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Altered Transcription Activation Specificity of a Mutant Form of *Bacillus subtilis* GltR, a LysR Family Member

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A mutation (*gltR24*) that allows *Bacillus subtilis* glutamate synthase (*gltAB*) gene expression in the absence of its positive regulator, GltC, was identified. Cloning and sequencing of the *gltR* gene revealed that the putative *gltR* product belongs to the LysR family of transcriptional regulators and is thus related to GltC. A null mutation in *gltR* had no effect on *gltAB* expression under any environmental condition tested, suggesting that *gltR24* is a gain-of-function mutation. GltR24-dependent transcription of *gltAB*, initiated at the same base pair as GltC-dependent transcription, was responsive to the nitrogen source in the medium and required the integrity of sequences upstream of the *gltAB* promoter that are also necessary for GltC-dependent expression. Expression of the *gltC* gene, transcribed divergently from *gltA* from an overlapping promoter, was not affected by GltR. Both wild-type GltR and GltR24 negatively regulated their own expression. The *gltR* gene was mapped to 233° on the *B. subtilis* chromosome, very close to the *azlB* locus.

Glutamate synthase, encoded by the *gltAB* genes, synthesizes glutamate from 2-ketoglutarate and glutamine and is an essential component of the major pathway for ammonia assimilation in *Bacillus subtilis* (39). Glutamate synthase gene expression is positively regulated in a nitrogen source-dependent manner by the product of the *gltC* gene. In the absence of GltC protein, *gltAB* transcription is drastically reduced under normally activating growth conditions, e.g., when ammonium ions are available as nitrogen source (7, 8). GltC may not be the only factor controlling *gltAB* expression, however, since the low, residual expression of *gltA* in a *gltC* mutant decreases further under nonactivating growth conditions, e.g., when glutamate or proline serves as sole nitrogen source (5). We sought to identify a second factor involved in *gltAB* regulation by looking for mutants with elevated expression of *gltA* despite the absence of GltC.

GltC belongs to the LysR family of bacterial transcriptional regulators (20, 38), a family which now comprises about 100 members. The members of this family share some common properties (38): (i) they have near their N termini a conserved helix-turn-helix region that is thought to be responsible for DNA binding; (ii) their target genes are frequently linked to and transcribed divergently from the genes for the LysR-type regulators; (iii) binding sites for many LysR-type proteins have a consensus sequence T-N_{11}-A, with elements of dyad symmetry around the two conserved nucleotides, often separated by an (A+T)-rich core; centers of these sequences are located near position −65 with respect to the transcription start sites of the target genes; (iv) for some LysR proteins the binding site extends beyond the consensus site or they have an additional binding site between the consensus sequence and the transcription start site of the target gene; and (v) the genes encoding LysR-type proteins are frequently negatively autoregulated. All of these properties apply to GltC (7, 8).

The common arrangement and sequence similarities of regulatory regions involved in binding of LysR-type proteins and the conservation of putative DNA-binding motifs of these regulators raise the possibility of cross-talk between different members of the family. Analysis of the available databases shows that six of these proteins have been identified in *B. subtilis*. We describe here a mutation in the *gltR* gene that allows GltR, a new member of the LysR family, to activate transcription of *gltAB* in the absence of the normal regulator, GltC.

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MATERIALS AND METHODS

Bacterial strains and culture media. Bacterial strains used in this study are listed in Table 1. *B. subtilis* strains were grown at 37°C in TSS minimal medium with 0.5% glucose and 0.2% nitrogen source or in DS nutrient broth medium, supplemented with appropriate antibiotics (15). Strain BB91 is a derivative of LG200 in which the *cat* marker at the *amyE* locus was substituted by an *spc* marker; it was created by transforming LG200 with pJL62 (Table 2) and selecting for the Spc^r Cam^s phenotype. Strains carrying *gltC*/*gltA* or *gltR* transcriptional fusions at the *amyE* locus were isolated after transforming strain SMY with derivatives of pLG102 (Table 2), selecting for resistance to chloramphenicol, and screening for loss of α-amylase production, which indicated a double-crossover recombination event. The Amy phenotype was assayed with colonies grown overnight on TBAB–0.2% starch plates (15). Derivatives of strain SMY carrying an additional copy of the wild-type *gltR* gene or its *gltR24* allele at the *thrC* locus of the chromosome as a result of a double-crossover, homologous recombination event were constructed by transforming SMY with pBB603 or pBB604 (see below), selecting for the Ery^r Spc^s phenotype, and screening for threonine-dependent growth in minimal medium. The azaleucine resistance (Azl^r) phenotype was determined on TSS-agar plates containing 100 to 200 μg of 4-aza-DL-leucine (Sigma Chemical Corporation) per ml. L broth with appropriate antibiotics (29) was used for growth of *Escherichia coli* strains.

DNA manipulations and transformation. Methods for plasmid isolation, use of restriction and DNA modification enzymes, DNA ligation, and PCR were as described by Sambrook et al. (36). *B. subtilis* chromosomal DNA was isolated by a published procedure (15). Preparation of electroporation-competent *E. coli* cells and plasmid transformation with a Bio-Rad GenePulser apparatus (Bio-Rad Laboratories) were as described by Dower et al. (11). Transformation of *B. subtilis* by chromosomal or plasmid DNA was performed according to the method of Dubnau and Davidoff-Abelson (12). Hybridizations to membrane-bound DNA fragments (Southern blots) were with Nytran membranes (Schleicher & Schuell) by the protocol provided by the supplier. ^{32}P-labelled DNA probes were generated with the Ambion DECAprime DNA labelling kit (Ambion, Inc.).

Cloning of the *gltR* region. To clone the region of DNA that includes the *gltR24* mutation, we first integrated a pJPM1-based library of random 0.5- to 2.0-kb pieces of wild-type *B. subtilis* chromosomal DNA (40) into the chromosome of strain BB632 (*gltC150*::Tn*917 gltR24*). Chromosomal DNA was isolated from the pooled Cam^r transformants and used to transform strain BB92 (*gltC150*::Tn*917*), selecting for cotransformation of Cam^r (the pJPM1 marker) and Glt^+ (the *gltR24* mutation) phenotypes. Individual Cam^r Glt^+ strains were used for chromosomal DNA isolation, and the linkage between Cam^r and Glt^+ was determined after retransformation of BB92.

