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Regulation of *Bacillus subtilis* Glutamate Synthase Genes by the Nitrogen Source

DIAN E. BOHANNON, MARK S. ROSENKRANTZ,† AND ABRAHAM L. SONENSHEIN*

Department of Molecular Biology and Microbiology, Tufts University Health Sciences Campus, Boston, Massachusetts 02111

Received 9 April 1985/Accepted 8 June 1985

The wild-type alleles of the *gltA292* and *gltB1* mutations of *Bacillus subtilis* have been identified in banks of *B. subtilis* DNA cloned in phage lambda. These mutations are thought to define the genes for the two subunits of glutamate synthase. Sequences having transforming activity for each allele were subcloned in plasmids and used as hybridization probes for measurements of the rates of synthesis and steady-state levels of *glt* mRNAs under different growth conditions. For both *gltA* and *gltB*, the level of mRNA varied according to the nitrogen source in the growth medium, to an extent sufficient to explain the variation in glutamate synthase activity under the same conditions. Two start points for mRNA synthesis were detected within the cloned DNA, one of which corresponded to the *gltA* locus. The other start point appears to define a transcription unit, separate from *gltA* and *gltB*, within which mutations cause loss of glutamate synthase activity.

Glutamate synthase (L-glutamate:$NADP^+$ oxidoreductase [transaminating], EC 1.4.1.13) is required for synthesis of glutamate and, in conjunction with glutamine synthetase, for assimilation of ammonium ion into cellular metabolic pathways. Mutations in this enzyme cause glutamate auxotrophy, which can be relieved by addition to the medium of glutamate, aspartate (a substrate for glutamate:2-ketoglutarate transaminase), or glutamine (which is converted to glutamate by the action of glutaminase).

The specific activity of glutamate synthase in *Bacillus subtilis* varies considerably in cells grown in media containing different nitrogen sources (24, 28). It is not known to what extent this reflects alterations in enzyme activity or changes in gene expression. It is also not known whether this regulation is a reflection of a global control mechanism affecting many genes involved in nitrogen metabolism. In the *Escherichia* spp.-*Klebsiella* spp. group of gram-negative bacteria, the *ntr* gene products regulate a large number of nitrogen metabolism genes, including the gene for glutamine synthetase and genes involved in intracellular production of ammonia (20). Even in this well-characterized system, however, it is unclear whether the genes for glutamate synthase are subject to *ntr* regulation.

The construction by Ferrari et al. (12) of a collection of recombinant coliphage λ clones containing large segments of the *B. subtilis* chromosome has made possible the purification and amplification of a number of genetic loci. We report here the subcloning in *Escherichia coli* of *B. subtilis* DNA that includes the wild-type alleles of *gltA* and *gltB*, the genes which apparently code for the two subunits of glutamate synthase (9). Deshpande and Kane (9) have shown that an extract of a *gltB1* mutant complements in vitro an extract of a *gltA292* mutant. These authors have therefore concluded that *gltA292* and *gltB1* define two cistrons whose products constitute the two subunits of glutamate synthase.

We have correlated the transforming activity for *gltA* and *gltB* with regions of cloned DNA and shown that transcription of these regions is subject to regulation by the nitrogen source in the growth medium. The difference in steady-state levels of *gltA* and *gltB* mRNA in cells grown in media containing different nitrogen sources is sufficient to account for the differences in specific activity of glutamate synthase seen under the same conditions.

MATERIALS AND METHODS

Bacterial and phage strains. *B. subtilis* strains SMY, SF31 (*gltA292 trpC2 hutH1 metC*), and UTB602 (*gltB1 leu met his*) were obtained from P. Schaeffer, S. Fisher, and J. Kane, respectively. Mutants PY150 and PY194, both of which are glutamate auxotrophs caused by insertion of Tn*917*, were from P. Youngman. *E. coli* strain MM294 (*hsdR endA thi*) was from R. Losick, and strain DP50supF (*dapD8 lacY Δgal-uvrB ΔthyA nalA hsdS supE44 supF58*) was from J. Hoch. Plasmids pBR325 (4) and pTR262 (25) were obtained from K. Dharmalingam and T. Roberts, respectively.

Two pools of hybrid λ phages carrying *B. subtilis* DNA were made available to us by J. Hoch. One pool was made from the left and right arms of λ Charon 4A DNA ligated to *B. subtilis* DNA that had been partially methylated before cleavage with *Eco*RI (3). The other pool had the left and right arms of λgtWES.λB DNA (18) ligated to *B. subtilis* DNA that had been partially cleaved with *Eco*RI (J. Hoch, personal communication). In both cases, a 10- to 20-kilobase (kb) size class of *B. subtilis* DNA was used for ligation. Growth of λ phage derivatives has been described previously (13).

DNA extraction. DNA of lambda phages was prepared for transformation or enzymatic digestion by phenol extraction (21) or by treatment of virions with formamide (7). For transformation of *B. subtilis*, it was sufficient to treat virions with 0.02% Triton X-100 and 5 mM EDTA (pH 8), for 15 min at 65°C. As noted by Ferrari et al. (12), untreated phage stocks also had transforming activity. Chromosomal DNA of *B. subtilis* was extracted as described previously (30).

Two methods were used to extract plasmids from *E. coli*. Small amounts of DNA suitable for restriction analysis and transformation of *B. subtilis* were prepared from colonies or 1- to 3-ml cultures by the method of Holmes and Quigley (17). For detailed analysis, subcloning, and transformation

* Corresponding author.
† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.

of *E. coli*, the method of Birnboim and Doly (2) was used. The plasmid DNA was further purified by centrifugation to equilibrium in a gradient of CsCl containing ethidium bromide (500 μg/ml) (5).

