNA indicated that the 5' end of the *glsA-glnT* transcript was 95 bp upstream of the translation initiation site for *glsA*. This mapping allowed us to predict the sequences of the -10 and -35 regions of the *glsA-glnT* promoter as TATACA and TTGTAT, respectively, with a 17-bp gap, which is likely recognized by σ^A-RNA polymerase (14).

In addition, we found a ρ-independent terminator located between 2,544 and 2,576 bases from the initiation base for *glsA-glnT* transcription (Fig. 1A), the location of which was immediately downstream of *glnT* and coincided well with the appearance of the 2.5-kb long *glsA-glnT* transcript (Fig. 1B).

In vitro localization of the binding site for GlnL in the *glsA-glnT* promoter region. The *glsA-glnT* transcription induced by glutamine was positively regulated by the response regulator of GlnL, which is cognate to the sensor kinase of GlnK (Fig. 1B). To determine if GlnL binds to the *glsA-glnT* promoter region for its activation by means of gel retardation assaying, we induced the His-tagged GlnL protein in *E. coli* BL21(DE3) cells carrying plasmid pGLNL by the addition of IPTG and purified it by nickel affinity chromatography to homogeneity (Fig. 3A). As shown in Fig. 3B, the probe spanning bases -205 to +25 became increasingly retarded as the amount of His-tagged GlnL in the assay mixture increased, forming a band corresponding to a protein-DNA complex. The results clearly indicated that the His-tagged GlnL protein bound to the promoter region between bases -205 and +25. The equilibrium dissociation constant for the interaction of His-tagged GlnL with the probe under our assay conditions was roughly estimated to be 500 nM. Thus, a half shift of the probe was observed when the protein/DNA concentration ratio was 625; this suggests that, as demonstrated for other response regulators (22), GlnL must be phosphorylated to be fully active in DNA binding.

To raise the affinity of His-tagged GlnL for its DNA-binding site to physiologically relevant levels, we treated the protein with acetylphosphate, which acts as a substrate for autophosphorylation of many response regulators (33). As shown in Fig. 3C, acetylphosphate-treated GlnL exhibited only a 1.5-fold increase in binding affinity to the *glsA-glnT* promoter region compared to that treated without acetylphosphate. However, the equilibrium dissociation constant for GlnL protein treated without acetylphosphate was 600 nM, which was higher than that of the untreated protein, possibly due to partial denaturation of the GlnL protein during this treatment.

To further localize the GlnL-binding site, DNase I footprinting was performed with the same probe as that used for gel retardation analysis. As shown in Fig. 4A, specific interaction of His-tagged GlnL with the coding and noncoding strands was

FIG. 3. Gel retardation analysis of GlnL interaction with the *glsA-glnT* promoter region. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of pure His-tagged GlnL. The purified His-tagged GlnL protein (lane 1) and a crude protein extract of cells of *E. coli* BL21(DE3) bearing plasmid pGLNL exposed to IPTG (lane 2) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 12.5% gel. The preparation of the crude protein extract and purification of the His-tagged GlnL are described in the text. The arrow indicates His-tagged GlnL. (B) Gel retardation analysis of the binding of His-tagged GlnL to the 230-bp probe containing its recognition site. The analysis was performed as described in the text. The probe (0.02 pmol) was incubated with 0, 0.9, 1.7, 3.4, 6.9, and 13.7 pmol of His-tagged GlnL (lanes 1, 2, 3, 4, 5, and 6, respectively) in a 25- μ l reaction mixture. The addition of more His-tagged GlnL to the reaction mixture was not performed due to the low concentration of the protein preparation. The His-tagged GlnL/probe complexes (bound) and free probe (free) are indicated on the right. (C) The increased binding affinity of autophosphorylated His-tagged GlnL to the probe. His-tagged GlnL was treated in the absence and presence of 50 mM acetylphosphate, respectively (GlnL treated and GlnL~P treated), as described in the text. The probe (0.02 pmol) was incubated with 0, 3.3, 6.6, 9.9, 13.2, and 16.5 pmol of His-tagged GlnL treated without and with acetylphosphate (lanes 1 to 6) in a 25- μ l reaction mixture. Lane 7 contains the probe incubated with 33.0 pmol of His-tagged GlnL treated without acetylphosphate.