TABLE 1. Bacterial strains used

Strain	Genotype	Source or reference
E. coli JM107	*endA1 gyrA96 thi hsdR17* (r_K^- m_K^+) *supE44 relA1* λ$^-$ Δ(*lac-proAB*) e14$^-$/F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15	45
B. subtilis		
SMY	Wild type	P. Schaeffer
SMY-S	Δ*gltC*::*spc*	7
SX150	*gltC150*::Tn*917*	8
PY194	*gltA194*::Tn*917*	9
LG200	Δ*amyE*::[*cat*Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)]	7
LG200-S	Δ*amyE*::[*cat*Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] Δ*gltC*::*spc*	7
LG221	Δ*amyE*::[*cat* Φ(*gltCp21′-gusA*) Φ(*gltAp21′-lacZ*)]	SMY × pLG221
LG223	Δ*amyE*::[*cat* Φ(*gltCp23′-gusA*) Φ(*gltAp23′-lacZ*)]	SMY × pLG223
LG806	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)]	SMY × pLG806
BB91	Δ*amyE*::[*cat*::*spc* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)]	LG200 × pJL62
BB92	Δ*amyE*::[*cat*::*spc* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] *gltC150*::Tn*917*	BB91 × DNA of SX150
BB602	Δ*amyE*::[*cat* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] Δ*gltC*::*spc gltR24*	Glt$^+$ spontaneous mutant of LG200-S
BB603	Δ*gltC*::*spc gltR24*	SMY-S × DNA of B602
BB605	*gltC150*::Tn*917 gltR24*	BB603 × DNA of SX150
BB606	Δ*amyE*::[*cat* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] *gltA194*::Tn*917 gltR24*	BB602 × DNA of PY194
BB609	Δ*amyE*::[*cat* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] *gltR24*	BB606 × DNA of SMY
BB614	Δ*amyE*::[*cat* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] Δ*gltC*::*spc gltR24*	BB603 × DNA of LG200
BB632	Δ*amyE*::[*cat*::*spc* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] *gltC150*::Tn*917 gltR24*	BB605 × DNA of BB91
BB645	Δ*amyE*::[*cat*::*spc* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] *gltR24*	BB609 × DNA of BB91
BB649	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)] *gltR24*	BB645 × DNA of LG806
BB651	*gltR*::[pBB434 (*cat*)]	SMY × pBB434
BB666	*thrC*::(*erm gltR24*)	SMY × pBB604
BB667	*thrC*::(*erm gltR*$^+$)	SMY × pBB603
BB669	Δ*amyE*::[*cat* Φ(*gltC′-gusA*) Φ(*gltA′-lacZ*)] Δ*gltC*::*spc gltR24 thrC*::(*erm gltR*$^+$)	BB614 × DNA of BB667
BB672	Δ*gltR*::*neo*	SMY × pBB460
BB678	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)] Δ*gltR*::*neo*	LG806 × DNA of BB672
BB697	*gltR*::[pBB434 (*cat*)] pBB617 (*gltR′neo*)	BB651 × pBB617
BB817	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)] *gltR24 thrC*::(*erm gltR*$^+$)	BB649 × DNA of BB667
BB828	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)] Δ*gltR*::*neo thrC*::(*erm gltR*$^+$)	BB678 × DNA of BB667
BB829	Δ*amyE*::[*cat* Φ(*gltR′-gusA*)] Δ*gltR*::*neo thrC*::(*erm gltR24*)	BB678 × DNA of BB666
CU744	*azlB101 trpC2*	46
IS58	*lys pheA12*	41
BB34	*azlB101 pheA12*	IS58 × DNA of CU744

A strain in which the two markers were about 40% linked by transformation was chosen for cloning of the *gltR24* locus. Chromosomal DNA from this strain was digested with *Sac*I, self-ligated, and introduced by electroporation into *E. coli* cells. The isolated plasmid pBB401 had a 2.35-kb insert of chromosomal DNA (Fig. 1).

Deletion of the leftmost 1.8-kb fragment of the *B. subtilis* DNA insert in pBB401 using the vector *Pst*I site and the *Nsi*I$_1$ site of the insert created pBB407, which was integrated again into the chromosome of *B. subtilis* BB632. The chromosomal DNA of the resulting strain was digested with *Eco*RI, self-ligated, and introduced into *E. coli* cells. The isolated plasmid pBB408 had a 1.3-kb insert of chromosomal DNA (Fig. 1).

The rightmost 0.20-kb *Pst*I$_1$-*Eco*RI$_2$ piece of the chromosomal insert of pBB408, the *Eco*RI site of which was filled in with the large (Klenow) fragment of *E. coli* DNA polymerase I, was subcloned in pJPM1 cut with *Pst*I and *Hin*cII. The resulting plasmid, pBB410, was integrated into the *B. subtilis* chromosome. The chromosomal DNA of this strain was digested with *Sac*I, self-ligated, and introduced into *E. coli* cells. The isolated plasmid pBB411 had a 9.8-kb insert of chromosomal DNA (Fig. 1).

TABLE 2. Plasmids used

Plasmid	Markers	Source or reference
pJPM1	*bla cat lacZ′*	31
pIPC10	*neo gltC*$^+$	7
pIPC100	*neo gltC′*	7
pJL62	*bla cat*::*spc*	27
pBEST501	*bla neo*	22
pBS(−)	*bla lacZ′*	Stratagene, Inc.
pBluescript SK(−)	*bla lacZ′*	Stratagene, Inc.
pDG1664	*bla spc erm*	18
pBB603	*bla spc erm gltR*$^+$	This work
pBB604	*bla spc erm gltR24*	This work
pMK3-1	*bla neo lacZ′*	48
pBB617	*bla neo lacZ′ gltR′*	This work
pLG102	*bla cat ′lacZ ′gusA*	7
pLG806	*bla cat* Φ(*gltR′-gusA*)	This work
pLG221	*bla cat* Φ(*gltCp21′-gusA*) Φ(*gltAp21′-lacZ*)	This work
pLG223	*bla cat* Φ(*gltCp23′-gusA*) Φ(*gltAp23′-lacZ*)	This work

Other plasmid constructions. All the plasmids presented in Fig. 1 are derivatives of pJPM1 or pBluescript SK(−) and are either described above and below or were constructed by deleting or subcloning fragments of the *gltR* region. Other plasmids used in this work are described in Table 2.

Plasmid pLG806 was constructed in several steps. A blunt-ended 0.54-kb *Sty*I-*Nde*I fragment, containing the entire intergenic region between *gltR* and *orf129* (Fig. 1) and a terminal GTCGACTCTAGAGGATC sequence, originating from the pBS(−) polylinker and creating a *Bam*HI site at the *Sty*I junction, was subcloned between the *Sal*I and *Sma*I sites of pBluescript SK(−). The 0.56-kb *Bam*HI-*Bam*HI fragment of this plasmid, pBB454, including the same *Sty*I-*Nde*I fragment of *B. subtilis* DNA, was subcloned in both orientations in the bidirectional promoter-probe, integrative vector pLG102, cleaved with *Bgl*II. One of the two resulting plasmids, pLG806, carries a *gltR-gusA* fusion.

pBB603 and pBB604, containing the entire *gltR* and *gltR24* genes, were constructed by cloning the 1.4-kb *Bgl*II-*Hin*dIII$_5$ fragments of pBB435 and pBB426 (Fig. 1), respectively, in pDG1664, cleaved with *Bam*HI and *Hin*dIII.

pBB617, containing a truncated *gltR* gene, was constructed by cloning the 0.77-kb *Nae*I-*Hin*dIII$_5$ segment, excised from pBB450 (Fig. 1) as a *Bam*HI-*Hin*dIII fragment, in a multicopy *E. coli-B. subtilis* shuttle vector, pMK3-1, a derivative of pMK3 (43) with a unique *Eco*RI site.

