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Functional Roles of the Conserved Glu304 Loop of *Bacillus subtilis* Glutamine Synthetase[▽]

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The enzymatic activity of *Bacillus subtilis* glutamine synthetase (GS), which catalyzes the synthesis of glutamine from ammonium and glutamate, is regulated by glutamine feedback inhibition. The feedback-inhibited form of *B. subtilis* GS regulates the DNA-binding activities of the TnrA and GlnR nitrogen transcriptional factors. Bacterial GS proteins contain a flexible seven-residue loop, the Glu304 flap, that closes over the glutamate entrance to the active site. Amino acid substitutions in Glu304 flap residues were examined for their effects on gene regulation, enzymatic activity, and feedback inhibition. Substitutions in five of the Glu304 loop residues resulted in constitutive expression of both TnrA- and GlnR-regulated genes, indicating that this flap is important for regulating the activity of these transcription factors. The residues in the highly conserved Glu304 flap appear to be optimized for glutamate binding because mutant enzymes with substitutions in five of the flap residues had increased glutamate K_m values compared to that for wild-type GS. The E304A and E304D substitutions increased the ammonium K_m values compared to that for wild-type GS and conferred high-level resistance to inhibition by glutamine, glycine, and methionine sulfoximine. A model for the role of the Glu304 residue in glutamine feedback inhibition is proposed.

The ATP-dependent enzyme glutamine synthetase (GS) is the only known enzyme that is capable of the *de novo* synthesis of glutamine. Although GS is a metalloenzyme that can utilize Mg^{2+} or Mn^{2+} for *in vitro* catalytic activity, the Mg^{2+}-dependent biosynthetic reaction is the physiologically relevant enzymatic activity (38, 46). The biosynthesis of glutamine involves the initial formation of γ-glutamyl phosphate from ATP and glutamate (Fig. 1) (33, 34). The second step involves the formation of a transition state complex that forms from a nucleophilic attack by ammonia on γ-glutamyl phosphate (Fig. 1). The subsequent release of phosphate results in the formation of glutamine.

Since glutamine is a key metabolite in nitrogen physiology, both the synthesis and the activity of GS are tightly regulated to maintain adequate glutamine levels for optimal growth. While low GS levels are found in cells growing rapidly with excess nitrogen, high GS levels are present in cells grown with limiting nitrogen. The enzymatic activity of GS is regulated by a variety of mechanisms. For instance, the Mg^{2+}-dependent activity of GS from the low-G+C Gram-positive bacterium *Bacillus subtilis* is regulated by glutamine feedback inhibition (11). In contrast, the Mg^{2+}-dependent activity of *Escherichia coli* GS is regulated by adenylylation, the covalent attachment of AMP to a specific tyrosine residue (38). The adenylylated form of the *E. coli* GS enzyme can be feedback inhibited by nine nitrogen-containing metabolites but not by glutamine (38, 43). It is not understood why the GS proteins from *E. coli* and *B. subtilis* have different sensitivities to feedback inhibition by glutamine.

While multiple GS isozymes are present in procaryotes (35), the most common bacterial GS enzyme, GSI, is a multimeric protein that contains 12 identical subunits arranged as two face-to-face hexameric rings (13). GSI enzymes have 12 active sites that are situated at the subunit interfaces within the hexameric rings. Each active site has a cylindrical structure where glutamate and ATP enter the active site from different ends of the cylinder (30). The only GS enzyme in *B. subtilis* is a GSI isozyme (40). Crystallographic studies of enzyme-ligand complexes for the *Salmonella enterica* serovar Typhimurium and *Mycobacterium tuberculosis* GSI proteins suggest that the synthesis of glutamine involves a series of polypeptide loop and residue side-chain movements (13, 21). Of particular interest is a highly conserved 7-amino-acid-residue loop, the glutamate binding flap, which is located adjacent to the glutamate entrance to the active site. Because the position numbers of conserved residues are different for the GSI proteins from different organisms, the *B. subtilis* GS residue numbers are used exclusively in this report for clarity. The glutamate binding flap of *B. subtilis* GS corresponds to residues 300 to 306 and is called the Glu304 flap. The glutamate binding flap closes over the glutamate entrance to the active site and protects the unstable intermediates formed during the catalytic reaction from aberrant hydrolysis by water (13).