Purification of λ *glt* phages. Approximately 1,000 plaques of each of two lambda phage libraries of *B. subtilis* DNA were picked and transferred in groups of about 100 to duplicate plates seeded with *E. coli* strain DP50 *supF*. The next day, one plate of each set was harvested for phage; the other served as a master plate. DNA was extracted from the lysates of each set of plaques and used to transform (6) competent cells of *B. subtilis* auxotrophs to prototrophy.

If DNA of a particular subpool was able to transform a given allele, the 100 plaques of that subpool were inoculated in groups of 8 to 10 onto fresh pairs of indicator plates. If transformation with DNA extracted from pools of 8 to 10 plaques was again positive, individual plaques were tested for transforming activity. In this way, we obtained purified phages that carried the wild-type alleles of *gltA292* (8) and *gltB1* (9). It should be noted that after identification of a particular plaque as having transforming activity, it was necessary to replate and test a number of single plaques to obtain a pure clone.

Large-scale isolation of *B. subtilis* RNA. Strain SMY grown in TSS medium (14) containing various nitrogen sources was harvested in the midlogarithmic phase. As described by Fisher et al. (13), RNA was extracted from protoplasts with phenol and purified over a column of cellulose (15) or precipitated with LiCl from an extract prepared by passage through a French pressure cell (36). RNA was stored at −70°C as an ethanol precipitate. As judged by agarose gel electrophoresis, RNA was intact and contained <1% contaminating DNA.

Isolation of pulse-labeled RNA from *B. subtilis*. *B. subtilis* strain SMY was grown in TSS medium with 0.25 mM K_2HPO_4 and various nitrogen sources until growth slowed due to phosphate exhaustion. Three milliliters of cells (100 to 150 Klett units) was mixed with 3 to 6 mCi of carrier-free ^{32}P-PO_4 (New England Nuclear Corp.; 20 mCi/ml in 20 mM HCl) and shaken at 37°C for 5 min. Cells were then poured over 1.5 ml of frozen 121 medium (11) to stop incorporation and RNA degradation. RNA was extracted from protoplasts and the mRNA-rRNA fraction was purified by cellulose column chromatography (15).

Preparation of ^{32}P-labeled plasmid DNA. Nick translation reactions contained (in 20 μl) 0.5 μg of DNA, 100 μCi of one α-^{32}P-labeled deoxynucleoside triphosphate (New England Nuclear Corp.; 3,200 Ci/mmol, 10 mCi/ml, in Tricine), 50 mM Tris-hydrochloride (pH 7.2), 10 mM $MgSO_4$, 1 mM dithiothreitol, 100 μM each of the remaining deoxynucleoside triphosphates, 4 U of *E. coli* DNA polymerase I (Bethesda Research Laboratories, Inc.), and 1 μl of DNase I (Worthington Diagnostics; 0.1 μg/ml, DPFF grade). The reaction was incubated at 14°C for 30 min. Labeled DNA was separated from unincorporated substrate by two precipitations with ethanol and passage through a column of Sephadex G-100 in 1 mM Tris-hydrochloride (pH 8)–0.1 mM EDTA.

To label DNA at protruding 5′ ends, restriction endonuclease-treated plasmids or purified fragments were treated with calf intestinal alkaline phosphatase (Boehringer Mannheim Biochemicals) at 37°C. Reactions contained 50 mM Tris-hydrochloride (pH 9.0), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM spermidine, and 0.04 U of calf intestinal alkaline phosphatase. After 30 min, a second dose of 0.04 U of calf intestinal alkaline phosphatase was added. The reaction was terminated 30 min later by incubation at 65°C for 15 min, followed by three phenol extractions and three $CHCl_3$ extractions. After ethanol precipitation, dephosphorylated DNA was suspended in water and labeled by the action of T4 polynucleotide kinase (New England Biolabs, Inc.). Reactions (50 μl) contained 50 mM Tris-hydrochloride (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM EDTA, 0.1 mM spermidine, 100 to 150 μCi [γ-^{32}P]ATP (New England Nuclear Corp.; 3,200 Ci/mmole, 10 mCi/ml, in Tricine), and 4 to 8 U of kinase. After 30 min at 37°C, reactions were terminated by ethanol precipitation in the presence of carrier *Saccharomyces cerevisiae* RNA. Unincorporated radioactive material was removed by multiple ethanol precipitations.

Hybridization techniques. DNA fragments separated on 1% agarose gels (20 cm long, 5 to 8 mm thick) were transferred to nitrocellulose (Millipore Corp; HAHY) as described by Southern (31). After overnight blotting, the nitrocellulose sheets were baked for 2 h at 80°C in a vacuum oven and stored for short periods at 4°C.

For hybridization, blots were wetted one side at a time in 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M trisodium citrate), prehybridized for at least 2 h at 37°C in 10 to 20 ml of 5× SSC containing 50% formamide [deionized with AG501-X8 (D) mixed bed resin; Bio-Rad Laboratories], 0.5% sodium dodecyl sulfate, 0.1% Ficoll, 0.1% polyvinylpyrollidone, 0.1% bovine serum albumin, and 200 to 500 μg of sheared, denatured calf thymus or salmon sperm DNA. The radioactive RNA probe or heat-denatured DNA probe was added to the same hybridization solution, and blots were incubated in sealed plastic bags for 16 to 48 h at 37°C. After hybridization, the solution containing the probe was removed and frozen for possible reuse, and nitrocellulose membranes were washed three times at 22°C for 15 to 30 min each time by shaking in 100 ml of 2× SSC plus 0.1% sodium dodecyl sulfate and three times at 50°C for 15 to 30 min each time in 0.2× SSC plus 0.1% sodium dodecyl sulfate. Nitrocellulose membranes were then dried on Whatman 3MM paper and subjected to autoradiography with Kodak XAR-5 film and DuPont Cronex Lightning Plus intensifying screens at −70°C. Dot blots, prepared by the method of Thomas (32), have been described previously (13).