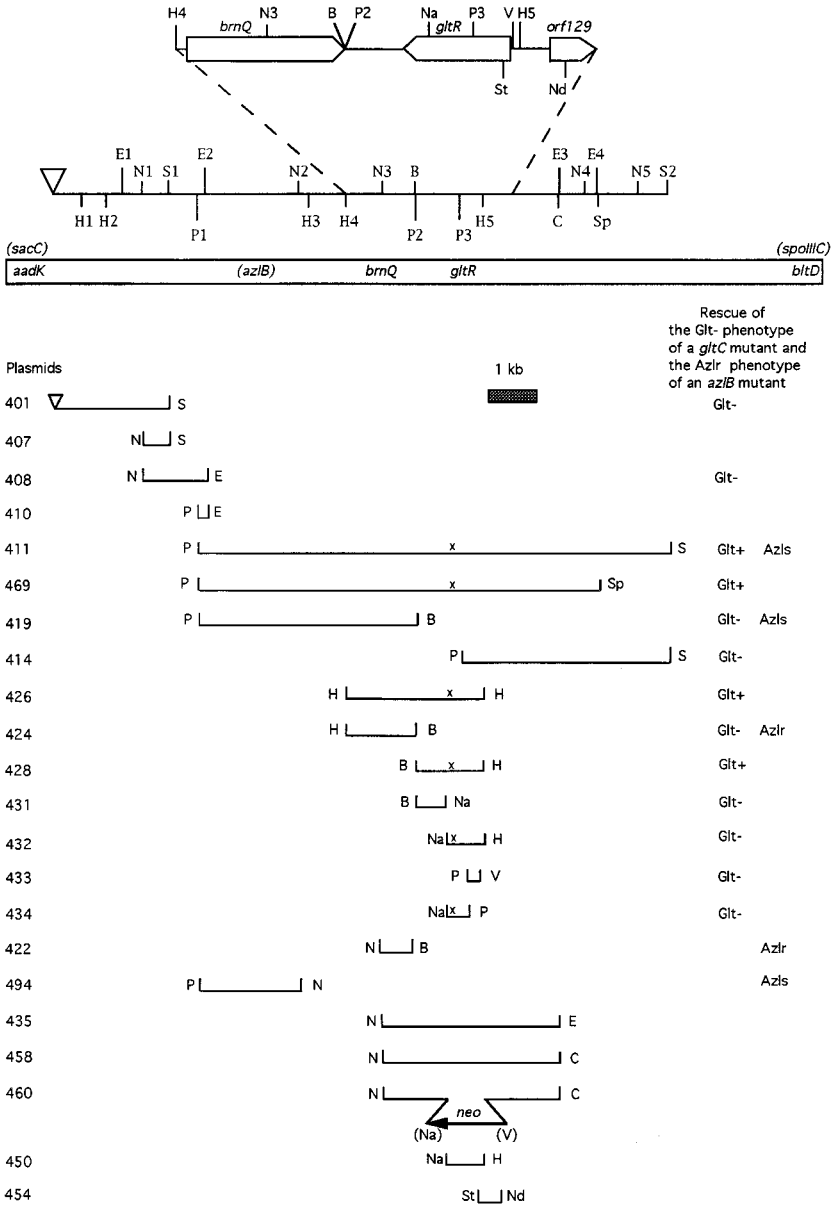

FIG. 1. Restriction map of the *gltR* region and scheme of *gltR* deletion mapping. Restriction sites are abbreviated as follows: B, *Bgl*II, C, *Cla*I, E, *Eco*RI, H, *Hin*dIII, N, *Nsi*I, Na, *Nae*I, Nd, *Nde*I, P, *Pst*I, S, *Sac*I, Sp, *Spe*I, St, *Sty*I, V, *Eco*RV. Only relevant *Cla*I, *Eco*RV, *Nde*I, and *Sty*I sites are shown. Inverted triangle indicates the site of the original integration in the chromosome of a pJPM1 derivative, used for cloning of the *gltR* region. To check for the presence of the *gltR24* mutation, various plasmids were used to transform strain BB92 (Glt^-) to chloramphenicol resistance followed by scoring for the Glt^+ phenotype. The small x marks the presence and approximate location of the *gltR24* mutation in some plasmids. Presence of the wild-type *azlB* allele within some of the plasmids was tested for after transformation of strain BB34 (Azl^r) to Cam^r and then scoring for sensitivity to 4-azaleucine. Plasmids pBB450 and pBB454 are derivatives of pBluescript SK(−). All other plasmids are derivatives of pJPM1.

Construction of a *gltR* null mutant. A deletion-insertion within the *gltR* gene was created by replacing the 0.70-kb *Nae*I-*Eco*RV fragment of pBB458 (Fig. 1) with the 1.3-kb *neo* cassette, excised from pBEST501 as a *Sma*I fragment. The deletion removes 79% of the *gltR* coding region, including the putative helix-turn-helix motif of GltR. One of the two resulting plasmids, pBB460 (Fig. 1), in which the orientation of the *neo* gene coincided with the orientation of *gltR*, was introduced into *B. subtilis* SMY, and Neo^r Cam^s transformants, arising from double crossover homologous recombination, were selected. The replacement of the chromosomal *gltR* gene by the Δ*gltR*::*neo* allele in strain BB672 was confirmed by sizing the PCR fragments generated from the *gltR* locus.

RNA isolation and primer extension. Cells of *B. subtilis* were grown in TSS-glucose-ammonia medium until late exponential phase. Pelleted cells from a 5-ml culture were resuspended in 0.1 ml of 20% sucrose–150 mM NaCl–1 mM EDTA and treated with lysozyme (0.4 mg/ml) for 20 min at 37°C. One milliliter of Trizol reagent (Gibco BRL, Life Technologies) was added and RNA was extracted according to the manufacturer's instructions and quantified by spectrophotometry. Primer extension experiments were performed as described previously (23) with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc., St. Petersburg, Fla.) or, according to the manufacturer's protocol, with Superscript II (Gibco BRL, Life Technologies). The oligonucleotide oBB13 (7) served as a primer specific for *gltA*. For *gltR*, two primers, oBB14 (5′-CTTGG AAATACTGCCTTCACG) and oBB29 (5′-CGTTACATTCGATTGTGC), corresponding to positions 105 to 85 and 145 to 127 with respect to the *gltR* transcription start point, respectively (see below), were used. All oligonucleotides used in this work were synthesized by M. Berne, Tufts University Protein and Nucleic Acid Analysis Facility.