The GS inhibitor L-methionine-*S*-sulfoximine (MetSox) binds to the glutamate substrate site (30). In the presence of ATP, MetSox is phosphorylated by GS to form a transition state analogue that results in essentially irreversible inhibition of enzymatic activity (29, 42). Crystallographic studies of *S*. Typhimurium GS have shown that the binding of unphosphorylated MetSox induces the Glu304 flap to close over the entrance to the active site so that the side chain of the Glu304 residue interacts with MetSox (Fig. 2) (30). In contrast to its interaction with MetSox, the Glu304 residue of *S*. Typhimurium GS does not make contact with glutamine bound at the active site (30).

B. subtilis GS is a moonlighting protein which, in addition to

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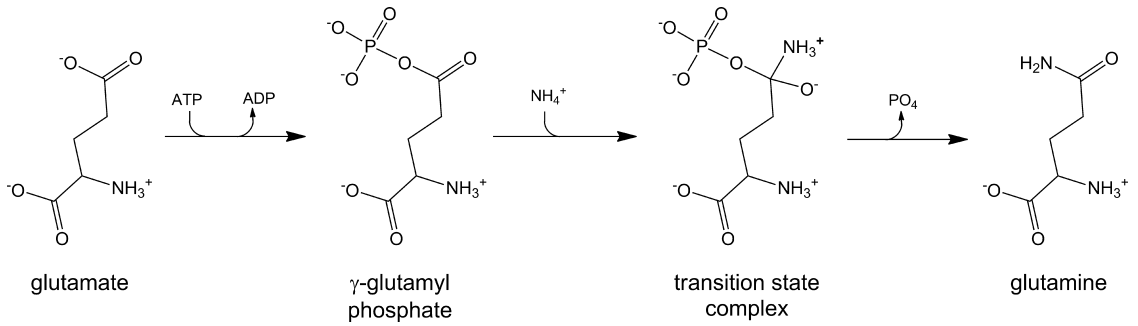

FIG. 1. Glutamine synthetase reaction mechanism.

its enzymatic function, has a critical role in the control of gene expression. Nitrogen regulation in *B. subtilis* is mediated by the TnrA and GlnR transcription factors (14). The feedback-inhibited form of GS (FBI-GS), which is present during growth with excess nitrogen, modulates the DNA-binding activities of TnrA and GlnR through direct protein-protein interactions. TnrA and GlnR are active under different nutritional growth conditions. GlnR is a transcriptional repressor that is functional in cells grown with excess nitrogen sources (7, 39). FBI-GS functions as a chaperone that activates GlnR by stabilizing GlnR-DNA complexes (17). TnrA activates and represses transcription during growth with limited nitrogen sources (4, 45, 48). FBI-GS forms a stable complex with TnrA that inhibits TnrA DNA-binding activity in cells grown with excess nitrogen (47). A previous study described two GS mutants with amino acid substitutions in the Glu304 flap, G302D and P306H, that were defective in regulating TnrA and GlnR (15). Since glutamine acts as a feedback inhibitor of *B. subtilis* GS by binding to the glutamate substrate site (16, 46), conformational changes in the Glu304 flap presumably occur during feedback inhibition. Taken together, these observations led to the proposal that TnrA and GlnR interact with the feedback-inhibited form of GS near the glutamate entrance to the active site (15, 16).

Although the Glu304 residue of *E. coli* GS has been examined in kinetic and mutagenic studies (1), the functions of the other residues in the Glu304 flap have not been similarly analyzed. In this report, amino acid substitutions in the Glu304 flap of *B. subtilis* GS were generated to examine the roles of Glu304 flap residues in enzymatic activity, feedback inhibition, and regulation of TnrA and GlnR activity. Characterization of the resulting mutant GS proteins demonstrated that the Glu304 flap plays a significant role in regulating the activity of TnrA and GlnR. Moreover, the Glu304 residue was found to be necessary for inhibition by glutamine.