To map the 5′ ends of transcripts, the S1 nuclease technique of Berk and Sharp (1) was employed. DNA probes labeled at 5′ termini (10,000 to 50,000 cpm) were mixed with various amounts of *B. subtilis* RNA. *S. cerevisiae* RNA was used to bring all mixtures to equal nucleic acid concentration. Ethanol-precipitated nucleic acids were suspended in 10 or 20 μl of hybridization buffer containing 80% deionized formamide, 40 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (pH 6.5), 0.4 M NaCl, and 1 mM EDTA. Nucleic acids were denatured by treatment at 80°C for 5 min in sealed glass capillary pipettes and then incubated overnight at 45°C. Hybridization was terminated by dilution into 10 volumes of ice-cold S1 digestion buffer (0.25 M NaCl, 50 mM sodium acetate [pH 4.5], 5 mM $ZnSO_4$). S1 nuclease (Bethesda Research Laboratories, Inc.) was added (0.3 to 0.5 U/μg of RNA), and the reactions were incubated at 37°C for 30 min. Reactions were terminated by the addition of EDTA to 25 mM and ethanol precipitation in the presence of carrier *S. cerevisiae* RNA. Washed pellets were dried and suspended in 3 to 5 μl of 80% formamide, 0.089 M Tris, 0.089 M boric acid, 0.2 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol. Samples were heated to 90°C for 5 min, cooled on ice for 5 min, and subjected to electrophoresis through a 6 or 8% polyacrylamide gel containing 8 M urea.

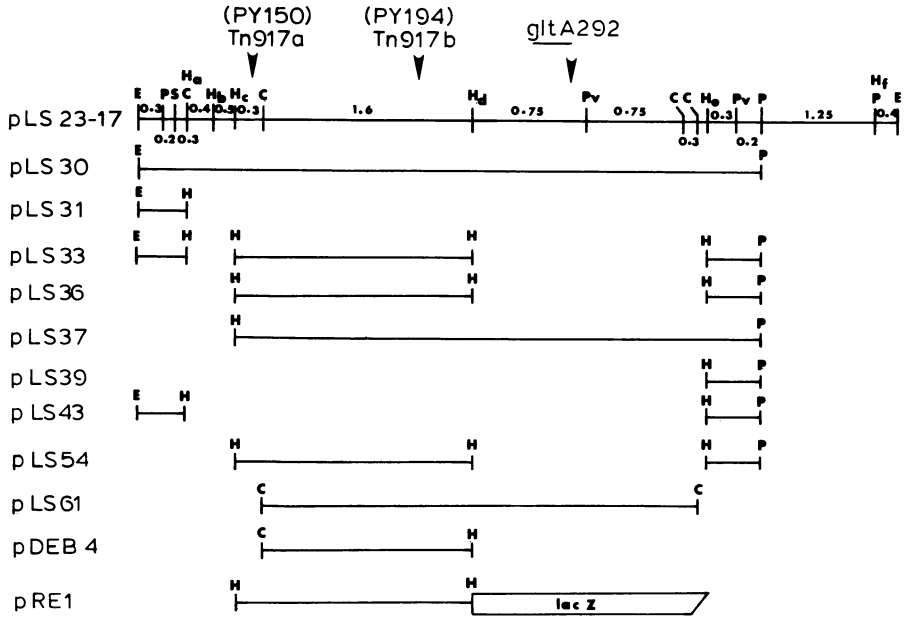

FIG. 1. Restriction map of the *gltA* region. The top line shows a physical map of the *B. subtilis* DNA contained within pLS23-17 (Amp^r Tet^r), a pBR325 derivative that gives Glt$^+$ transformants with all *gltA* mutants tested. The locations of some *gltA* mutations are indicated. The various *Hin*dIII sites have been identified with subscripts a through f. The remaining lines show the *B. subtilis* DNA content of various subclones of pLS23-17. To construct pLS30 (Tet^r Amp^s), pLS23-17 was partially cleaved with *Pst*I and religated. pLS31, pLS36, and pLS39 (all Tet^r) were formed by ligation of *Hin*dIII fragments of pLS30 to pTR262. pLS33, pLS37, and pLS43 (all Tet^r) were found after digestion of pLS30 with *Hin*dIII and religation. To create pLS54, pLS37 was cut with *Hin*dIII and religated. pLS61 (Amp^r Tet^s) was formed by cloning *Cla*I fragments of pLS37 in pBR322. In some cases, the relative orientations of segments of the *gltA* region became inverted during subcloning. For instance, in pLS37, the *Hin*dIII fragments of 1.9 and 1.8 kb are in the opposite orientation to that shown with respect to the 0.5-kb *Hin*dIII-*Pst*I fragment. In pLS61, the 0.3-kb *Cla*I fragment lies to the left of the 3.0-kb *Cla*I fragment. pRE1 (Amp^r Tet^r) carries a fusion of the *gltA* promoter region (1.9-kb *Hin*dIII fragment) to the *E. coli lacZ* gene. It was constructed by ligating together pLS54 and pCED6 (10), each of which had been cut with *Hin*dIII. Restriction sites are abbreviated as follows: C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Pv, *Pvu*II; S, *Sal*I.

Dried gels were exposed to Kodak XAR-5 film and DuPont Cronex Lightning Plus intensifying screens at −70°C.

RESULTS

Cloning of the *gltA* allele. A λ phage whose DNA transformed *B. subtilis* strain SF31 (*gltA292*) to Glt$^+$ was isolated from the λgtWES bank and named λgtWES-10-9. Upon cleavage by endonuclease *Eco*RI, this DNA yielded fragments of approximately 7.5, 5.5, and 4.85 kb, as well as the left and right arms of λgtWES (22 and 14 kb, respectively [18]). Transformation with *B. subtilis* DNA fragments extracted from agarose gels showed that the 7.5-kb fragment carried the transforming activity for *gltA*$^+$. This was confirmed by subcloning the 7.5-kb *Eco*RI fragment in pBR325, creating plasmid pLS23-17 (Fig. 1). The *gltA* region is known to be near the terminus of DNA replication (16); pLS23-17 has been shown to hybridize to a restriction fragment that contains the terminus (33). After establishing a rudimentary restriction map of pLS23-17, we subcloned the region of Glt$^+$ transforming activity. These smaller plasmids are described by their restriction maps in Fig. 1.