Isolation of mutations in the *gltC-gltA* regulatory region. Plasmid pIPC100 was used as the template for oligonucleotide-mediated site-directed mutagenesis by the method of Kunkel et al. (24), as described previously (7). The mutagenic oligonucleotide 5′-CTCAAAATXAGATAGATG, where **X** represents an equimolar mixture of dATP, dCTP, and dTTP, was used to create the substitutions *gltAp21* and *gltAp23* (see below). Both mutations were confirmed by DNA

TABLE 3. Effect of the *gltR24* mutation on activities of *gltA* and *gltC* fusions in glucose minimal medium

Strain genotype[b]	*gltA-lacZ* promoter type	β-Galactosidase activity (*gltA-lacZ* fusion)[a]			β-Glucuronidase activity (*gltC-gusA* fusion)[a]		
		NH_4Cl	NH_4Cl + Pro	Pro	NH_4Cl	NH_4Cl + Pro	Pro
glt$^+$	Wild type	75.8[c]	49.6	<0.6	0.7	0.5	0.8
glt$^+$	C(−67)A	38.7	20.8	<0.8	1.0	0.7	1.1
glt$^+$	C(−67)G	34.7	16.9	<0.4	2.0	1.7	2.2
Δ*gltC*::*spc*	Wild type	NA[d]	0.9	≤0.3	NA	7.1	8.8
Δ*gltC*::*spc*	C(−67)A	NA	0.9		NA	6.4	
Δ*gltC*::*spc*	C(−67)G	NA	0.9		NA	5.6	
Δ*gltC*::*spc gltR24*	Wild type	34.2	12.5	1.6	6.4	5.7	4.8
Δ*gltC*::*spc gltR24*	C(−67)A	28.8			7.1		
Δ*gltC*::*spc gltR24*	C(−67)G	43.7		1.8	6.6		4.2
Δ*gltC*::*spc gltR24 thrC*::*gltR*$^+$	Wild type	NA	5.6		NA		7.9

[a] Cells were grown in TSS-glucose minimal medium with different nitrogen sources; threonine (100 μg/ml) and valine (40 μg/ml) (27) were also added for a *thrC* mutant. Enzyme activity was assayed and expressed in units as described in Materials and Methods.
[b] Strains LG200, LG221, and LG223 and their Δ*gltC*::*spc*, *gltR24*, or *thrC*::*gltR*$^+$ derivatives were used.
[c] All of the numbers are averages of at least two experiments, and the mean errors did not exceed 20%.
[d] NA, not applicable (*gltC* null mutants are unable to grow unless provided with a source of glutamate).

sequencing of the entire *gltCA* regulatory region. The bidirectional promoter fusion plasmids pLG221 and pLG223 were constructed by subcloning the mutated *gltCA* regulatory regions in pLG102, as described previously (7).

DNA sequencing. DNA fragments containing the wild-type or mutant *gltR* regions or mutations in the *gltCA* regulatory region were sequenced by the dideoxy chain termination method of Sanger et al. (37) using vector- or *gltR* region-specific oligonucleotides as primers. Plasmid double-stranded DNA to be sequenced was purified by modifications of the boiling (19) or mini-alkaline (38) lysis protocols, or by using the Wizard miniprep purification kit (Promega Corp.). A Sequenase reagent kit (United States Biochemical Corp.) was used according to the protocol of the manufacturer.

DNA and protein sequences were analyzed by using DNA Strider, the package of programs provided by the University of Wisconsin Genetics Computer Group (10), and the BLAST program (2).

Enzyme assays. β-Galactosidase and β-glucuronidase activities were determined in Z buffer (29) as described previously (7).

Nucleotide sequence accession number. The nucleotide sequence of the *gltR* region reported here has been assigned GenBank accession number U79494.

RESULTS

Isolation of *trans*-acting Glt$^+$ pseudorevertants. *B. subtilis* LG200-S (Δ*gltC*::*spc*), which is unable to grow without a source of glutamate and cannot express its *gltA-lacZ* fusion, forms white colonies on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Spontaneous *trans*-acting Glt$^+$ pseudorevertants of LG200-S were selected as colonies that grew on minimal glucose-ammonia plates and turned blue in the presence of X-Gal, i.e., were capable of expressing simultaneously the *gltAB* genes and the *gltA-lacZ* fusion. We demonstrated by transformation crosses that one of these strains, BB602, had a single mutation conferring both phenotypes. This mutation, called *gltR24*, was unlinked to either the *gltCAB* locus or the *amyE* locus (at which the *gltA-lacZ* fusion was integrated), i.e., it was an extragenic suppressor mutation for the Δ*gltC*::*spc* allele. The same mutation also suppressed the *gltC150*::Tn*917* allele. Five additional, independent *trans*-acting Glt$^+$ mutants were isolated from strains LG200-S (Δ*gltC*::*spc*) or BB92 (*gltC150*::Tn*917*). All of these mutations suppressed both *gltC* null alleles.

***gltR24*-dependent expression of the *gltA* gene.** The generation time of the Δ*gltC*::*spc gltR24* double mutant in minimal glucose-ammonia medium (~70 min) was only slightly longer than that of the wild-type strain (~60 min); *gltC* mutants cannot grow at all in this medium. When the medium contained glutamate or proline the growth rate of the double mutant was identical to that of either *glt*$^+$ or Δ*gltC*::*spc* strains (data not shown). *gltA-lacZ* expression in the Δ*gltC*::*spc gltR24* double mutant under normally activating growth conditions (when ammonia served as sole nitrogen source) constituted 45% of the wild-type level (Table 3, lines 1 and 7). Under partially activating conditions (in the presence of ammonia plus proline as nitrogen sources) expression was >10-fold higher than in the Δ*gltC*::*spc* strain and constituted 25% of the expression level in wild-type cells (Table 3, lines 1, 4, and 7). Low *gltA* expression was seen in the presence of glutamate or proline (Table 3, lines 1 and 7, and data not shown). Thus, *gltR24*-dependent expression of *gltA* was regulated by the nature of nitrogen source in the medium in a manner similar to that for GltC-dependent expression in wild-type cells.

A mutant *gltA-lacZ* fusion containing a defect in the −10 region of the *gltA* promoter (nucleotides TGAT at positions from −7 to −4 with respect to the *gltA* transcription start point were replaced by ACTA) (5) was completely inactive both in *glt*$^+$ and in Δ*gltC*::*spc gltR24* strains, indicating that the same promoter is likely to be active in both strains (data not shown). This deduction was confirmed by primer extension analysis (Fig. 2A), which showed that *gltA* mRNA has the same apparent 5′ end as a result of either GltC-dependent (7) or GltR24-dependent transcription.