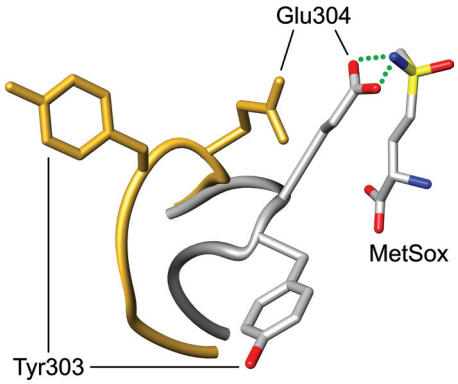

FIG. 2. MetSox-induced structural changes in the Glu304 flap. The crystallographic models of *S.* Typhimurium GS in the presence (gray) and absence (gold) of MetSox are superimposed. The peptide backbones of the Glu304 flaps (residues 300 to 306) are depicted as smooth tubes. In the GS-MetSox complex, the residue side chains and MetSox are color coded by atom type: oxygen in red, nitrogen in blue, carbon in gray, and sulfur in yellow. *B. subtilis* GS residue Glu304 corresponds to *S.* Typhimurium GS residue Glu327. This figure was prepared with UCSF Chimera (37).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study are listed in Table 1. Methods for the cultivation of bacteria in the minimal medium of Neidhardt et al. (36) have been described previously (3). All cultures contained 0.5% glucose and a nitrogen source with a concentration of 0.2%. Minimal medium agar plates were prepared as previously described (8). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was added to agar plates to give a final concentration of 40 μg/ml.

Plasmids. GS overexpression plasmids containing the mutant *glnA* genes were constructed as previously described (15). Plasmid pGLN211 is a clone of the *glnRA* region where the entire *glnA* coding sequence was replaced with a tetracycline resistance gene. The previously described pGLN206 plasmid contains DNA sequences from both the upstream and downstream regions adjacent to the

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

Strain	Genotype[a]	Reference or source
168	*trpC2*	This laboratory
SF62	*tnrA62*::Tn*917 trpC2*	45
SF218G	*glnA*(*spc*) *trpC2*	168 × pGLN218
SF300	*amtB29*::Tn*917-lacZ trpC2*	16
SF300G7	*amtB29*::Tn*917-lacZ* Δ*glnA207*::*spc trpC2*	16
SF416	*amyE*::((*amtB-lacZ*)*416 neo*) *trpC2*	45
SF416G11	*amyE*::((*amtB-lacZ*)*416 neo*) Δ*glnA211*::*tet trpC2*	SF416 × pGLN211
SF17	*amyE*::((*glnRA-lacZ*)*17 neo*) *trpC2*	45
SF17G11	*amyE*::((*glnRA-lacZ*)*17 neo*) Δ*glnA211*::*tet trpC2*	SF17 × pGLN211
SF17G11T	*amyE*::((*glnRA-lacZ*)*17 neo*) Δ*glnA211*::*tet tnrA62*::Tn*917 trpC2*	SF17G11 × SF62

[a] The *amtB-glnK* operon was formerly called *nrgAB*. The genotype symbols are described by Biaudet et al. (5).

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	DNA sequence (5′ → 3′)	Location
GMCAAT31	ATCTGACGTCGCCGCTCCATAATTTATTACC	54 bp downstream of *glnA*
GMCAAT32	TAGCGACGTCAATAAATTATGGAGCGGCACTG	46 bp downstream of *glnA*
GMCBAM32	TCGAGGATCCAGTTATCAGCAAGACAAATTCG	380 bp upstream of *glnA*
GMCKPN32	TCGAGGTACCAGTTATCAGCAAGACAAATTCG	380 bp upstream of *glnA*
GMCUPS22	AGTTATCAGCAAGACAAATTCG	380 bp upstream of *glnA*
GMCHIN30	ATCGAAGCTTTAAGAGTCATCTATAACCGC	456 bp downstream of *glnA*
GMCXHO30	ATGCCTCGAGTAAGAGTCATCTATAACCGC	456 bp downstream of *glnA*
GMCDNS20	TAAGAGTCATCTATAACCGC	456 bp downstream of *glnA*

B. subtilis glnA gene that were inserted into pJDC9 (9) and separated by a PstI restriction site (16). pGLN211 was constructed by inserting a tetracycline resistance gene cassette from pBEST309 (27) into the PstI site of pGLN206.