Functional analysis of the *glsA* and *glnT* genes. As the *glsA-glnT* operon is specifically induced by glutamine in the culture medium, we examined if the two genes are actually involved in glutamine utilization. To verify that *glsA* encodes glutaminase, we cloned and expressed it in *E. coli*. A reaction mixture containing glutamine was incubated with a protein extract prepared from cells bearing either plasmid pGLSA possessing *glsA* or vector pET-22b, both of which had been grown with IPTG, and then submitted to paper chromatography, together with authentic glutamine and glutamate solutions (Fig. 5). A ninhydrin spot corresponding to glutamate was observed on the paper chromatogram only on incubation with the protein extract from cells bearing plasmid pGLSA (Fig. 5, lane 4), clearly indicating that GlsA is a glutaminase.

Glutaminase produces glutamate from glutamine; glutamate synthase, whose large subunit is encoded by *gltA* (4, 7), forms

two glutamate molecules from glutamine and α -ketoglutarate through the aminotransferase reaction. However, not only strain YBGJd carrying the pMUTIN disruption of the *glsA* gene encoding glutaminase but also strain 1A71 possessing the *gltA1* mutation grew in MM medium containing glutamine as the sole nitrogen source, as well as the wild-type strain 168 (Fig. 6). Thus, we constructed strain FU801 harboring both the *glsA::pMUTIN* and *gltA1* mutations to see if it could grow on glutamine as the sole nitrogen source. As shown in Fig. 6, strain FU801 grew slowly with a prolonged doubling time, in contrast to the two strains carrying *glsA::pMUTIN* and *gltA1*, respectively. These results indicated that the *glsA* gene is actually involved in glutamine utilization.

A BLAST similarity search regarding the GlnT protein indicated that this protein belongs to the sodium:alanine symporter family (24). Since another constituent of the *glsA-glnT*

A **B**

TGCTTTTGACCCAGAACATGAGGTTTTTATAAATCTTACTGCTTATTTT**CAAAATGTTTTT**GTCTATTTT**TGTATGA**
 ACGAAAACCTGGGTCTTGTACTCCAAAAATATTTAGAATGACGAATAAAAGTT**TTTACAAAAACAGCATAAAACATACT**
 -10 +1
TTCTGTAGTCTCCATTATACAGTAATTAACCTTCCGCTATTATGAAAGCGTTTAACGCCCCCTTAGCCTTCGTTTT
AAGACATCAGAGGTAATATGTCATTAATATGGAAGGCGATAAACTTTCGCAAATTGCGGGGGAATCGGAAGCAAAA
 -35
 GATACACCTCAGTCCTTTAAGAAAAAATCAGTGAGGAGGAATT**CGATG**
 CTATGTGGAGTCAGGAAATCTTTTTTTAGTCACTCCTCCTTAAGCTAC

FIG. 4. DNase I footprinting analysis of the interaction of GlnL with the *glsA-glnT* region. (A) The analysis was performed as described in the text. The left and right panels are DNase I footprints of the 5'-end-labeled coding and noncoding strands of the DNA probe, respectively. Lanes 1 to 4 contained 0.04 pmol of the 32 P-labeled probe DNA in the reaction mixture (50 μ l). Lane 1 contained no His-tagged GlnL. Lanes 2, 3, and 4 contained 27.4, 20.5, and 13.7 pmol of His-tagged GlnL. Lanes G, A, T, and C contained the products of the respective sequencing reactions performed with the same primers as those used for the probe preparation. The protected areas of the coding and noncoding strands are boxed. (B) The nucleotide sequences of the coding and noncoding strands of the region upstream of the *glsA-glnT* promoter, with the protected sequences (in boldface type), and the -35 and -10 regions of the *glsA-glnT* promoter (+1 is the transcription initiation base) indicated. The boxed T and ATG are the transcription initiation base and translation initiation codon, respectively. Arrows indicate directly repeated sequences (TTTTGT).

operon, *glsA*, was found to encode glutaminase, which is involved in glutamine utilization, it is highly likely that the *glnT* gene encodes the sodium:glutamine symporter. To determine if the GlnT protein is a sodium:glutamine symporter, we measured glutamine uptake by cells of strains YBGJd (*glsA*::pMUTIN) and YBGHd (*glnT*::pMUTIN) (Fig. 7). The *glnT* gene was disrupted through the integration of plasmid pMUTIN, resulting in strain YBGHd, whereas it was under the control of an IPTG-inducible *Pspac* promoter in strain YBGJd. As shown in Fig. 7, glutamine uptake into YBGJd cells was significantly induced by IPTG in the culture medium. At a glutamine concentration of 500 μ M, rates of glutamine uptake were calculated to be 12.1 ± 0.4 and 8.0 ± 0.4 nmol/min per OD₆₀₀ unit with and without IPTG induction, respectively. But glutamine uptake into YBGHd cells was not induced by IPTG and remained at a level similar to that into YBGJd cells not exposed to IPTG. These results suggested that the *glnT* gene encodes a

glutamine transporter, probably a sodium:glutamine symporter.