Location of *gltA292* and *gltA*::Tn*917* mutations. Each of the plasmids shown in Fig. 1 was used to transform *B. subtilis* strain SF31 (Table 1). Only those plasmids (pLS23-17, pLS30, pLS37, pLS61) that contained DNA from the right side of *Hin*dIII site *d* were able to give Glt$^+$ transformants. Cleavage of these plasmids with *Hin*dIII destroyed Glt$^+$ transforming activity. The results indicate that the *gltA292* mutation lies very close to this *Hin*dIII site or between the *Hin*dIII site and the nearest *Cla*I site to the right.

The transposon Tn*917*, which occurs in natural isolates of *Streptococcus* spp. and can function in *B. subtilis*, has a high propensity for generating glutamate-requiring mutants of *B. subtilis* (34; S. Zahler, personal communication). For some

TABLE 1. Transformation of glutamate auxotrophs by plasmids[a]

Plasmid[b]	Recipient strain				
	SF31 (*gltA292*)	PY150 (*glt*::Tn*917*)	PY194 (*glt*::Tn*917*)	CU3278 (*glt*::Tn*917*)	UTB602 (*gltB1*)
pLS23-17	+	ND	+	+	−
pLS30	+	+	ND	+	ND
pLS31	−	−	ND	−	ND
pLS33	−	+	ND	−	ND
pLS36	−	+	ND	ND	ND
pLS37	+	+	+	+	ND
pLS39	−	−	ND	−	ND
pLS43	−	−	ND	ND	ND
pLS54	ND	+	+	−	ND
pLS61	+	−	+	+	ND
pRE1	ND	+	ND	−	ND
pCS23	−	−	−	−	+
pCS24	ND	ND	ND	ND	+
pLS24-1	−	−	−	−	+

[a] Competent cells of the indicated recipient strain were transformed with plasmid DNAs and tested for ability to given Glt$^+$ transformants. +, >1,000 transformants per μg of donor DNA; −, <10 transformants per μg; ND, not done.

[b] Plasmids used are described in Fig. 1 and 3.

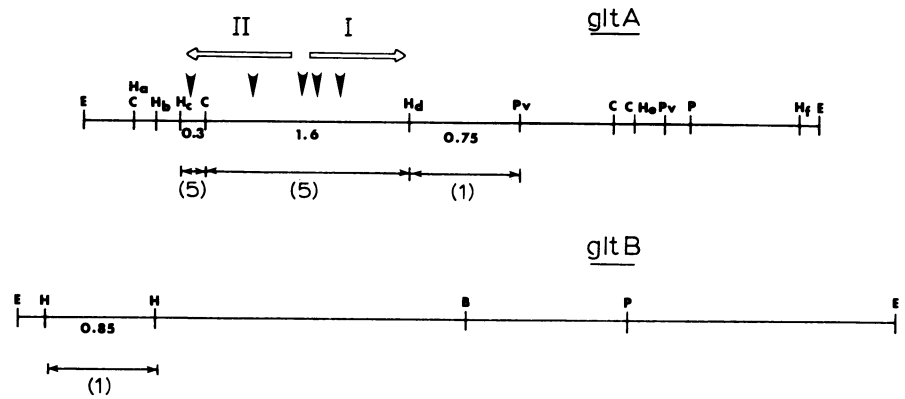

FIG. 2. Sites of insertion of Tn*917* in the *gltA* and *gltB* regions contained in pLS23-17 and pCS23, respectively. This figure shows the locations of Tn*917* insertions in the *B. subtilis* chromosome which caused a Glt$^-$ phenotype. All of the mutations recombine with various cloned *glt* DNAs to generate Glt$^+$ transformants. Closed arrows indicate specific positions of transposon insertions mapped within a restriction fragment; the numbers within parentheses refer to the total number of independent insertions within a particular fragment. The open arrows indicate the direction and minimum extent of transcriptional units found in the *gltA* region.

of these mutants the transposon has been shown to have inserted in the vicinity of *gltA* (S. Zahler, personal communication). Tn*917*-induced glutamate auxotrophs were transformed to Glt$^+$ by various plasmids (Table 1). The *glt*::Tn*917* mutation in strain CU3278, provided by S. Zahler, mapped to the right of *Hin*dIII site *d*. Strain PY194 appeared to have a Tn*917* insertion in the *Cla*I-*Hin*dIII segment to the left of *Hin*dIII site *d*, since it could be transformed to Glt$^+$ by both pLS54 and pLS61. In PY150, the site of Tn*917* insertion appeared to be in the *Hin*dIII-*Cla*I segment to the right of *Hin*dIII site *c* (Fig. 1), since transformation to Glt$^+$ was obtained with pLS54 and pRE1, but not with pLS61. To prove the sites of insertion, we purified chromosomal DNA from PY150 and PY194, cleaved the DNA with various restriction endonucleases, and subjected it to electrophoresis in agarose. After transfer to nitrocellulose by the method of Southern (31), the fragments were hybridized to nick-translated pLS54 or pLS61 DNA. Wild-type DNA showed the hybridization pattern expected from the restriction maps of pLS23-17 and pLS30. Mutants PY150 and PY194, however, had inserts in the middle of a 0.3-kb *Hin*dIII-*Cla*I fragment and in a 1.6-kb *Cla*I-*Hin*dIII fragment, respectively (data not shown; Figure 1). The latter insertion was approximately 0.6 kb to the left of the *Hin*dIII site. A number of additional Glt$^-$ mutants caused by insertion of Tn*917* were obtained from J. Perkins and P. Youngman and mapped genetically, by recombination with various subclones of the *gltA* region, and physically. Three insertions were near the site of insertion in PY194, but were located approximately 0.4, 0.7, and 1.0 kb to the left of *Hin*dIII site *d* (Fig. 2). Four other Tn*917*-induced *glt* mutations were in the same 0.3-kb *Hin*dIII-*Cla*I segment as was the mutation in PY150. It may be noted that, although the *gltA* region is a hot spot for Tn*917* insertion, no two insertions tested were at exactly the same site.