Plasmid pGLN218, which contains the *glnRA* region with a spectinomycin resistance gene inserted immediately downstream of the *glnA* gene, was constructed in three steps. A DNA fragment containing sequences located immediately downstream of the *glnA* gene was prepared by PCR amplification with primers GMCAAT32 and GMCHIN30 (Table 2) and inserted into pLEW424 (44) as an AatII-HindIII fragment to construct pGLN215. A DNA fragment containing the 3′ end of the *glnR* gene and the entire *glnA* gene was prepared by PCR amplification with primers GMCKPN32 and GMCAAT31 (Table 2) and inserted into pGLN215 as a KpnI-AatII fragment to give pGLN216. Plasmid pGLN218 was constructed by inserting a spectinomycin resistance gene cassette (22) into the AatII site of pGLN216.

Mutagenesis. pGLN209 is an *E. coli* plasmid that contains the entire *B. subtilis glnA* gene and a neomycin resistance gene that is selectable in both *E. coli* and *B. subtilis* (16). Plasmid pGLN209 was mutagenized by propagation in the *E. coli* mutator strain XL1-Red (Stratagene). Strain SF300G7 (Δ*glnA207*::*spc*) was transformed with mutagenized pGLN209 DNA with selection for Gln on glucose minimal medium plates containing X-Gal and an excess nitrogen source (either ammonium chloride or glutamate plus ammonium chloride). Gln^+ transformants that resulted from the integration of *glnA* into its chromosomal locus by a double-crossover event were identified by the absence of both plasmid-encoded neomycin resistance and chromosomal-encoded spectinomycin resistance. This mutant isolation procedure was different from previously described mutant hunts in that it involved localized mutagenesis of the *glnA* gene with a screen that was not biased for the isolation of mutants encoding feedback-resistant *glnA* mutations (15, 16).

Site-directed mutagenesis of *glnA* was performed by PCR overlap extension (26). In this method, the first set of amplifications utilizes complementary mutagenic primers to produce two DNA fragments with overlapping ends where the targeted mutation is located within the overlapping DNA sequence. PCR amplification with chromosomal DNA of strain SF218G as the template was used to generate a DNA fragment of the 5′ end of *glnA* with the upstream primer GMCUPS22 (Table 2) and one mutagenic primer. A DNA fragment of the 3′ end of *glnA* was produced with the downstream primer GMCDNS20 (Table 2) and the complementary mutagenic primer. The reaction products from these two amplifications were used as templates, together with primers GMCBAM32 and GMCXHO30 (Table 2), to generate a full-length *glnA* DNA fragment by PCR amplification. The final PCR product was cloned into the erythromycin-resistant plasmid pLEW424 as a BamHI-XhoI DNA fragment and subsequently sequenced to confirm the success of the mutagenesis. All of the mutant plasmids generated by this protocol have a spectinomycin resistance gene located immediately downstream of the *glnA* gene. To generate strains containing the mutant *glnA* genes and an *amtB-lacZ* fusion, mutant plasmid DNAs were used to transform strain SF416G11 (Δ*glnA211*::*tet*) with selection for spectinomycin resistance. Transformants that resulted from the integration of the mutant genes into the *glnA* chromosomal locus by a double-crossover event were identified by the absence of both plasmid-encoded erythromycin resistance and chromosomal-encoded tetracycline resistance. Chromosomal DNAs of the resulting strains were used to transform SF17G11T to obtain strains containing the mutant *glnA* genes and a *glnRA-lacZ* fusion.

Enzyme assays. β-Galactosidase was assayed in crude extracts prepared from cells grown to mid-log growth phase as described previously (3). The reported β-galactosidase levels were corrected for the endogenous activity present in cells containing the promoterless *lacZ* fusion vectors integrated into the *amyE* gene. One unit of β-galactosidase activity produced 1 nmol of *o*-nitrophenol per min.