As shown in Fig. 7, the cells of strain YBGHd (*glnT*::pMUTIN) still contained two-thirds the glutamine transporter activity detected in cells of strain YBGJd (*glsA*::pMUTIN) grown with IPTG, indicating that *B. subtilis* cells possess another glutamine transporter(s). *B. subtilis* likely possesses the ATP-binding cassette (ABC) transporter system (15) for glutamine, probably comprising GlnQHMP proteins, as was inferred from a BLAST search that showed that the GlnQH proteins (probable ATP- and glutamine-binding proteins) exhibited high similarities to the corresponding ABC glutamine transporter proteins of *B. stearothermophilus* (35; data not shown). Actually, strains YBGHd (*glnT*::pMUTIN) and FU707 (*glnQ*::cat) grew on glutamine as the sole nitrogen source, as well as the wild-type strain 168 (data not shown). So, we constructed strain FU708, doubly mutated in the *glnT* and *glnQ*

FIG. 5. Catalysis of deamination of glutamine to glutamate by the GlsA protein. After incubation of the reaction mixture containing glutamine with the crude protein extract prepared from *E. coli* BL21(DE3) cells carrying either plasmid pET-22b (lane 3) or pGLSA (lane 4) exposed to IPTG, an aliquot of the mixture was subjected to paper chromatography as described in the text. Lanes 1 and 2 contained authentic Na glutamate and glutamine, respectively. The spots were visualized by spraying a ninhydrin solution. Arrows indicate the positions of the glutamine and glutamate spots.

genes, but it grew as well on glutamine as the sole nitrogen source as the wild-type strain 168 did (data not shown). These results strongly suggest that *B. subtilis* possesses a glutamine transport system(s) other than the GlnQHMP complex and GlnT protein.

DISCUSSION

On comprehensive DNA microarray analysis of *B. subtilis* two-component regulatory systems (19), we revealed that glutamine in the medium is able to trigger expression of the *glsA-glnT* operon, which encodes glutaminase and the glutamine transporter, through signal transduction via a two-component regulatory system (GlnK-GlnL) (Fig. 1, 5, 6, and 7). The other candidate target genes for positive regulation of GlnK-GlnL, which had been indicated on DNA microarray analysis (19), were found to be false positives on in vivo and in vitro analysis (K. Asai and Y. Sadaie, unpublished data). On primer extension and retardation and DNase I footprinting analyses, we found that the GlnL protein interacts with a region (bases -13 to -56) in which a direct repeat, TTTTGTN 4TTTTGT, is present (Fig. 2, 3, and 4). We were unable to find this sequence on the *B. subtilis* genome, implying that the *glsA-glnT* operon might be the only target of the GlnK-GlnL system.

The sensor kinases of the two-component regulatory systems are classified into four groups according to the sequence around the phosphorylated histidine (9). The members of group

IV comprise four sensor kinases (CitS, DctS, MalK, and GlnK), and their cognate response regulators are CitT, DctR, MalR, and GlnL; the functions of all four two-component systems are now known to be in the utilization of citrate, fumarate and succinate, malate, and glutamine, respectively. The former three systems are especially involved in utilization of the intermediates of the tricarboxylic acid cycle, which is different in the case of the GlnK-GlnL system. It is notable that, except for GlnL and GlnK, the sensor kinases (CitS, DctR, and MalK) and response regulators (CitT, DctR, and MalR) belong to the CitA and CitB subfamilies in *Klebsiella pneumoniae*, respectively (18).

Using the SOSUI system (16) to predict the transmembrane segments for the GlnK protein (387 amino acids) and a search for its protein sequence motif (<http://motif.genome.jp>) revealed that this protein possesses four transmembrane segments at the N terminal, as reported previously (9), and a histidine phosphotransferase domain (106 amino acids) at the C terminal. To examine if the external domains of GlnK resemble substrate-binding sites of transport proteins, we subjected the amino acid sequences of the corresponding regions to the protein similarity search using the BLASTP 2.2.3 program (2), which revealed no significant similarity to any protein. On the other hand, the GlnL protein (314 amino acids) carries a response regulator receiver domain (193 amino acids) at the N terminal, which was detected with the above motif search, and a helix-turn-helix motif consisting of 22 amino acids (amino acids 216 to 237), which was predicted by NPS@ (network protein sequence analysis) (6).

