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Positive Regulation of Glutamate Biosynthesis in *Bacillus subtilis*

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Nitrogen source regulation of glutamate synthase activity in *Bacillus subtilis* occurs at the level of transcription of the *gltA* and *gltB* genes, which encode the two subunits of the enzyme. We show here that transcription of *gltA* requires the product of *gltC*, a gene whose transcription is divergent from that of *gltA* and whose transcriptional control sequences overlap those of *gltA*. *gltC* mutants had decreased, aberrantly regulated levels of glutamate synthase activity and decreased *gltA* mRNA. The *gltC* gene product could act in *trans* to complement both these defects. In addition, the *gltC* gene product repressed its own transcription. The DNA sequence of *gltC* revealed that its putative product is very similar to a number of positive regulatory proteins from gram-negative bacteria (the LysR family).

Glutamate synthase (glutamine:2-oxoglutarate amidotransferase; GOGAT) is a key enzyme of nitrogen metabolism in *Bacillus subtilis*. It catalyzes the synthesis of glutamate and, together with glutamine synthetase, is responsible for the sole pathway for assimilation of ammonium into organic compounds. GOGAT activity is regulated by the available nitrogen source; it is high in cells grown with ammonia as the nitrogen source and low in cells grown with glutamate as the nitrogen source (30, 37). We and others have shown previously (4, 31) that these variations in enzyme activity are due to changes in the rate of synthesis and steady-state levels of transcripts of *gltA* and *gltB*, the genes that code for the large and small subunits of GOGAT, respectively (14). We show here that the product of a third gene, *gltC*, is required for expression from the *gltA* promoter. We postulate that the product of *gltC* is a positive transcription factor that acts at the *gltA* promoter to stimulate transcription under conditions of limiting glutamate.

The detailed mechanism by which the genes for glutamate synthase are regulated is not known for any species. While global control of nitrogen metabolism (*ntr* system; see reference 22 for a review) has been well described for enteric organisms, this system does not appear to be involved directly in the regulation of GOGAT gene expression in *Escherichia coli*. GOGAT levels in *E. coli* are repressed by growth in glutamate, but, in contrast to the situation in *B. subtilis*, are equally derepressed by growth in ammonia or glutamine (7). GOGAT levels in *Salmonella typhimurium* are regulated in a complex, poorly understood manner by a growth rate-limiting nitrogen source (6). In *S. typhimurium*, the large and small subunits of GOGAT are encoded by *gltB* and *gltD*, respectively; these two genes are apparently cotranscribed (21). The genes encoding the subunits of *E. coli* GOGAT, also called *gltB* and *gltD*, have been cloned (13) and recently sequenced (28). A third gene, *gltF*, downstream from and possibly cotranscribed with *gltB* and *gltD*, does not appear to be involved in GOGAT regulation, but rather in *ntr* regulation (7). *gltF* mutants are unable to fully derepress genes which respond to *glnF* (*ntrA*) and *glnG* (*ntrC*) under conditions of nitrogen limitation. This phenotype has been observed previously for *Klebsiella pneumoniae nac* mutants (3).

The situation in *B. subtilis* seems to be considerably different from that in the enteric bacteria in that *B. subtilis* has a much more limited response to nitrogen availability (37; S. Fisher, personal communication) and, as shown below, GOGAT gene expression is dependent on a positive regulator that is itself transcribed from a divergent but overlapping promoter site. The *B. subtilis glt* system does have at least one important similarity to regulatory systems from gram-negative bacteria, however. The predicted amino acid sequence of GltC indicates that it is closely related to a family of positive regulatory proteins that includes LysR, IlvY, and CysB of *E. coli*.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used are listed in Table 1. *E. coli* and *B. subtilis* were grown in Luria-Bertani (LB) broth (25); *B. subtilis* was also grown in TSS medium (16), with the final concentration of the nitrogen source at 0.2%. For some experiments, *E. coli* was grown in M9 minimal medium (25). Drugs were used at the following concentrations: ampicillin, 100 μg/ml;chloramphenicol, 30 μg/ml (for *E. coli*) or 5 μg/ml (for *B. subtilis*); kanamycin, 25 μg/ml (for *E. coli*) or 5 μg/ml (for *B. subtilis*); spectinomycin (Spc), 200 μg/ml; erythromycin, 1 μg/ml; and lincomycin, 25 μg/ml. Cells to be grown with a combination of erythromycin and lincomycin were first induced by growth for 2 h in LB containing 0.15 μg of erythromycin per ml. All cultures were grown at 37°C unless otherwise indicated. Growth of cultures was monitored by the use of a Klett-Summerson colorimeter (green filter).

Plasmid construction. pDEB19 was constructed by cloning the 1.9-kilobase (kb) *Hin*dIII fragment of pLS30 (4) into the *Hin*dIII site of pBR322. This *Hin*dIII fragment includes the promoters for *gltA* and *gltC* and about 900 base pairs (bp) of DNA downstream of each promoter (the insert of pDEB19 is the same as that in pDEB819; see Fig. 1). To make pDEB19S, the 63-bp *Cla*I fragment within the 1.9-kb insert of pDEB19 was replaced with a synthetic, self-complementary oligonucleotide (5′-CGCGAGCTCG-3′) containing a *Sac*I site. The predicted construct was verified by DNA sequencing. The mutation was designated *gltC99*.

pDEB8 was constructed by substituting the polylinker region (*Eco*RI to *Hin*dIII) of M13mp8 for the 0.73-kb *Eco*RI-*Hin*dIII fragment of ptrpBGI (38). pDEB811, -816, -819 and -844 represent inserts into pDEB8 of the following DNAs,

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TABLE 1. Bacterial strains

Strain	Genotype	Reference or source
B. subtilis		
SMY	Wild type	Lab strain
SX150	*gltC150*::Tn*917*	4
SX15011	*gltC150*::Tn*917 amyE*::pDEB811	SX150 × pDEB811[a]
SCS	*gltC99*	See text
SCS11	*gltC99 amyE*::pDEB811	SCS × pDEB811[a]
E. coli RV	Δ*lacX174 thi*	M. Malamy

[a] pDEB811 does not replicate in *B. subtilis*. It produces stable transformants by recombination with the chromosome (see the text).

respectively: the 1.1-kb *Hin*dIII-*Nsi*I fragment of pDEB19 (inserted at the *Hin*dIII and *Pst*I sites of pDEB8), the 1.6-kb *Sac*I-*Hin*dIII fragment of pDEB19S (inserted at the *Sac*I and *Hin*dIII sites of pDEB8), the 1.9-kb *Hin*dIII fragment of pDEB19 (inserted at the *Hin*dIII site of pDEB8), and the 4.4-kb *Eco*RI-*Pvu*II fragment of pLS30 (inserted at the *Eco*RI and *Sac*I sites of pDEB8 with the use of an adaptor molecule consisting of two synthetic oligonucleotides of the following sequences: 5′-GGATCCGAGCT-3′ and 5′-CGGA TCC-3′). Oligonucleotides used for cloning were synthesized with an Applied Biosystems machine.