Identification of *gltB*. A purified λ phage whose DNA was able to transform strain UTB602 (*gltB1*) to Glt$^+$ was purified from the λ Charon 4A bank and designated λ Charon 4A-112-1. Upon cleavage by *Eco*RI this phage proved to carry fragments of *B. subtilis* DNA having sizes of 5.5 and 8.5 kb. Subcloning in pBR325 indicated that the fragment of 8.5 kb carried the wild-type allele of *gltB1*. Two *gltB*$^+$ plasmids were obtained at this step. One, pCS23, contained the 8.5-kb *Eco*RI fragment of λ Charon 4A-112-1. The other, pCS24, carried an *Eco*RI fragment of only 7.1 kb. Detailed restriction analysis led us to conclude that pCS24 was similar to pCS23, but had its insert in the opposite orientation and had undergone a deletion of 1.4 kb of *B. subtilis* DNA, removing a *Bam*HI site. Restriction maps of the *B. subtilis* DNA inserts of pCS23 and pCS24 are shown in Fig. 3. We were able to narrow down the limits of the GltB$^+$ transforming activity by finding a spontaneous deletion mutant of pCS24 that had lost ampicillin resistance, but retained tetracycline resistance and Glt$^+$ transforming activity. In this way, we obtained pLS24-1, which has retained no more than 2.1 kb of *B. subtilis* DNA (Fig. 3), but still transforms strain UTB602 to Glt$^+$ (Table 1).

These subclones of the *gltB* region also recombine with

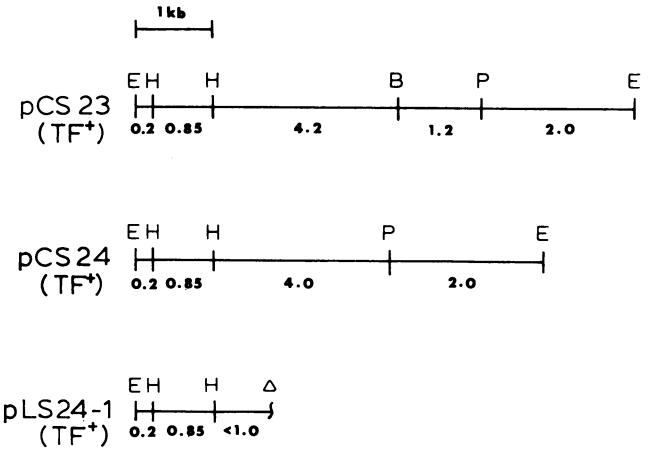

FIG. 3. Physical maps of *gltB* plasmids. Only the *B. subtilis* DNA is shown for plasmids that transform *gltB* mutants to Glt$^+$. In all cases the vector was pBR325 or a deletion derivative of pBR325. pCS23 and pCS24 (both Amp^r Tet^r) were created by cloning *Eco*RI fragments of λ Charon 4A-112-1 (*gltB*$^+$) into pBR325. Note that the *B. subtilis* DNA in pCS24 is deleted and inverted relative to that in pCS23. To isolate pLS24-1, pCS24 was cut with *Pst*I and transformed into *E. coli*, selecting Tet^r. Amp^s clones were isolated; one of these proved to carry pLS24-1, which presumably arose by deletion of pCS24. Restrictions sites are abbreviated as in Fig. 1. In addition, B stands for *Bam*HI.

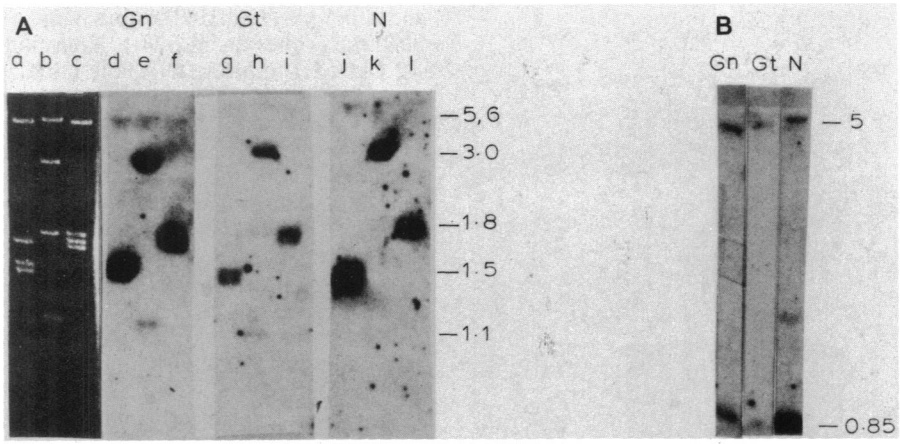

FIG. 4. Hybridization of pulse-labeled RNA to DNA from the *glt* regions. *B. subtilis* strain SMY was grown in defined medium containing various nitrogen sources and pulse-labeled with $^{32}PO_4$ as described in the text. RNA was purified and hybridized to restriction fragments of (A) *gltA* DNA or (B) *gltB* DNA. To the left of the autoradiogram in A is shown the restricted DNA that was blotted. The restricted DNAs were (A) pLS30 cut with *Hin*dIII and *Cla*I (lanes a, d, g, and j), with *Cla*I (lanes b, e, h, and k), and with *Hin*dIII (lanes c, f, i, and l) and (B) pLS24-1 cut with *Hin*dIII. The RNA sources were as follows: N, ammonia-grown cells; Gt, glutamate-grown cells; Gn, glutamine-grown cells.