The *gltC* gene is negatively autoregulated (7, 8). Though the *gltR24* mutation compensated for the lack of GltC with respect to activation of the *gltA* promoter, it did not significantly affect expression from the divergent *gltC* promoter (Table 3, lines 4 and 7).

The *gltR24* mutation had no effect on growth rate or *gltA-lacZ* and *gltC-gusA* expression in a *gltC*$^+$ strain (data not shown).

In vivo interaction of GltR24 with the *gltCA* regulatory region. GltC-mediated activation of *gltA* expression is contingent on the integrity of two dyad symmetry sequences, Box I and Box II, in the *gltCA* regulatory region (7) (in Fig. 3A and 4B this region is presented with respect to the *gltC* and *gltA* transcription start points, respectively). We measured expression of *gltA-lacZ* fusions containing mutations in this region in the Δ*gltC*::*spc gltR24* strain (GltR24-dependent expression) and compared it to similar data for a *gltC*$^+$ *gltR*$^+$ strain (GltC-dependent expression) (7). Figure 4A shows that most alterations of Box I and Box II decreased expression of mutant *gltA-lacZ* fusions in the Δ*gltC*::*spc gltR24* strain. The effects of individual mutations on GltC- or GltR24-dependent expression were not parallel, however. Two mutations [Ω(−57, 56)A

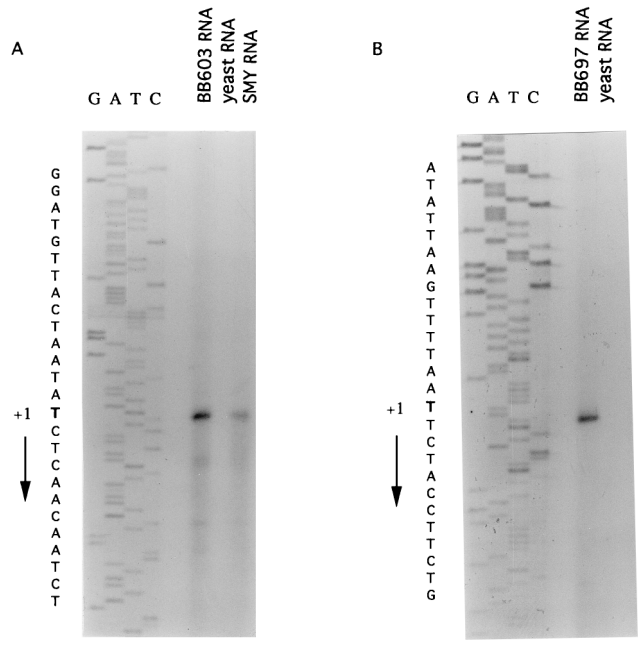

FIG. 2. Determination of transcription start sites. The apparent transcription start sites of *gltA* (Fig. 2A) and *gltR* (Fig. 2B) are outlined. The direction of transcription is shown by the arrows. Primers oBB13 and oBB14 were extended with avian myeloblastosis virus reverse transcriptase or Superscript II by using 20 to 40 μg of RNA from *B. subtilis* SMY, BB603 (Δ*gltC*::*spc gltR24*), and BB697 [*gltR*::pBB434 (pBB617 *gltR′*)] grown in glucose-ammonia minimal medium or from *Saccharomyces cerevisiae* (Sigma Chemical Company) as templates. The sequences of the template strands of plasmids pIPC10 and pBB454 obtained with oBB13 and oBB14 as primers, respectively, are shown to the left of each primer extension. Primer oBB29 gave the same apparent *gltR* mRNA 5′ end as did oBB14 (data not shown).

and T(−46)A] that severely affected GltC-dependent expression had little effect on GltR24-dependent expression (Fig. 4A).

Surprisingly, 9-bp insertions that increase the distance between the centers of Box I and Box II from two helical turns of DNA to three turns and greatly enhance GltC-dependent expression of such *gltA-lacZ* fusions (7) obliterated the ability of the GltR24 protein to compensate for the absence of GltC. In fact, any insertion of >3 bp between Box I and Box II abolished GltR24-dependent expression of mutant *gltA-lacZ* (data not shown).

Cloning and analysis of the *gltR* locus. The map of the 12.7-kb *gltR* region cloned from the chromosome in three consecutive steps (see Materials and Methods) is shown in Fig. 1. The presence of the *gltR24* mutation within each plasmid (all of which integrate by homologous recombination in or near *gltR*) was checked by testing for the appearance of Glt^+ colonies after transformation of BB92. In this way the mutation was localized to the *Bgl*II-*Hin*dIII$_5$ fragment. Neither the *Bgl*II-*Nae*I fragment of pBB431 nor the *Nae*I-*Hin*dIII$_5$ fragment of pBB432 could confer a Glt^+ phenotype to the *gltC* mutant, but after transformation with the latter plasmid we observed rare Glt^+ segregants of Glt^- transformants, apparently emerging due to gene conversion. We took this result as an indication that *gltR24* is located to the right of the *Nae*I site and very close to it.

To delineate the boundaries of the *gltR* transcriptional unit we used some of the constructed plasmids to transform strain BB632 (*gltC150*::Tn*917 gltR24*). Integration of pBB433 or pBB434 conferred a Glt^- phenotype to this strain, indicating insertional disruption of the *gltR24* locus. In contrast, integration of neither pBB431 nor pBB432 caused glutamate auxotrophy, indicating that the entire *gltR* gene resides between the *Bgl*II and *Hin*dIII$_5$ sites (Fig. 1).