To make pDEB10, the 286-bp *Bcl*I-*Nsi*I fragment of pLS30 (4) was purified and ligated to the *Bam*HI and *Pst*I sites of pMK3-1. pMK3-1 is identical to pMK3 (43) except that pMK3-1 has a single *Eco*RI site, in the polylinker region. Both plasmids are *E. coli* and *B. subtilis* shuttle vectors, as is pDEB10.

Construction of strain SCS. The *gltC99* allele was transferred to the wild-type *B. subtilis* chromosome by congression, by using a mixture of pDEB19S and chromosomal DNA from an Spc^r Glt$^+$ strain and selecting for Spc^r transformants. Spc^r Glt$^-$ transformants were screened by Southern analysis (data not shown) to verify replacement of the *Cla*I site by a *Sac*I site.

Transformation. Competent *E. coli* cells were prepared by the $CaCl_2$ procedure of Cohen et al. (11). *B. subtilis* was made competent and transformed by the method of Contente and Dubnau (12).

GOGAT assays. Cells grown in TSS medium were harvested when the culture turbidity reached 100 Klett units and washed with a solution containing 25 mM Tris hydrochloride (pH 7.9), 10 mM β-mercaptoethanol, 1 mM EDTA, and 200 mM KCl. The resulting cell pellet was suspended in 4 to 10% of the original culture volume of 25 mM Tris hydrochloride (pH 7.9)–10 mM β-mercaptoethanol–1 mM EDTA and disrupted by passage twice through a French pressure cell maintained at 20,000 lb/in^2. The extract was centrifuged for 10 min at 12,000 × *g* at 4°C and assayed within several hours. GOGAT activity, which was stable under these conditions, was measured by monitoring oxidation of NADPH at 340 nm. Reaction mixes (1 ml) contained 50 mM Tris hydrochloride (pH 8.0), 5 mM β-mercaptoethanol, 35 mM 2-ketoglutarate, 20 mM glutamine, and 0.1 mM NADPH. NADPH oxidase activity was measured in similar reaction mixes without added glutamine and was subtracted from apparent GOGAT activity. Protein was determined by a method based on Coomassie blue binding, with reagents supplied by Bio-Rad Laboratories; bovine serum albumin was used as a standard.

DNA extractions. Plasmid DNAs were purified from *E. coli* by a large-scale CsCl method (10) or by a rapid Triton lysis procedure. In the latter method, the pellet from a 10-ml overnight culture grown in LB containing the appropriate drug was suspended in 0.4 ml of STE (20% sucrose, 50 mM Tris hydrochloride [pH 8.0], 50 mM EDTA, pH 8.0). Lysozyme (0.1 ml; 4 mg/ml in STE) was added, and after incubation on ice for 5 min, 0.3 ml of a solution containing 0.1% Triton X-100, 10 mM Tris hydrochloride (pH 8.0), and 1 mM EDTA was added. After incubation for 15 min on ice, the mixture was centrifuged for 15 min at 4°C in an Eppendorf centrifuge. To the supernatant fluid was added 0.3 ml of phenol (previously equilibrated with 100 mM Tris hydrochloride [pH 8.0], 10 mM EDTA) and 0.3 ml of chloroform-isoamyl alcohol (24:1). After extraction, nucleic acids in the aqueous phase (ca. 0.5 ml) were precipitated by incubation for 10 min on dry ice following addition of 1 ml of 95% ethanol. The precipitate was collected, dried, and redissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA (TE).

Chromosomal DNA from *B. subtilis* was isolated from a 100-ml overnight culture in LB. The cells were lysed by treatment with lysozyme and extracted with phenol in the presence of sodium dodecyl sulfate (SDS). Nucleic acids in the aqueous phase were precipitated by addition of ice-cold isopropanol. The nucleic acids were treated with RNase, extracted once with phenol and three times with chloroform-isoamyl alcohol (24:1), and precipitated by the addition of sodium acetate (pH 7.5) to 0.4 M and ethanol to 65%. The pellet collected by centrifugation was rinsed with 70% ethanol, dried, dissolved in TE, and stored at 4°C.

RNA isolation. *B. subtilis* was grown to a culture turbidity of 100 Klett units in TSS medium containing the indicated nitrogen sources. Cultures were cooled by pouring over an equal volume of ice-cold 121 medium (15) and harvested by centrifugation for 5 min at 5,000 rpm at 4°C. Cells were suspended in a volume of 15 mM Tris hydrochloride (pH 8.0)–8 mM EDTA–17% sucrose equivalent to 1% of the culture volume and converted to protoplasts by incubation with lysozyme (500 μg/ml) at 4°C for 60 to 90 min. Protoplasts were pelleted by centrifugation at 5,000 rpm for 5 min at 4°C and lysed by the addition of 10 mM Tris hydrochloride (pH 8.0)–10 mM NaCl–3% diethylpyrocarbonate–1.5% SDS–0.03% trisodium citrate dihydrate. The resulting suspension was extracted with chloroform-isoamyl alcohol (24:1). The single-stranded nucleic acids in the aqueous phase were preferentially precipitated by incubation on ice for 60 min after addition of an equal volume of 4 M LiCl. This precipitate was collected by centrifugation at 9,000 rpm for 30 min at 4°C, dissolved in a volume of TE equivalent to 1% of the culture volume, and extracted with phenol-chloroform-isoamyl alcohol (25:24:1). Nucleic acids in the aqueous phase were precipitated by the addition of sodium acetate (pH 7.5) to 0.4 M and ethanol to 65%, collected by centrifugation, rinsed with 70% ethanol, dried, dissolved in TE, and stored at −20°C. As judged by agarose gel electrophoresis, RNAs were intact and contained less than 1% contaminating DNA.

Hybridization analyses. Agarose gel electrophoresis and blotting were performed as described previously (4), with the following exceptions. The membrane to which DNAs were blotted was Nytran (Schleicher & Schuell). Conditions for prehybridization, hybridization, and washing were those recommended by the manufacturer. Use of S1 nuclease to map transcripts was described previously (4).