certain other *glt* mutations. Zahler et al. (35) reported the mapping of a mutation designated *gapA2* (subsequently renamed *gltA2*). Although this mutation lies between *citK1* and *citB1*, it was not transformed to prototrophy by any of our clones from the *gltA* region. It was, however, transformed efficiently by pCS23, pCS24, and pLS24-1. Moreover, Zahler (personal communication) has found that *gapA2* is complemented by an SPβ transducing phage that also complements *gltB1*, but not by a phage that complements *gltA* mutations. We propose that this mutation be redesignated *gltB2*. In addition, a Tn*917*-induced *glt* mutation provided by Perkins and Youngman was rescued by pCS23 and pLS24-1. Thus three independent mutations causing a Glt$^-$ phenotype lie within the region of *B. subtilis* DNA carried on pLS24-1 and outside the region carried on any subclones from the *gltA* region. Although we have not shown by in vitro complementation that *gltB2* and the *gltB*::Tn*917* mutation are in a different cistron than is any *gltA* mutation, we have adopted the working hypothesis that they are in the same genetic locus as is *gltB1*.

It has been reported that *gltA* and *gltB* are not closely linked (9). The λ *gltA*$^+$ and λ *gltB*$^+$ phages we have isolated, in fact, do not show any cross-transformation. Moreover, none of the plasmid subclones for *gltA*$^+$ or *gltB*$^+$ transforms the other mutated allele. On the other hand, *gltA*$^+$-*gltB*$^+$ phages have been isolated from the λ Charon 4A bank by R. Fischer, J. Hoch, and J. Kane (personal communication) and by Monteiro et al. (22). Recent experiments in our laboratory have suggested that the *Eco*RI fragments cloned in pLS23-17 and pCS23 are contiguous in the *B. subtilis* chromosome (C. Mathiopoulos, D. E. Bohannon, and A. L. Sonenshein, unpublished data). These same experiments, however, indicate that the DNA cloned in pCS23 is not a true representation of the chromosomal DNA structure. Since pCS23 hybridizes to two *Eco*RI fragments of chromosomal DNA (5.5 and 9.0 kb), it must have arisen by a deletion event during cloning. Thus the *gltB*$^+$ clones we have isolated can be used for recombination experiments and as hybridization probes, but are not a faithful representation of the *gltB* gene.

Transcription of the *gltA* and *gltB* genes in vivo. To identify the transcription units corresponding to *gltA* and *gltB* and to study their regulation, we pulse-labeled RNA in wild-type cells growing in a minimal medium that contained glucose as the carbon source and various nitrogen sources. Glutamate synthase activity in *B. subtilis* is known to vary with the nitrogen source, the ratio of activities being 1:3:7 for cells grown in glutamate, glutamine, and ammonia, respectively, as the sole nitrogen source (24). The radioactive RNA was purified and hybridized to various restriction fragments from the *gltA* and *gltB* regions. For pLS30 (*gltA*$^+$), *Hin*dIII fragments of 1.9 and 1.8 kb, a *Cla*I fragment of 3 kb, and *Cla*I-*Hin*dIII fragments of 1.5 to 1.6 kb hybridized strongly to RNA from ammonia-grown cells or glutamine-grown cells, but much less well to RNA from glutamate-grown cells (Fig. 4A). Equal amounts of radioactivity were used in each of the hybridizations, and DNA was always in excess. A similar pattern of hybridization with respect to nitrogen

Fragment	Nitrogen source		
	Amm	Gln	Glt
A	-	-	-
B,C,D	-	+	+
D,E	++	++	+
F	++	++	+
F,G	+++	++	+
E,F,G	+++	++	+
F,G,H	++	++	+
G	++	+	+
G,H,I	++	+	+
I,J	+	-	-
A'	+	+	-
B'	+++	++	+

FIG. 5. Interpretation of hybridization experiments. The results of the experiment shown in Fig. 4 and additional experiments in which cloned DNAs were cut with the same and different restriction enzymes are summarized. Strength of hybridization by indicated segments of the *gltA* and *gltB* regions to various RNA preparations is denoted as +++ (strong), ++ (moderate), + (weak), or − (none detected).

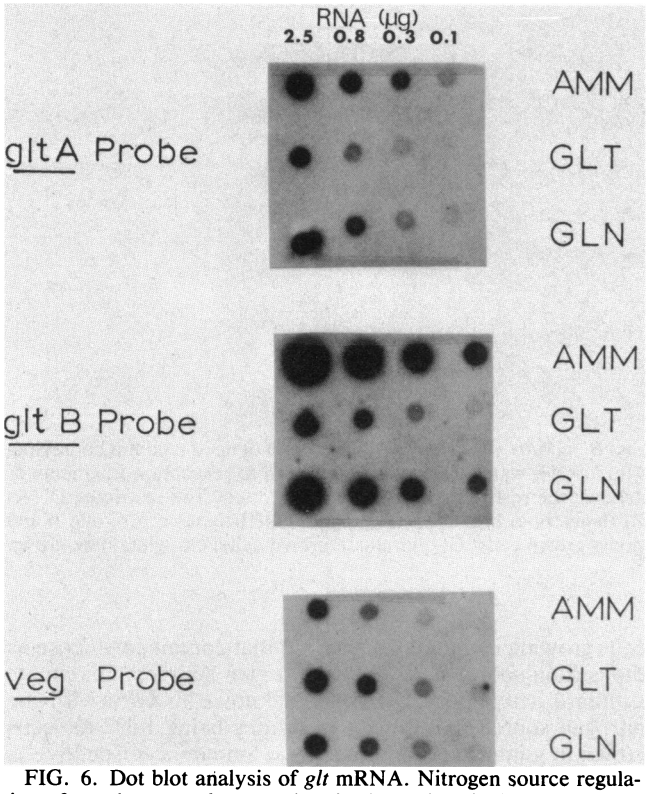

FIG. 6. Dot blot analysis of *glt* mRNA. Nitrogen source regulation of steady-state *glt* transcripts is shown in this dot blot experiment. RNA was purified from cells grown in minimal glucose medium with ammonia (AMM), glutamate (GLT), or glutamine (GLN) as the nitrogen source. Samples (5 μl) of serial threefold dilutions of RNA (2.5 mg/ml suspension) were baked on a nitrocellulose filter and probed with nick-translated plasmids containing subcloned (A) *gltA* (pLS39), (B) *gltB* (pLS24-1), or (C) a control gene (*veg*, pPH9) DNA.