To clone the wild-type *gltR* locus we integrated pBB422 into

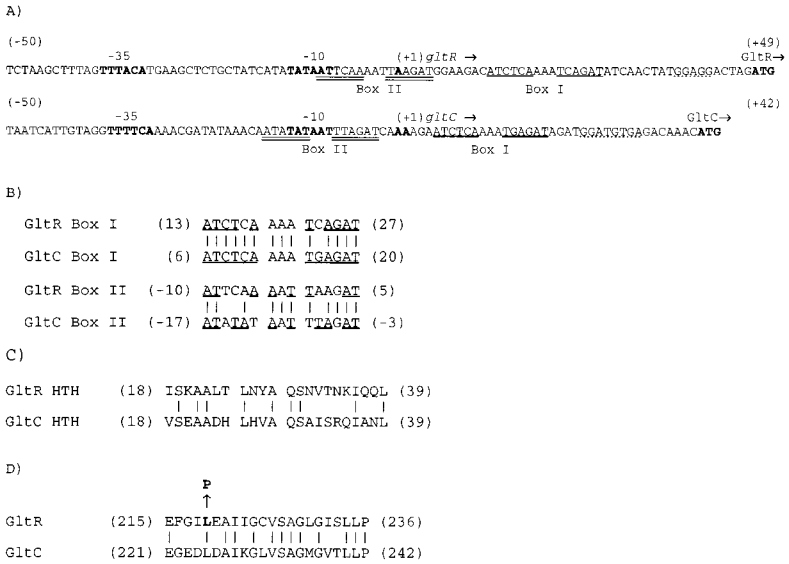

FIG. 3. (A) Comparison of *gltR* and *gltC* regulatory regions. Likely initiation codons and apparent transcription initiation sites of the *gltR* and *gltC* genes and their −10 and −35 regions are in bold; possible ribosomal binding sites are underlined with dots. Directions of transcription and translation are indicated by arrows. Box I sequences are underlined, and Box II sequences are double underlined. The coordinates are given with respect to the respective transcription start points. The sequence of the *gltC* promoter region was described previously (7). (B) Box I and Box II sequences upstream of the *gltR* and *gltC* coding regions. Symmetrical regions are underlined. Identical nucleotides are connected by a vertical bar. The coordinates are given with respect to the *gltR* and *gltC* transcription start points, respectively. (C) Putative helix-turn-helix domains of GltR and GltC. Identical amino acids are connected by a vertical bar. The coordinates indicate residue positions within the respective proteins. (D) Region of the GltR24 mutation and corresponding sequence of GltC. Identical amino acids are connected by a vertical bar. The site of the GltR24 mutation is shown by an arrow. The coordinates indicate residue positions within the respective proteins.

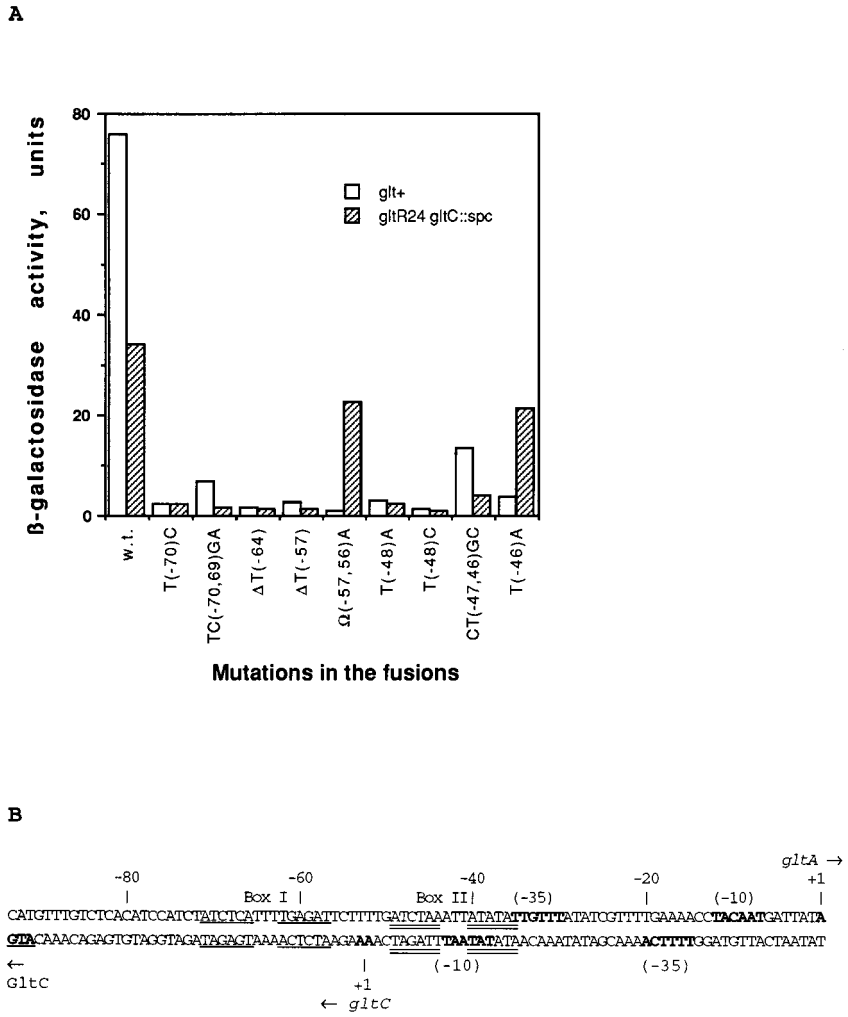

FIG. 4. (A) Effects of mutations in the *gltCA* regulatory region on *gltA-lacZ* expression. Strains LG200 and BB614 or their derivatives containing fusions with mutations in the *gltCA* regulatory region (7) were grown in glucose-ammonia minimal medium. Mutation coordinates are given with respect to the *gltA* transcription start point (see below). Enzyme activities are expressed in units as described in Materials and Methods. (B) Sequence of the *gltCA* regulatory region. A likely *gltC* initiation codon and apparent transcription initiation sites of the *gltA* and *gltC* genes and their −10 and −35 regions are in bold. Directions of transcription and translation are indicated by arrows. The Box I sequence is underlined, and the Box II sequence is double underlined. The coordinates are given with respect to the *gltA* transcription start point. The sequence of the *gltCA* regulatory region was reported previously (7).

the homologous chromosomal locus of strain SMY, next to *gltR*, and, after *Eco*RI digestion of the chromosomal DNA, self-ligation, and transformation of *E. coli* JM107 selecting for the Amp^r marker, we isolated pBB435, with the 3.7-kb $NsiI_3$-$EcoRI_3$ insert covering the entire *gltR* gene (Fig. 1).

Five independent Glt^+ suppressor mutations identified in strains LG200-S and BB92 were shown to be linked in transformation experiments with pBB422 integrated in the chromosome and were cloned in the same manner as the wild-type *gltR* locus.

DNA sequence of the *gltR* region. The sequence of the entire 2.83-kb $HindIII_4$-$HindIII_5$ wild-type fragment plus an additional 0.64 kb to the right of the $HindIII_5$ site (Fig. 1) was determined. An open reading frame of 296 amino acids (coordinates 2742 to 1855 with respect to the nucleotide sequence of the region) preceded by a putative ribosome binding site was identified between the *Bgl*II and $HindIII_5$ sites, overlapping the site of the *gltR24* mutation. The *gltR24* mutation and five other independent *gltR* mutations turned out to be the same single substitution of G for A at position 2087 (30 bp to the right of the *Nae*I site, as shown in Fig. 1), replacing Leu-219 of GltR by Pro (Fig. 3D). Computer analysis of the deduced GltR sequence (molecular mass, 32.9 kDa; pI = 7.2) showed that it belongs to the same LysR family of bacterial transcription regulators (20, 38) as GltC (7, 8). GltR is similar, though only moderately so, to many proteins of the family, including GltC (27% identity). The only protein with more pronounced similarity, SoxR of *Arthrobacter* sp. (32), is 43% identical to GltR; CbbR of *Rhodospirillum rubrum* (14) and Ipa89d of *B. subtilis* (16) are 28% identical to GltR.