DNA sequence analysis. Circular, double-stranded plasmid DNA was denatured with alkali (9) and sequenced by the chain termination method of Sanger et al. (35) with [α-^{35}S]dATP (New England Nuclear Corp.) and Sequenase (US Biochemical Corp.) as specified by the manufacturer. *gltC*-

TABLE 2. Specific activity of GOGAT in various strains

Strain	GOGAT sp act[a] (U/mg)		
	Glutamate	Glutamine	Ammonia
SMY	12.6	58.3	128
SX150	4.5	6.2	ND[b]
SX15011	13.4	58.7	ND
SCS	2.0	6.8	ND
SCS11	16.4	35.4	ND

[a] Specific activity is expressed in units per milligram of protein. One unit is equivalent to 1 nmol of NADPH cleaved per min. All cells were grown in TSS glucose-minimal medium with the indicated nitrogen source.
[b] ND, Not done.

specific oligonucleotides (16 to 18 bases) were synthesized with an Applied Biosystems machine. The DNA sequence products were separated by electrophoresis in polyacrylamide-urea gels and visualized by autoradiography with Kodak XAR film.

Complementation. *B. subtilis glt* mutants were complemented by the insertion of cloned DNAs at the *amyE* locus. This was accomplished by using pDEB8, a derivative of ptrpBGI, the insertion vector developed by Shimotsu and Henner (38). *B. subtilis* strains were transformed with derivatives of pDEB8 by using either covalently closed circular DNA or DNA which had been linearized by digestion with *Nru*I. In either case, approximately equal numbers of transformants were due to double or single crossovers. Chloramphenicol-resistant (Cam^r) transformants were screened for the ability to produce amylase, as judged by degradation of starch incorporated into agar plates; those that did not were assumed to result from a double crossover event at the *amyE* locus. Cam^r Amy^- transformants were tested for their Glt phenotype and the ability to express *glt* gene products. Only strains that showed 100% linkage between the *cam* and *amyE* markers were studied further. The structures of the insertions were also verified by Southern blot analysis (data not shown).

RESULTS

In previous work (4), we found that insertions of Tn*917* as far as 1 kbp upstream of the *gltA* promoter of *B. subtilis* resulted in a Glt^- phenotype. These insertions were found to be within a transcription unit divergent from *gltA*. Expression of this divergent transcript was not regulated by growth on different nitrogen sources, and therefore it did not correspond to either *gltA* or *gltB*. These results led us to the tentative conclusion that the upstream region contains a gene (*gltC*) that encodes a factor necessary for expression of *gltA* and *gltB*. We present evidence below (complementation tests and DNA sequencing analysis) to show that this is true.

Effect of *gltC* mutations on GOGAT activity. GOGAT activity is three- to fivefold higher in wild-type cells grown with glutamine as the nitrogen source than it is in cells grown with glutamate as the nitrogen source. Levels of GOGAT activity were measured in wild-type strain SMY and in two mutants, SX150 (*gltC150*::Tn*917*) and SCS (*gltC99*). All strains were grown in TSS glucose-minimal medium containing either glutamate or glutamine as the sole nitrogen source. Because Tn*917* insertions in the *glt* region cause glutamate auxotrophy, it was not possible to test GOGAT activity in such strains with ammonia as the sole nitrogen source. Mutations in the *gltC* region resulted in greatly decreased GOGAT activity (Table 2). In strain SX150, the residual activity was not regulated by the nitrogen source. Detailed restriction mapping and DNA sequencing revealed that, in SX150, the *gltC* coding sequence was interrupted (Fig. 1). GOGAT activity was also greatly decreased in strain SCS, in which a *Sac*I linker replaced a 63-bp *Cla*I fragment in *gltC* (Fig. 1 and Materials and Methods); partial response to the nitrogen source appeared to remain. (Low GOGAT levels made this point difficult to resolve.) This mutation will be shown below to cause production of a truncated *gltC* product.

Effect of *gltC*::Tn*917* insertion on *gltA* transcription. The effect of mutations in the *gltC* gene on GOGAT activity might be at the level of transcription, translation, or post-translational modification. To investigate this, we measured levels of *gltA* mRNA in SX150 and in the isogenic $gltC^+$ strain. The results of S1 mapping experiments (Fig. 2) indicated that strain SX150, carrying *gltC150*::Tn*917*, contained less than 10% of the wild-type steady-state level of *gltA* mRNA (compare lanes c and e). The residual ability to produce *gltA* mRNA may be at least partly responsive to the nitrogen source, since a detectable hybridization signal could be seen with RNA from glutamine-grown cells but not with RNA from glutamate-grown cells (compare lanes d and e and d′ and e′). GOGAT activity in this strain did not appear to be regulated to a significant extent by the nitrogen source (Table 2). In addition, GOGAT activity in strain SX150 (grown in glutamate medium) was not as severely reduced as was the *gltA* mRNA level. It is not known whether this difference is meaningful or a reflection of the difficulty of measuring low levels of GOGAT activity.

***The gltC* region codes for a diffusible product.** We subcloned sequences containing various parts of the *gltC* transcriptional unit by using the vector pDEB8. None of these sequences contained the entire *gltA* gene or any part of *gltB*. pDEB8 and its derivatives recombine at the *amyE* locus, creating cells that are Amy^- Cam^r and diploid for any DNA sequence cloned into pDEB8 (Fig. 3). The predicted recombination event was verified by genetic and physical mapping (data not shown). Recombinants carrying pDEB8 derivatives at the *amyE* locus were tested for their ability to complement the Tn*917* insertion mutation in strain SX150 as well as the *gltC99* mutation of strain SCS. (For all recombinants of strain SX150, retention of the Tn*917* insertion was verified by erythromycin-lincomycin resistance and the linkage of this resistance and the Glt^- marker in subsequent crosses.) As indicated in Fig. 1, pDEB844 and pDEB819 were able to complement the *gltC150*::Tn*917* mutation, showing that the *gltC* region codes for a diffusible product. Complementation resulted in Glt^+ strains which were indistinguishable from wild-type strains in terms of their growth rates in minimal glucose-ammonia medium (data not shown). pDEB816, however, was unable to complement the Tn*917* insertion mutation of SX150. This indicates that the minimum sequence needed for *gltC* complementation must extend to the left of the *Cla*I sites. Such a sequence was found to reside in pDEB811, which contains 1.1 kb of *B. subtilis glt* region DNA. This was the smallest insert tested that was able to complement SX150. This 1.1-kb insert complemented the *gltC99* mutation of strain SCS as well (Fig. 1). This region therefore defines the maximum sequence necessary for *gltC* activity. The 1.1-kb fragment carries ~1 kb of transcribed DNA, as well as ~170 bp upstream of the start point of the *gltC* transcript (4).