source could be seen with a 0.85-kb *Hin*dIII fragment of pCS24 (data not shown) and pLS24-1 (*gltB*$^+$) (Fig. 4B). The results of several experiments of this type are summarized in Fig. 5. A transcribed region whose regulation is consistent with that expected for glutamate synthase occupies the right half of pLS30 as drawn. This transcribed region is bordered on its left side by a weakly transcribed region. These results suggest that the *gltA* gene may occupy more than 2.5 kb of DNA. The *gltB* region includes a 0.85-kb segment that is transcribed at a higher rate in ammonia-grown cells than in glutamate-grown cells.

Glutamate synthase has not been purified from *B. subtilis*. The enzyme isolated from *Bacillus licheniformis*, however, has two subunits of 158,000 and 54,000 daltons (27). These would require about 4.3 and 1.5 kb of DNA-coding capacity, respectively. Given the relative sizes of the apparent transcription units for each locus, it is probable that the *gltA* locus corresponds to the large subunit of glutamate synthase and the *gltB* locus to the small subunit.

Steady-state levels of *glt* mRNA. The results with pulse-labeled RNA were difficult to quantitate reproducibly (perhaps because the transfer of DNA fragments of differing lengths from agarose to nitrocellulose occurs with varied efficiency). As a result, we turned to dot hybridization of radioactive DNA probes to steady-state RNA from cells grown in various nitrogen sources. Two probes were used for *gltA*, a 1.9-kb *Hin*dIII fragment of pRE1 (data not shown) and a 0.5-kb *Hin*dIII-*Pst*I fragment of pLS39 (Fig. 1). For *gltB*, the probe was pLS24-1, a plasmid that contains within <2.1 kb of *B. subtilis* DNA both GltB$^+$ transforming activity and a region that hybridized differentially to pulse-labeled RNAs from cells grown in different nitrogen sources.

The autoradiogram shown in Fig. 6 demonstrates that RNA from ammonia-grown cells contained a much larger proportion of *gltA* and *gltB* sequences than did RNA from glutamate-grown cells. Glutamine-grown cells had an intermediate level of *glt* RNA. As a control, we used pPH9 (S. F. J. LeGrice, C.-C. Shih, and A. L. Sonenshein, manuscript in preparation), a probe for the *veg* gene (23). This gene is not thought to be under nitrogen control; its use verified that all RNA preparations were similarly competent for hybridization. The approximate ratios of RNA content, as estimated by inspection of hybridization by serial dilutions of RNA, for cells grown in ammonia, glutamine, and glutamate media were 9:3:1 for *gltA* and 27:6:1 for *gltB*. This result indicates that the differences in specific activity of glutamate synthase for cells grown in the different media can be accounted for by differences in steady-state levels of *gltA* and *gltB* mRNA.

Transcription start points. Fusion of *gltA* DNA to the *lacZ* gene of *E. coli* in appropriate fusion plasmids suggested the existence of a promoter site for the *gltA* region located between *Hin*dIII sites *c* and *d* (R. Emond, D. E. Bohannon, C. E. Donnelly, and A. L. Sonenshein, unpublished results). This was confirmed by analysis of the 5′ ends of in vivo transcripts from the *B. subtilis* chromosomal *gltA* region. In fact, S1 mapping experiments revealed the presence of two transcripts in the *gltA* region (Fig. 7). These transcripts protected two fragments of a 1.9-kb *gltA* probe (pRE1) of 800 to 900 bases (Fig. 7A). Additional experiments showed that one of these transcripts (I) varied in abundance according to the nitrogen source, in a manner similar to that for glutamate synthase. When pRE1 was labeled at its *Hin*dIII sites and subsequently cut with *Cla*I, the DNA protected by the regulated transcript remained at a length of 900 bases (Fig. 7B). This indicates that the regulated transcript extends rightward (at least to *Hin*dIII site *d*) and correlates with the positions of *gltA* mutations. This transcript is depicted in Fig. 2. It was about fivefold more abundant in ammonia-grown cells than it was in glutamine-grown cells (Fig. 7B). In other experiments (data not shown), we found that there was ninefold more of transcipt I in ammonia-grown cells than in glutamate-grown cells. Finer mapping of the start point of transcript I (Fig. 7C) indicated that it begins 125 base pairs to the left of an *Hin*fI site and from a point located approximately 825 base pairs to the left of *Hin*dIII site *d*.

The second transcript was only slightly more abundant in ammonia-grown cells than it was in glutamine-grown cells (Fig. 7B). Transcript II is synthesized in the opposite direction from transcript I (i.e., to the left in Fig. 2), starting about 1,000 base pairs to the left of *Hin*dIII site *d*. This places the two transcriptional start points within about 200 base pairs of each other.