A potential open reading frame of 129 amino acids (molecular mass, 14.7 kDa; pI = 5.1) located upstream of *gltR* (coordinates 3076 to 3462) would be transcribed divergently from *gltR* (Fig. 1). Its most probable initiation codon, ATG, is preceded by a strong ribosomal binding site, though an in-frame TTG codon is located 66 bp upstream. The C-terminal part (39 amino acids) of Orf129 is very similar (72% identity) to a partially sequenced open reading frame downstream from the *ermD* (*ermK*) gene of *Bacillus licheniformis* (25). Since LysR

family members often regulate expression of upstream, divergently transcribed genes, *orf129* may be a target of GltR.

Downstream of *gltR* we found a convergent open reading frame (coordinates 97 to 1416) similar (38 to 42% identity at amino acid level) to several genes (*brnQ* of *Lactobacillus delbrueckii* and *Salmonella typhimurium*, *braB* and *braZ* of *Pseudomonas aeruginosa*) encoding low-affinity transport proteins for branched-chain amino acids (21, 34, 42). The product of this open reading frame, which we called *brnQ* (440 amino acids; molecular mass, 47.0 kDa; pI = 10.3), is predicted to have the 12 membrane-spanning domains common to the other members of this group (data not shown). A hypothetical open reading frame of 105 amino acids (coordinates 1377 to 1691) overlaps the *brnQ* gene and is separated from the *gltR* gene by a putative bidirectional transcriptional terminator (coordinates 1726 to 1759).

Phenotype of a *gltR* null mutant. A large DNA segment internal to the *gltR* gene was replaced by a neomycin resistance cassette (see Materials and Methods). When introduced into the *B. subtilis* chromosome, this mutation had no obvious effect on growth of cells in broth or in defined media. The Δ*gltR*::*neo* mutation did not affect expression of a *gltA-lacZ* fusion in either *gltC*$^+$ or Δ*gltC*::*spc* strains in any growth conditions tested (data not shown). Since this result shows that the wild-type GltR protein is neither necessary nor inhibitory for *gltA* expression or regulation, we assume that *gltR24* is a gain-of-function mutation, reflecting acquisition of a new transcription activation specificity by the mutant GltR protein.

Mapping and orientation of the *gltR* locus on the *B. subtilis* chromosome. ^{32}P-labelled fragments of the *gltR* region hybridized to two overlapping *spoIIIC*- and *sacC*-containing clones of the ordered YAC collection of *B. subtilis* chromosomal fragments (4), placing *gltR* between these two markers at 232° to 233° (data not shown). The *gltCAB* locus maps at 177° (3). In transformation experiments, the *gltR* locus was not linked to *spoIIIC* but was ~40% linked (data not shown) to the *blt* (formerly *acrA*) locus (1), which is located 18 kb clockwise from the *spoIIIC* gene (30). The *gltR* and *sacC* (28) loci were 15 to 20% linked (data not shown); *gltR* was not linked to *spoVB*.

The Δ*gltR*::*neo* marker was also ~70% linked (data not shown) to the *azlB* locus, determining resistance to a leucine analog, 4-azaleucine (44). Partial sequencing of DNA beyond the ends of the 12.7-kb segment in Fig. 1 showed that the *bltD* gene is located ~3 kb beyond the *Sac*I$_2$ site (5) and that the *aadK* gene (33), reported to be closely linked to *azlB* and between *azlB* and *sacC*, is ~1 kb beyond the left end of the 12.7-kb DNA segment (5). In addition, the *Pst*I$_1$-*Bgl*II and *Pst*I$_1$-*Nsi*I$_2$ fragments carried by pBB419 and pBB494, respectively, but not the *Hin*dIII$_4$-*Bgl*II fragment carried by pBB424 (Fig. 1), rescued the *azlB101* mutation. This places the *azlB* locus to the left of *brnQ* and establishes the counterclockwise gene order *sacC-aadK-azlB-brnQ-gltR-blt* (Fig. 1).

Determination of the 5′ end of the *gltR* mRNA. We were unable to obtain any primer extension product for chromosomally derived *gltR* mRNA, which presumably reflected its low level of expression. When the *gltR* gene, truncated at the 3′ end to prevent negative autoregulation (see below), was amplified on a multicopy plasmid in a *gltR* null mutant, a likely transcription start point for *gltR* was identified as the A residue at position 2788 of the *gltR* region sequence (Fig. 2B). Sequences with moderate similarity to −10 and −35 regions of σ^A-type promoters can be identified immediately upstream of the putative *gltR* transcription start point (Fig. 3A).

The promoter region of *gltR* contains two 15-bp sequences that bear high similarity to dyad symmetry regions (Box I and Box II) that overlap the *gltC* promoter and are required for regulation of *gltA* and *gltC* (7). *gltR* Box I differs in only one position from *gltC* Box I, and *gltR* Box II is identical in 10 out of 15 positions to *gltC* Box II and to Boxes I of both genes (Fig. 3B); both *gltR* box sequences conform to the consensus site for LysR-type proteins (17).

TABLE 4. Effects of *gltR* mutations on activity of a *gltR-gusA* fusion in glucose minimal medium[a]

Strain	Genotype	β-Glucuronidase activity with the following nitrogen source	
		NH_4Cl	Pro
BB806	*glt*$^+$	0.2[b]	0.4
BB678	Δ*gltR*::*neo*	3.7	7.2
BB649	*gltR24*	0.8	1.2
BB817	*gltR24 thrC*::*gltR*$^+$	0.3	
BB828	Δ*gltR*::*neo thrC*::*gltR*$^+$	0.1	
BB829	Δ*gltR*::*neo thrC*::*gltR24*	0.4	

[a] Cells were grown in TSS-glucose minimal medium with different nitrogen sources; threonine (100 μg/ml) and valine (40 μg/ml) (26) were also added for BB817, BB828, and BB829. β-Glucuronidase activity was assayed and expressed in units as described in Materials and Methods.

[b] All of the numbers are averages of at least two experiments, and the mean errors did not exceed 20%.

Autoregulation of *gltR* expression. Transcriptional fusion of the 540-bp fragment that includes the promoter region of *gltR* to the *gusA* reporter gene was constructed as described in Materials and Methods. Expression of the *gltR-gusA* fusion was elevated >10-fold in the Δ*gltR*::*neo* mutant, indicating negative autoregulation of *gltR* (Table 4). In the *gltR24* mutant, *gltR-gusA* expression was partially derepressed (Table 4). *gltR-gusA* expression was only slightly dependent on the nitrogen source in the growth medium (Table 4) and was not affected by the presence of hyperactive alleles of *gltC* described previously (6), overexpression of wild-type GltC, or the absence of GltC (data not shown).