Complementation of *gltC* mutants results in regulated transcription of *gltA*. In order to verify that the complementing

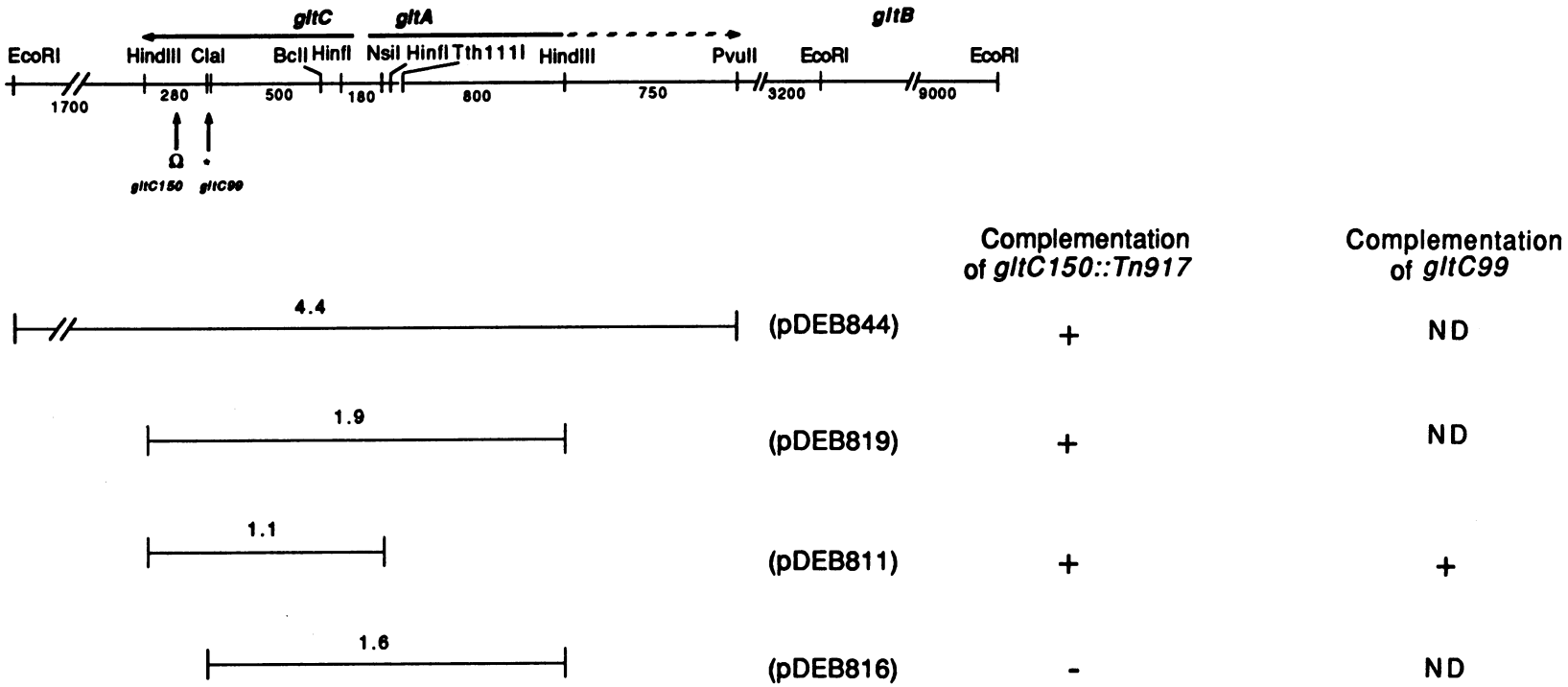

FIG. 1. Map and complementation analysis of the *glt* region of the *B. subtilis* chromosome. The top line shows the relationship between the *glt* genes, the in vivo transcriptional start points and the extent of the known transcripts, and the physical map of the *glt* regulon. The approximate distances between restriction sites are indicated in base pairs. Only relevant restriction sites are shown. The symbol Ω indicates sites of insertion of Tn*917*. The insertion Ω150 is present in strains SX150 and SX15011. The star (★) indicates the small *Cla*I fragment replaced by a *Sac*I adaptor molecule to create the *gltC99* mutation (see Materials and Methods). The bottom four lines describe the results of complementation analyses of different Glt$^-$ strains with the indicated regions of *glt* DNA. The symbol + indicates that a strain was complemented to Glt$^+$; the symbol − indicates that the strain remained Glt$^-$. ND, Not done.

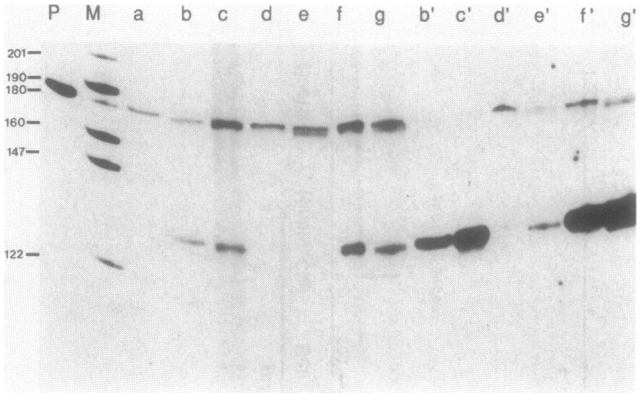

FIG. 2. S1 analysis of *gltA* transcription in various *gltC* backgrounds. The probe (~10^5 cpm for lanes a to g') was a 185-bp *Hin*fI fragment labeled at the 5' ends. This probe detects only *gltA* transcription. Lanes: a, probe DNA treated in the presence of yeast RNA; b and c, probe DNA protected by 18 and 54 μg, respectively, of RNA from strain SMY (*gltC*$^+$) cells; d and e, probe DNA protected by 18 and 54 μg, respectively, of RNA from strain SX150 (*gltC150*::Tn*917*) cells; f and g, probe DNA protected by 18 and 54 μg, respectively, of RNA from strain SX15011 (*gltC150*::Tn*917 amyE*::*gltC*$^+$). Lanes b through g represent cells grown in TSS minimal glucose-glutamate medium; lanes b' through g' represent cells grown in TSS minimal glucose-glutamine medium. Lane M contains molecular mass markers; lane P contains untreated probe (~1,000 cpm). Sizes are shown in nucleotides.