GlnK probably senses glutamine in the medium, because it possesses four transmembrane segments, as described above. It is most likely that GlnK autophosphorylated at a histidine residue transfers a phosphoryl group to an aspartate residue of

FIG. 6. Some defect in utilization of glutamine as the nitrogen source in *B. subtilis* strain mutated in the *gltA* and *glsA* genes, which encoded glutamate synthase and glutaminase, respectively. Cells of strains 168 (open circles), YBGJd (*glsA*::pMUTIN) (open squares), 1A71 (*gltA1*) (open triangles), and FU801 (*glsA*::pMUTIN *gltA1*) (closed circles) were grown on TBABG plates at 30°C for 14 h. Then the cells were inoculated, to an OD₆₀₀ of 0.05, into MM medium (38) containing 13.6 mM glutamine as the sole nitrogen source and incubated at 37°C with shaking.

FIG. 7. Glutamine transport into cells of the pMUTIN integrant strains YBGJd (*glsA*::pMUTIN) and YBGHd (*glnT*::pMUTIN). Cells of strains YBGJd (bars 1 and 2) and YBGHd (bars 3 and 4) were grown with (bars 1 and 3) or without (bars 2 and 4) IPTG, and the glutamine transport into cells was measured as described in the text. The values are averages with standard deviations and were obtained in duplicate experiments.

GlnL, which positively regulates the expression of the *glsA-glnT* operon through binding to the promoter region (Fig. 3 and 4). The *glsA-glnT* operon is adjacent to *glnKL* (Fig. 1A) but is transcribed diversely with respect to the direction of transcription. A search for orthologs of the *glnK*, *L*, *glsA*, and *glnT* genes in complete genome sequences of bacteria in the MBGD database (<http://mbgd.genome.ad.jp>) revealed that *glsA* orthologs are adjacent to oppositely directed *glnKL* ones in the *Bacillus cereus*, *Bacillus thuringiensis*, and *Bacillus halodurans* genomes, as well as in the *B. subtilis* genome, implying that these *glnKL* orthologs might possibly be involved in glutamine transport and utilization in these bacilli.

Acetylphosphate-treated GlnL exhibited only a 1.5-fold increase in binding affinity for the *glsA-glnT* promoter region. Hence, it is uncertain whether or not acetylphosphate acts as an autophosphorylation substrate, as reported for other response regulators (1, 21, 33). Of the *B. subtilis* response regulators, the binding affinity of ComA (25) and CitT (36) to the targets was reported to be enhanced approximately 4 and 1.2 fold, respectively. *B. subtilis* cultures growing in the presence of excess carbohydrate normally excrete acetate as one of the major by-products during exponential growth phase (27). The *ackA* and *pta* genes encode acetate kinase and phosphotransacetylase, respectively, which catalyze the conversion of acetyl-coenzyme A to acetate via an acetyl phosphate intermediate and are direct targets of CcpA-dependent positive regulation occurring during glycolic cell growth (23, 31). Thus, the in vivo concentration of acetyl phosphate is supposed to be elevated during cell growth in the presence of excess carbohydrate, which might cause autophosphorylation of several response regulators to adjust to these physiological conditions, just as in *E. coli* (33). Further investigation of autophosphorylation of the response regulator of *B. subtilis* would be necessary to unveil the physiological role of the autophosphorylation of the response regulators.

B. subtilis appears to contain multiple systems for glutamine utilization, as inferred from the fact that mutant strains deficient in *glsA* and *glnT*, respectively, were able to normally utilize

glutamine as the sole nitrogen source. Glutamine serves as an amino acid for protein synthesis and a nitrogen donor for the synthesis of various nitrogenous molecules in the cell (10), so this organism has developed several compensatory systems for glutamine supply and utilization to maintain the concentration of glutamine in the cell. Glutamine is now known to be metabolized not only by glutamate synthetase encoded by *gltA* but also by glutaminase encoded by *glsA*. Moreover, *B. subtilis* strains doubly mutated in the *glsA* and *gltA* genes were able to grow on glutamine, although the doubling time was longer than those of the singly mutated strains (Fig. 6). This suggests that a glutamine utilization enzyme(s) other than glutaminase and glutamate synthetase should be present in *B. subtilis*, such as another candidate glutaminase (YlaM) and/or nitrogen-donating enzymes for the synthesis of various nitrogenous molecules in the cell. On the other hand, this organism was shown to possess a glutamine transporter (GlnT), probably an sodium:glutamine symporter (Fig. 7). Besides GlnT, this organism likely contains an ATP-binding cassette transporter system for glutamine, probably comprising GlnQHMP proteins; GlnQ is a probable glutamine-binding protein. However, even a strain doubly mutated in *glnQ* and *glnT* grew well on glutamine as the sole nitrogen source, like wild-type strain 168, indicating that *B. subtilis* possesses an unidentified glutamine transport system(s) other than the GlnQHMP complex and GlnT proteins.

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