Dominance analysis of *gltR* alleles. As expected, the Δ*gltR*::*neo* null mutation was recessive to either wild-type or *gltR24* alleles (integrated at the *gltR* or *thrC* loci of the chromosome) with respect to either *gltR* autoregulation (Table 4) or (for *gltR24*) *gltA-lacZ* activation (data not shown). On the other hand, the ability of GltR24 to activate *gltA-lacZ* was decreased by the presence of an additional wild-type copy of the *gltR* gene at the *thrC* locus (Table 3, line 10). In fact, such a strain even lost its ability to grow without a source of glutamate. This result indicates that the *gltR24* mutation is partially recessive to (codominant with) the wild-type allele with respect to *gltA* regulation.

Effect of C(−67) mutations on *gltA* activation by GltR. As noted above (Fig. 3B), *gltC* Box I, essential for GltR24-dependent activation of *gltA*, is different from *gltR* Box I at only one position: G(16) with respect to the *gltC* transcription start point, corresponding to C(−67) with respect to the *gltA* transcription start point (see also Fig. 4B). We created *gltA* fusions in which C at position −67 was replaced by G (the *gltAp23* mutation), making *gltC* Box I identical to *gltR* Box I, or by T (*gltAp21*). These alterations had little effect on GltR24-dependent expression of *gltA* (Table 3, lines 7 to 9) and reduced *gltA* expression twofold in a *gltC*$^+$ *gltR*$^+$ strain (Table 3, lines 1 to 3). The residual expression of these mutant fusions in a wild-type strain was still dependent on the presence of GltC (Table 3, lines 4 to 6), indicating that wild-type GltR cannot substitute for GltC function even when box I is mutated. In accord with this result, when the *gltAp23* mutation was crossed onto the

chromosome of a *gltC* mutant upstream of the *gltAB* genes, it did not allow the wild-type GltR protein to compensate for the lack of GltC and relieve glutamate auxotrophy.

Neither of the *gltAp* mutations allowed GltR24 or wild-type GltR to repress the mutant *gltC-gusA* fusions in the absence of GltC (Table 3, lines 4 to 9, and data not shown).

DISCUSSION

Two models could explain how the *gltR24* mutation leads to activation of transcription of *gltAB*, in the absence of GltC. First, wild-type GltR may be intrinsically defective in one or more steps in transcription activation of the *gltA* promoter (e.g., protein-DNA interaction, protein-protein interaction, and protein-effector interaction); the mutation may correct the hypothetical defect. This would represent nonphysiological cross-talk of two functionally similar proteins. It was shown previously that several other pairs of LysR-type proteins from different bacterial species (GsvA and AmpR) or different strains of the same species (NocR and OccR), or even from the same strain if a protein is overexpressed (ClcR and CatR), are able to cross-activate their target genes in vivo (13, 35, 45). Moreover, multiple species of NodD proteins, coexisting in the same cell and activating the same genes, are abundantly described in rhizobia (44). In most of these cases the similarity between the cross-talking proteins is relatively high and the sequences of their helix-turn-helix DNA-binding motifs are similar. Neither the overall similarity nor the similarity of the helix-turn-helix regions (Fig. 3C) of GltR and GltC is particularly striking.

Alternatively, GltR, like most regulatory proteins, may exist in both active and inactive conformations whose interconversion normally requires an effector molecule. In the latter case, the *gltR24* mutation, a very specific alteration of GltR, may permit the protein to assume its transcription-stimulating conformation whether the effector is present or not. This implies that wild-type GltR would activate transcription of *gltAB* if provided with the right effector molecule under environmental conditions as yet untested. Interestingly, the site within GltR (Leu-219) at which the mutation occurred is within a region (residues 217 to 244) in which mutations in other LysR family members lead to constitutively active or inactive proteins (38), though none of them affects the exact position corresponding to Leu-219 of GltR. In GltC this position is occupied by leucine (Leu-225), as in wild-type GltR, and not by proline, as in GltR24. The specific function of this conserved region remains unknown (38).

The possibility that wild-type GltR is a completely inactive protein is refuted by its ability to negatively regulate its own gene and by the codominance of the wild-type *gltR* and *gltR24* alleles. We hypothesize that Box I- and Box II-like sequences in the *gltR* regulatory region are binding sites for GltR and that the ability of GltR24 to activate *gltA* is due to its binding to the similar Box I-Box II region upstream of *gltA*. The Box I- and Box II-like sequences in the *gltR* regulatory region overlap the presumed RNA polymerase binding site, providing an apparent mechanism for *gltR* autoregulation. On the other hand, a requirement for interaction of GltR24 with the Box I and Box II sequences of the *gltCA* regulatory region for positive regulation of *gltA* is in some ways paradoxical. First, while GltR24 can substitute for GltC in positive regulation of *gltA*, it interacts with the *gltCA* regulatory region in a way different from that described for GltC; GltR24 can activate some *gltA-lacZ* fusions that have mutations in Box I or Box II that prevent activation by GltC but, unlike GltC, GltR24 does not tolerate separation of Box I and Box II sequences by one helical turn of DNA. Second, GltR24 is not able to replace GltC with respect to repression of *gltC*. We have assumed that repression of *gltC* requires only interaction of a regulatory protein (GltC, in wild-type cells) with Box I, which overlaps the *gltC* promoter (7). GltR24 (and wild-type GltR) do repress *gltR* expression, presumably through interaction with a sequence that is nearly identical to the *gltCA* Box I. The single-base-pair difference between *gltC*-Box I and *gltR*-Box I sequences cannot be solely responsible for the inability of wild-type GltR to activate *gltA* or the inability of both wild-type and mutant GltR to repress *gltC*, since converting the *gltCA* Box I to the exact *gltR* Box I sequence did not permit *gltA* activation by wild-type GltR or repression of *gltC* by GltR or GltR24.

Some of the inferences derived here from genetic and physiological studies can be verified by in vitro experiments with purified GltR proteins. Our initial results indicate that partially purified GltR and GltR24 both bind, but in somewhat different ways, to the *gltR* and *gltC-gltA* regulatory regions (5). Competition for binding may explain the codominance of the *gltR24* mutation. Further analysis of these interactions is likely to clarify the mechanism by which the *gltR24* mutation bypasses the need for GltC in glutamate synthase expression and the reason why GltR24-dependent expression of *gltA* is regulated by the nitrogen source of the medium. We are also testing the possible involvement of other factors in nitrogen source-dependent regulation of *gltA*.

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