DNA at the *amyE* locus was functioning similarly to wild-type *glt* DNA, we measured transcription of *gltA* in complemented strains by the S1 mapping technique. *gltA* transcription was restored in strain SX15011 (Fig. 2, compare lanes d and f). Transcription occurred from the normal startpoint (compare lane b, SMY, with lane f, SX15011). Furthermore, *gltA* transcription in SX15011 was regulated similarly to that in wild-type cells during growth in different nitrogen sources (for example, compare lanes b and b' with lanes f and f'). (It should be noted that for strain SX15011, this experiment measures transcription from two copies of the *gltA* promoter, the one at the *gltA* locus and the one carried at *amyE*.) We conclude that at least one *trans*-acting product of the *gltC* region is necessary for transcription from the *gltA* promoter.

In order to test the hypothesis that this *trans*-acting factor was bound to the *gltC*/*gltA* intergenic region, we sought to titrate this factor by introducing into *B. subtilis* multiple copies of this region. Strain SMY (wild type) was transformed to Kan^r with DNA of either pMK3-1 (the vector) or pDEB10 (vector plus a 286-bp *Bcl*I-*Nsi*I fragment), and the resulting transformants were tested for their Glt phenotype. All measurements were made in the presence of kanamycin to maintain selection for the plasmids. Strain SMY(pMK3-1) grew well in TSS-glucose minimal medium containing either ammonia (doubling time, 2.6 h) or glutamate (doubling time, 3.6 h) as the nitrogen source. Strain SMY(pDEB10), however, was able to grow only in the presence of glutamate (doubling time, 3.8 h); no growth was observed in TSS-glucose minimal medium containing ammonia as the sole nitrogen source (doubling time, ⩾8 h). These growth rates

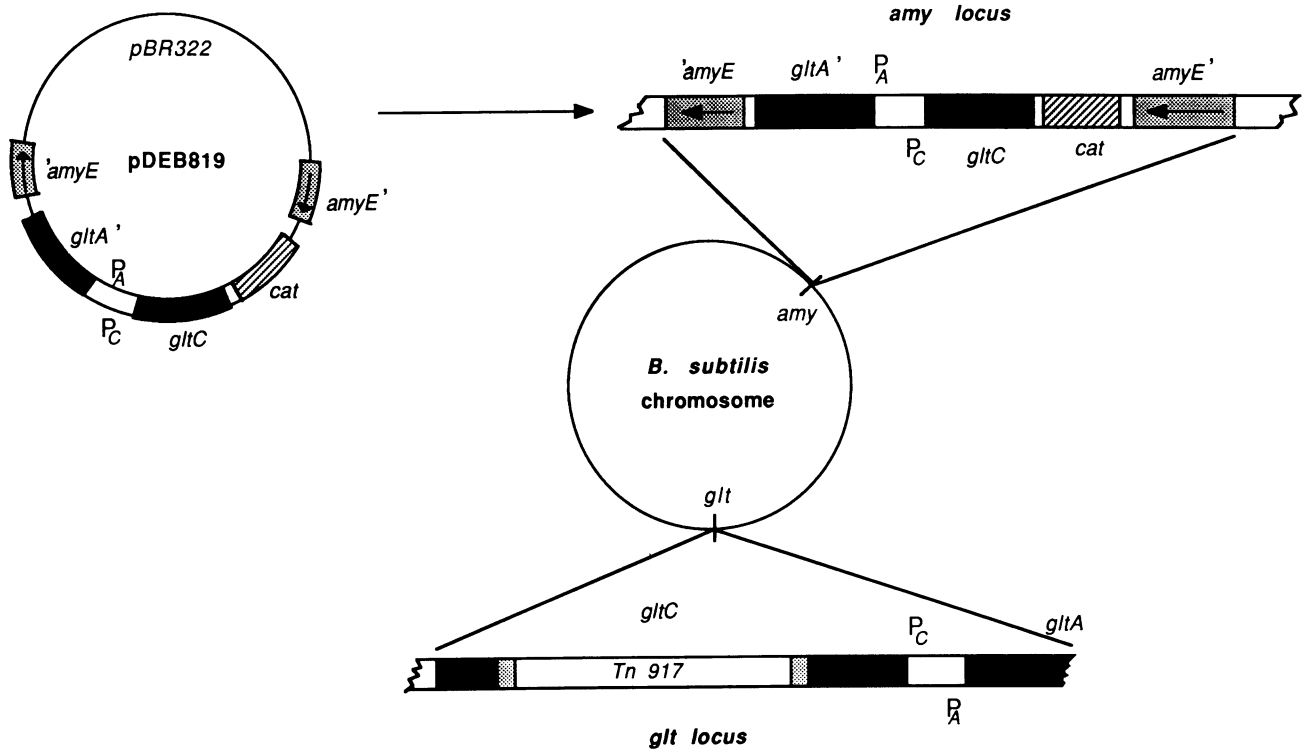

FIG. 3. *gltC* complementation system. pDEB819 is a derivative of pDEB8 that contains a 1.9-kb *Hin*dIII insert (see Materials and Methods). Transformation to Cam^r of a *gltC* mutant by pDEB819 results in the formation of a stable strain diploid for the *gltC* gene. Cam^r Amy$^-$ transformants are the result of a double crossover event replacing part of the wild-type *amyE* gene with the *cam* gene and *gltC* DNA cloned in pDEB819. P_A and P_C refer to the promoters for *gltA* and *gltC*, respectively.

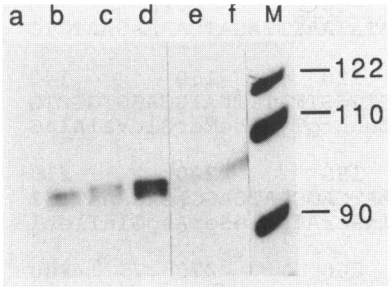

FIG. 4. S1 analysis of transcripts of the *gltC* gene. The 5′-end-labeled probe (a 333-bp *Bcl*I-*Tth*111I fragment much larger than the protected fragments) is not shown here. Lanes: a and b, probe protected by 9 and 27 μg, respectively, of RNA from SMY (*gltC*$^+$) cells; c and d, probe protected by 9 and 27 μg, respectively, of RNA from SX150 (*gltC150*::Tn*917*) cells; e and f, probe protected by 9 and 27 μg, respectively, of RNA from strain SX15011 (*gltC150*::Tn*917 amyE*::*gltC*$^+$); M, marker DNAs, shown next to their sizes (in nucleotides). All RNAs were from cells grown in TSS glucose-glutamine minimal medium.

were reflected in colony size on plates of TSS-glucose minimal medium; strain SMY(pDEB10) behaved as a glutamate auxotroph under these conditions. A simple interpretation of these results is that the 286-bp segment of *B. subtilis* DNA that includes the promoters for *gltA* and *gltC* contains at least one site which, in high copy number, titrates a positive regulatory protein (presumably GltC).