DISCUSSION

Using transformation of glutamate auxotrophs as an initial assay, we have identified regions of cloned *B. subtilis* DNA that correspond to the wild-type alleles of *gltA* and *gltB*. These same regions correlate with DNA segments whose transcription in vivo is regulated according to the nitrogen source in the growth medium. This is the first evidence that expression of glutamate synthase is regulated at the transcriptional level and is, to our knowledge, one of only two

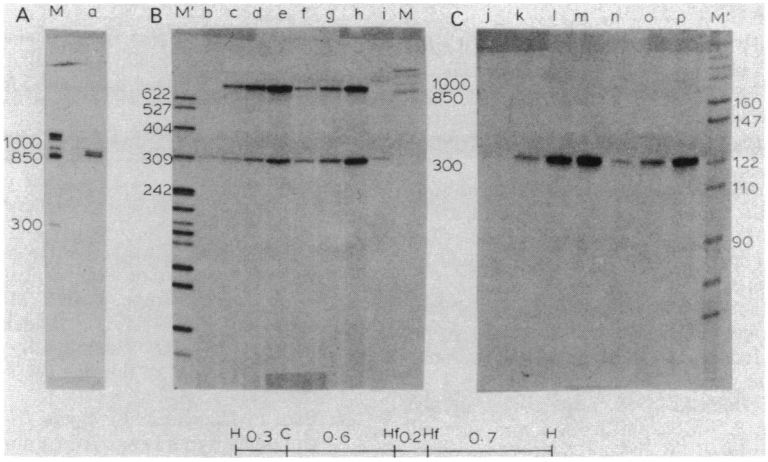

FIG. 7. S1 mapping of *gltA* transcripts. Steady-state RNA was used to protect end-labeled fragments from the *gltA* region, shown at the bottom of the figure. Restriction sites are abbreviated as in Fig. 1. In addition, Hf stands for *Hin*fI. The region shown corresponds to that located between *Hin*dIII sites *c* and *d* in Fig. 1. The rightmost *Hin*fI site is actually two sites located 29 base pairs apart. End-labeled molecular weight markers were pCS24 cut with *Hin*dIII (M) and pBR322 cut with *Hpa*II (M'). These photographs depict autoradiograms of polyacrylamide gels after electrophoresis of the products of S1 nuclease digestion. (A) Lane a, 50 μg of RNA from glutamine-grown cells was used to protect from S1 nuclease digestion the 1.9-kb *Hin*dIII fragment of pRE1 (shown below) labeled at both 5′ ends. (B) To identify the directions of these transcripts, the same probe was cut with *Cla*I after labeling, yielding 0.3- and 1.6-kb fragments, each of which was labeled at its *Hin*dIII end. They were hybridized to RNA isolated from cells grown in medium containing either ammonia or glutamine as a nitrogen source. Lanes: b, DNA hybridized to *S. cerevisiae* RNA (the faint protected band at 300 bases presumably reflects reannealed probe DNA); c, d, and e, DNA protected by 1, 3, and 9 μg of RNA from ammonia-grown cells, respectively; f, g, and h, DNA protected by 3, 9, and 27 μg of RNA from glutamine-grown cells, respectively; i, probe before treatment with S1 nuclease. (C) To measure more accurately the startpoint of the rightward transcript, the purified 1.6-kb fragment was digested with *Hin*fI before end labeling. As seen from the diagram at the bottom of the figure, fragments of 0.6, 0.2, and 0.7 kb were generated. Their relative orientations were mapped in separate experiments. Lanes: j, probe DNA protected by *S. cerevisiae* RNA; k, l, and m, DNA protected by 3, 9, and 27 μg of RNA from ammonia-grown cells, respectively; n, o, and p DNA protected by 9, 27, and 81 μg of RNA from glutamine-grown cells, respectively.

examples of regulation of transcription of a growth gene of a gram-positive bacterium in response to the nitrogen source in the growth medium. (The other example is the glutamine synthetase gene of *B. subtilis* [13]). The mechanism of this regulation is entirely unknown. It may be that glutamate synthase expression is simply controlled by a gene-specific regulator that responds to the intracellular pool of ammonia or glutamate or both. Alternatively, *gltA* and *gltB* may be members of a larger group of genes whose expression is controlled by a global mechanism that affects genes involved in nitrogen metabolism. However, the total number of genes subject to this supposed regulation is probably small. In surveys of enzymes that produce ammonia from amino acids, neither Schreier et al. (28) nor S. H. Fisher (personal communication) was able to find any regulation by the nitrogen source. This reflects a major difference in regulation of metabolism between *B. subtilis* and the *Escherichia* spp.-*Klebsiella* spp. group of gram-negative bacteria. On the other hand, endospore formation in *Bacillus* spp. and *Clostridium* spp. is regulated by the availability of nitrogen-containing compounds (26, 29). Thus, understanding how *gltA* and *gltB* are controlled may contribute to our understanding of how the process of sporulation is initiated.

We have found two transcripts that originate in the *gltA* region, one of which is regulated by the nitrogen source and crosses the region within which the prototypical *gltA* mutation, *gltA292*, lies. For these reasons we conclude tentatively that this rightward transcript corresponds to *gltA* mRNA. The leftward transcript is unlikely to correspond to *gltB*, since it is not appreciably regulated by the nitrogen source. Why mutations in the region coding for this transcript cause glutamate auxotrophy is unknown; it is conceivable that this region codes for a factor required for the expression of *gltA* or *gltB* or both. It should be noted that the *gltA* and *gltB* mutations used are assumed to be in the structural genes for this enzyme, but have not been proved to be so. We have not yet looked for any transcripts that might begin within the *gltB* region.

To determine whether our subclones of *gltA* and *gltB* contained the entire coding sequences of the respective genes, we transformed a glutamate auxotroph of *E. coli* with various chimeric plasmids. Only one locus for glutamate synthase (*gltB*) has been identified in *E. coli*, although a cloned DNA sequence of 8.55 kb appears to code for both subunits of the enzyme (19). A mutation at this locus was not complemented by any of the *glt* subclones of *B.subtilis*. We do not know whether this means that our plasmids lack an entire *glt* cistron or whether both *Bacillus* spp. subunits must be present to complement the *E. coli* defect. Determination of the mechanism of regulation of *gltA* and *gltB* will require analysis of in vitro transcription and isolation of regulatory mutants. We are currently analyzing the promoter site for *gltA* and using a *gltA-lacZ* fusion plasmid to advance this process.

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