Regulation of *gltC* expression. The transcript encoding *gltC* is not appreciably regulated by the nitrogen source in wild-type cells (4). In strain SX150 (*gltC150*::Tn*917*), on the other hand, the steady-state level of *gltC* transcript was dramatically increased, as demonstrated in the S1 mapping experiment shown in Fig. 4 (compare lanes a and b with c and d). In this same insertion strain, the level of the *gltA* transcript was decreased at least 10-fold (Fig. 2). Disruption of the *gltC* gene by Tn*917* therefore appears to have opposite effects on transcription of *gltC* and *gltA*. This is reinforced by the observation that complementation of the *gltC150*::Tn*917* mutation by insertion of wild-type DNA at the *amyE* locus restored *gltA* and *gltC* transcripts to wild-type levels (S1 mapping data shown in Fig. 4). On the basis of these experiments, we suggest that the *gltC* gene product negatively regulates its own transcription.

The *gltC* sequence. Both strands of the *gltC*-encoding 1.1-kb *Hin*dIII-*Nsi*I fragment were sequenced; most of the sequence is shown in Fig. 5. Note that the orientation is opposite to that shown in Fig. 1. Putative −10 and −35 recognition sequences for the major vegetative (Eσ^{43}) form of *B. subtilis* RNA polymerase are indicated, as are the transcriptional starts mapped by S1 nuclease protection.

Within this *gltC* region, only a single substantial open reading frame was detected. The first potential start codon indicated would direct the synthesis of a 34,795-dalton (Da) protein. This start codon is preceded by a sequence that has only a poor match with the ribosome-binding site identified by Shine and Dalgarno (39). Other potential start codons are preceded by potential ribosome-binding sites that have even less similarity to consensus sequences. The true start codon will only be identified when GltC is purified from *B. subtilis* and its amino-terminal sequence is determined. The sequence of the *gltC99* mutant was also determined. The mutation generated a frameshift, resulting in termination after the 200th amino acid of the *gltC99* product (19 amino acids after the frameshift). In fact, a comparison of GltC proteins expressed from *gltC*$^+$ and *gltC99* alleles in a T7 RNA polymerase-directed system in *E. coli* (44) showed that the mutant gene encoded a truncated protein (data not shown).

The sequence of the predicted GltC protein was compared with those of other proteins in the data bases at the Georgetown University facility, by the protocol of Lipman and Pearson (20). This procedure identifies evolutionarily related proteins and indicates the significance of their relatedness. Regions of significant homology were identified in several proteins, including those encoded by *spoIIGB* of *B. subtilis*, *nodD*, *nodD1*, and *nodD2* of *Rhizobium meliloti*, *nodD* of *Bradyrhizobium* sp. strain ANU 289, *ilvY* of *E. coli*, and *cysB* of *E. coli* and *Salmonella typhimurium*. The homology to the *spoIIGB* gene product, a sigma factor (σ^{29} or σ^{E}) of *B. subtilis* (40), was restricted to a 32-amino-acid stretch that corresponds to the so-called "helix-turn-helix" region of the DNA-binding domain common to all sigma factors (17).

Larger and more significant regions of homology were observed in the NodD proteins of two nitrogen-fixing organisms, *R. meliloti* and *Bradyrhizobium* sp. strain ANU289. In this case, significant similarity extended over the amino-terminal two-thirds of the proteins; ~21% of the residues were identical, while ~21% were functionally conservative changes, giving an overall similarity of ~42%. The NodD protein of *R. meliloti* has been identified as a positive factor for the transcription of *nodABC*, from which it is divergently transcribed (27). The predicted primary sequence of GltC is homologous over its entire length to the IlvY and CysB proteins. In each of these, ~27% of the residues are identical, while ~24% are conservative changes, for an overall similarity of >50%. In *E. coli*, both proteins have been determined to be positive transcriptional factors, IlvY for the divergently transcribed gene *ilvC* (48, 49) and CysB for several unlinked *cys* genes (23). NodD, IlvY, and CysB have been recently identified as members of a family of proteins (the LysR family) from gram-negative bacteria (18). A comparison of the predicted sequence of GltC with that of LysR and a consensus sequence for the LysR family (18) is shown in Fig. 6. It is evident that GltC is similar to LysR, especially in the amino-terminal region, and that it contains most of the highly conserved residues found in the LysR family. All of the positive transcriptional factors in this family have sizes in the range of 30,000 to 40,000 Da, similar to the mobility observed for GltC (data not shown).

Sequence of the *gltC*/*gltA* intergenic region. The DNA sequence of a 286-bp *Bcl*I-*Nsi*I fragment from pDEB10 containing the *gltC*/*gltA* intergenic region is presented in Fig. 7. The *gltA* promoter region has sequences (−35, TTGTTT; −10, TACAAT) that are similar to the consensus sequences for the Eσ^{43} form of *B. subtilis* RNA polymerase at positions consistent with the apparent start point of transcription (here at position 167) as determined by nuclease mapping of in vivo RNA. The fit of these sequences to the consensus is relatively poor by *B. subtilis* standards (26). In *E. coli*, positively regulated promoters are frequently weak promoters (32). The −35 sequence (TTTTCA) for *gltC* is found between the *gltA* −35 and −10 sequences and on the opposite strand. This hexanucleotide is followed by the appropriately spaced −10 consensus sequence (TATAAT). The positions of these sequences are consistent with transcription beginning at one of the A residues at positions 114 and 115, as predicted by S1 mapping experiments.

An interesting feature of this region is the presence of several copies of a direct repeat with the consensus sequence 5′-ATATTGTTT-3′. These repeats are indicated in

```
        10         20         30         40         50         60▲▲       70
AACAACTCTATAATCATTGTAGGTTTTCAAAACGATATAAACAATATATAATTTAGATCAAAAGAATCTC
          ▼
        80         90        100        110        120        130        140
AAAATGAGATAGATGGATGTGAGACAAACATGGAGCCTGCGCCAACTGCGTTATTTTATGGAGGTGGCTG
            MetAspValArgGlnThrTrpSerLeuArgGlnLeuArgTyrPheMetGluValAlaG

       150        160        170        180        190        200        210
AAAGAGAACACGTTTCAGAAGCCGCTGATCATTTGCATGTGGCCCAATCAGCAATCAGCAGACAAATTGC
luArgGluHisValSerGluAlaAlaAspHisLeuHisValAlaGlnSerAlaIleSerArgGlnIleAl

       220        230        240        250        260        270        280
CAATCTTGAAGAAGAATTAAATGTGACCTTATTTGAGCGTGAAGGGAGAAATATCAAACTCACGCCAATC
aAsnLeuGluGluGluLeuAsnValThrLeuPheGluArgGluGlyArgAsnIleLysLeuThrProIle

       290        300        310        320        330        340        350
GGAAAAGAATTTTTAATTCATGTGAAAACGGCGATGAAAGCCATTGATTATGCGAAAGAGCAAATTGATG
GlyLysGluPheLeuIleHisValLysThrAlaMetLysAlaIleAspTyrAlaLysGluGlnIleAspG

       360        370        380        390        400        410        420
AGTATCTTGACCCGCATCGCGGAACGGTAAAGATCGGCTTTCCTACAAGCCTCGCCAGCCAGCTTTTGCC
luTyrLeuAspProHisArgGlyThrValLysIleGlyPheProThrSerLeuAlaSerGlnLeuLeuPr

       430        440        450        460        470        480        490
GACTGTCATTTCAGCGTTTAAAGAAGAATATCCGCACGTCGAATTTTTGCTGCGCCAAGGCTCCTATAAG
oThrValIleSerAlaPheLysGluGluTyrProHisValGluPheLeuLeuArgGlnGlySerTyrLys

       500        510        520        530        540        550        560
TTTCTGATTGAAGCTGTCAGAAACCGCGATATTGATCTGGCCTTATTAGGGCCGGTGCCGACGAATTTCT
PheLeuIleGluAlaValArgAsnArgAspIleAspLeuAlaLeuLeuGlyProValProThrAsnPheS

       570        580        590        600        610        620        630
CTGACATAACGGGAAAAATATTATTTACTGAAAAAATTTACGCGCTTGTTCCATTAAATCATCCGCTTGC
erAspIleThrGlyLysIleLeuPheThrGluLysIleTyrAlaLeuValProLeuAsnHisProLeuAl

       640        650        660        670        680        690        700
TAAACAAAAAAACGGTTCATTTAATCGATTTGCGCAACGACCAATTTGTATTGTTCCCGGAAGGATTTGTA
aLysGlnLysThrValHisLeuIleAspLeuArgAsnAspGlnPheValLeuPheProGluGlyPheVal

       710        720        730        740        750        760        770
CTTAGAGAGATGGCAATCGATACTTGCAAACAAGCAGGCTTTGCTCCTCTCGTTTCCACGGAGGGTGAGG
LeuArgGluMetAlaIleAspThrCysLysGlnAlaGlyPheAlaProLeuValSerThrGluGlyGluA

       780        790        800        810        820        830        840
ATTTGGATGCGATCAAAGGGTTAGTGTCCGCAGGAATGGGCGTTACCCTTCTGCCTGAAAGTACTTTTGC
spLeuAspAlaIleLysGlyLeuValSerAlaGlyMetGlyValThrLeuLeuProGluSerThrPheAl

       850        860        870        880        890        900        910
TGAAACAACACCTCGTTTTACTGTGAAAATTCCAATTGAGTTCCCTCAAGTAAAACGGACTGTCGGAATC
aGluThrThrProArgPheThrValLysIleProIleGluPheProGlnValLysArgThrValGlyIle

       920        930        940        950        960        970        980
ATTAAACCGAAAAATAGAGAGCTTGCGCCTTCCGCGAATGATTTTTATGAGTTTGTCATTCAATTTTTCT
IleLysProLysAsnArgGluLeuAlaProSerAlaAsnAspPheTyrGluPheValIleGlnPhePheS

       990       1000       1010       1020       1030
CTAAGCTGGAGCAGTATCAATAAAAAAAATGAACCCGAGCTTCTATATAGAAGCTT
erLysLeuGluGlnTyrGln
```

FIG. 5. Nucleotide and predicted protein sequence of the *gltC* gene. Only the nontemplate strand is shown. Note that the orientation is opposite that shown in Fig. 1. The putative promoters for *gltA* and *gltC* are underlined and overlined, respectively. The mapped transcriptional start points are indicated by open triangles.

Fig. 7. In all, nine copies of this sequence appear in the region of DNA representing the 5′ ends of the *gltA* and *gltC* genes. Two copies of the repeat (nine of nine and eight of nine match to the consensus) are found between the −35 and −10 sequences of *gltC*, overlapping the −35 region of *gltA*. The remaining copies have poorer matches to the consensus and are located between positions +100 of *gltC* and +20 of *gltA* (relative to the respective start points of transcription, as indicated in Fig. 7).

DISCUSSION

The evidence presented here suggests that the previously observed nitrogen source-dependent regulation of the genes for glutamate synthase, *gltA* and *gltB*, requires a *trans*-acting positive regulator encoded by the *gltC* locus. This region is itself transcribed from a promoter that is divergent from but overlapping with the *gltA* promoter. In addition to increasing expression from the *gltA* promoter, the *gltC* product (pre-

FIG. 6. Similarity of *B. subtilis* GltC and the LysR family of proteins. The predicted sequence of GltC was aligned with that of LysR (41) and with a consensus (Cons.) sequence for members of the LysR family (18). Numbers above the consensus sequence represent the frequency of the consensus residue in nine members of the family; frequencies of three or less are not shown (18). The single-letter code for amino acids is used. Dashes represent gaps introduced to maximize sequence similarities. Double dots indicate amino acid identity; single dots indicate a conservative change according to functional groupings (D and E; F, Y, and W; I, L, and V; K and R; N and Q; S and T).

sumed at present to be a 35,000-Da polypeptide) negatively regulates its own transcription in a manner that is independent of the nitrogen source.

To account for these observations, we propose that GltC binds to a site(s) within the *gltA/gltC* promoter region, limiting the frequency of initiation from its own promoter in order to maintain a low but constant intracellular concentration of its own transcript and product. Under conditions of limiting glutamate, a conformational change is induced in GltC, such that it acts to stimulate the association of RNA polymerase with the *gltA* promoter, increasing the level of transcription of the glutamate synthase structural genes. The conformational change in GltC does not alter either its DNA-binding properties or its ability to repress *gltC* transcription. Promoter occlusion does not explain the role of GltC in regulating *gltA* transcription, since *gltC* expression does not vary under conditions which alter *gltA* expression 10-fold (i.e., growth in ammonia versus growth in glutamate). The idea that GltC stimulates transcription at the *gltA* promoter by interacting with RNA polymerase is consistent with the observation by Ryu and co-workers (33, 34) that certain Rif^r RNA polymerase mutants are specifically reduced in GOGAT activity. Since we do not yet know whether the contiguous genes *gltA* and *gltB* constitute an operon, regulation by GltC of *gltB* expression could occur at the *gltA* promoter or at a separate *gltB* promoter; in either case, the mechanism of GltC action could be equivalent.

The mutations in *gltC* that we have studied all cause substantial decreases in *gltA* expression, but vary slightly in their responsiveness to changes in the nitrogen source. This raises the possibility that an additional factor acts in concert with GltC to inform it of the glutamate status of the cell. While we cannot rule this out, it is at least as likely that ability to interact with DNA and responsiveness to a nitrogen signal are both encoded in *gltC*. We do not know the identity of the metabolite that might inform GltC of the availability of glutamate inside the cell. Deshpande et al. (14) have suggested that glutamine, a substrate of GOGAT, is the metabolite whose intracellular concentration most directly controls GOGAT synthesis.

The model presented here predicts that there is a site(s) for GltC binding in the *gltA/gltC* promoter region. Candidates for such a site(s) are the direct repeats (5′-ATATTGTTT-3′) located within this region. DNA protection studies with purified GltC would permit determination of the actual binding sites. The site of GltC action is very likely to be within the *Bcl*I-*Nsi*I fragment whose sequence is shown in Fig. 7, since multiple copies of this DNA in wild-type *B. subtilis* render the strain a glutamate auxotroph, as if they were titrating a positive factor (that is, GltC).

Comparison of the putative GltC sequence with that of other proteins revealed that it is very similar to LysR, CysB, and IlvY of *E. coli* and NodD of *R. meliloti*. It has been recently noted that these proteins belong to a group known as the LysR family of bacterial activator proteins (18). This communication identifies GltC as a member of the LysR family and extends the original observation of Henikoff et al. (18) by including a protein of gram-positive origin. It is

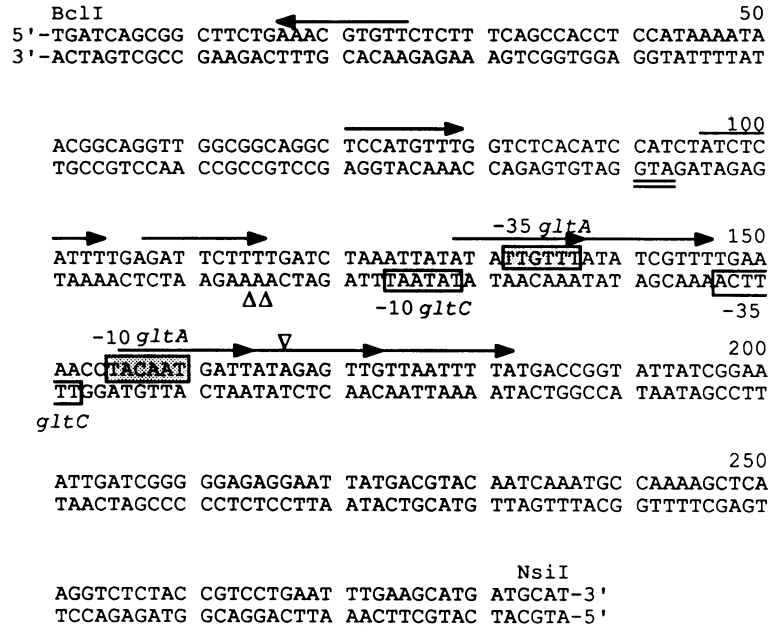

FIG. 7. Nucleotide sequence of the 286-bp *Bcl*I-*Nsi*I fragment containing the *glt* intergenic region of *B. subtilis*. The sequence has the same orientation as in Fig. 1, with *gltA* to the right and *gltC* to the left. Indicated are the transcriptional start points determined by nuclease mapping for *gltA* (open triangle) and *gltC* (solid triangles). The putative promoter signals are boxed. The first start codon of the *gltC* open reading frame is doubly underlined. Occurrences of the sequence 5'-ATATTGTTT-3' are indicated by an arrow over the nucleotides; the possible significance of this sequence is discussed in the text.

interesting that a 9-bp sequence that occurs several times in the *gltA/gltC* regulatory region is similar to sequences found in the target DNAs of several other LysR family proteins (Table 3). One might imagine that these sequences represent versions of a common binding site whose variations permit specificity of activator protein-DNA interactions. In the *ilvY/ilvC* intergenic region, versions of this sequence appear in inverted repeats and have been shown to be binding sites for IlvY (49).

The *gltC* gene is autoregulated; this is a common theme for regulatory genes in gram-negative bacteria (e.g., λ *c*I [29], *pifC* [24], *dnaA* [5]) and is also true for the *B. subtilis* phage ϕ105 repressor gene (46). This theme also appears in the regulation of the *B. subtilis gln* operon, in which both *glnA* and the upstream gene *glnR* are negative regulators of *glnRA* expression (H. J. Schreier, S. Brown, K. Hirshi, J. Nomellini, and A. L. Sonenshein, J. Mol. Biol., in press) and in the expression of the *outB* gene, which appears to be a *cis*-acting autorepressor (1). It may be significant that *gltC* and *gltA* are in divergent transcription units, one of which encodes an autorepressed positive regulator of the other. This arrangement may turn out to be a common motif in regulation of amino acid biosynthesis (examples include *lysR/A* [41, 42], *ilvC/Y* [48, 49], *metR/E* [45], *asnC/A* [19] of *E. coli*, and *trpI/BA* of *Pseudomonas aeruginosa* [8]), as well as in other regulatory systems (2).

TABLE 3. Repeated sequences in positively regulated systems[a]

Sequence (5'→3')	System	Reference
ATATTGTTT	*gltC/A*	This work
ATATCGTTT	*gltC/A*	This work
AGATTCTTT	*gltC/A*	This work
ACAATGATT	*gltC/A*	This work
ATAGAGTTG	*gltC/A*	This work
ATCTCATTG	*gltC/A*	This work
TTAATTTTA	*gltC/A*	This work
TCCATGTTT	*gltC/A*	This work
AACACGTTT	*gltC/A*	This work
ATATTGTTT	*gltC/A* consensus	
ATATCATTT	*lysR/A*	41
ATATTTTTT	*lysR/A*	41
ATTCTTTTT	*lysR/A*	41
TATCAATTT	*ilvY/C*	48
AATAAATTT	*ilvY/C*	48
TTTTTTATG	*asnC/A*	19
TTATTGAAT	*asnC/A*	19
TTATTGCAT	*asnC/A*	19
AAATTGATT	*nodD/ABC*	36
AAATTGATT	*nodD/FE*	36

[a] The consensus sequence is 5'-ATATTGTTT-3'.

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