The specific activities of the biosynthetic and transferase GS reactions were determined as previously described (15). The kinetic constants for glutamate, ATP, and hydroxylamine were measured by assaying the production of γ-glutamylhydroxamate (46). The ammonium K_m values were determined by measuring the production of inorganic phosphate that resulted from the hydrolysis of ATP (19). To minimize the effects of glutamine product inhibition, these assays were performed at 25°C and the reaction time was limited to 7.5 min. Assays to produce the ammonium saturation curves contained glutamate and ATP concentrations of 100 and 7.5 mM, respectively. The levels of inhibition of the Mg^{2+}-dependent biosynthetic reaction by glutamine, AMP, and MetSox were determined with glutamate and ATP concentrations of 150 and 18 mM, respectively (46). Glycine inhibition of the Mn^{2+}-dependent biosynthetic reaction was determined with glutamate and ATP concentrations of 100 and 7.5 mM, respectively.

Protein purification and analysis. Overexpression and purification of TnrA and GS were performed as previously described (47). The purified proteins were greater than 98% homogeneous as judged by Coomassie blue staining of SDS protein gels. The conditions for the gel mobility shift assay used to analyze the DNA-binding activities of TnrA have been reported previously (47).

Protein modeling. Although the crystal structure of the *S*. Typhimurium GS-MetSox complex has been determined (30), the atomic coordinates for this model are not present in the Protein Data Bank (PDB). Therefore, the *S*. Typhimurium GS-phosphinothricin structure (PDB entry 1FPY) was used to model the GS-MetSox complex (20). Since MetSox and phosphinothricin are isosteric, the appropriate atoms in phosphinothricin were modified to give MetSox. As proposed by Gill and Eisenberg (20), the nitrogen atom of the MetSox sulfonimide group was oriented so that it was able to form a hydrogen bond with the carboxylate side chain of Glu304. To prepare Fig. 2, the native *S*. Typhimurium GS structure (PDB entry 1F52) was structurally aligned with the GS-MetSox complex.

RESULTS

Isolation and characterization of *glnA* mutations. Two different approaches were used to generate alterations in residues located in the Glu304 flap of *glnA*. In the first method, *glnA* mutations that resulted in constitutive TnrA regulation were isolated by localized mutagenesis of *glnA* followed by screening for constitutive expression of the TnrA-activated *amtB-glnK* operon. In this procedure, plasmid DNA containing the *B. subtilis glnA* gene was first mutagenized by propagation in an *E. coli* mutator strain. The mutagenized plasmid DNA was used to transform a *glnA* null mutant of *B. subtilis* with selection for Gln. This selection procedure requires all of the mutants to retain sufficient GS biosynthetic activity so that they can grow on medium that lacks glutamine. The *B. subtilis* strain contained a TnrA-activated *amtB-lacZ* fusion so that mutants with constitutive expression of the *amtB* promoter could be visualized as blue colonies on plates containing excess nitrogen and X-Gal, a chromogenic substrate for β-galactosidase. Sequence analysis identified seven different amino acid substitutions in the Glu304 flap (G302D, G302V, G302S, Y303H, Y303C, E304A, and E304D). Although six additional substitutions were also isolated (D27N, N34Y, L42P, F60L, D198N, and

TABLE 3. TnrA- and GlnR-dependent regulation in wild-type and *glnA* mutant strains

glnA allele	Amino acid substitution	β-Galactosidase level[a]			
		amtB-lacZ fusion strain grown on:		*glnRA-lacZ* fusion strain grown on:	
		Glutamine	Glutamate	Glutamine	Glutamate
Wild type		0.03	125	0.3	42
glnA(*V300A*)	Val300→Ala	3.6	54	8.6	54
glnA(*P301A*)	Pro301→Ala	0.09	121	1.1	30
glnA(*G302A*)	Gly302→Ala	25	124	2.2	31
glnA(*Y303A*)	Tyr303→Ala	161	96	41	26
glnA(*Y303L*)	Tyr303→Leu	58	103	54	32
glnA(*Y303F*)	Tyr303→Phe	0.01	117	0.7	32
glnA(*Y303H*)	Tyr303→His	22	87	5.1	31
glnA(*E304A*)	Glu304→Ala	125	NG[b]	36	NG
glnA(*E304D*)	Glu304→Asp	115	NG	51	NG
glnA(*A305G*)	Ala305→Gly	39	47	30	22
glnA(*P306A*)	Pro306→Ala	0.01	60	4.5	23

[a] All values are enzyme units per mg of protein and are the averages of the results from three or more determinations which did not vary by more than 20%. TnrA-dependent regulation was examined by determining β-galactosidase levels in cells containing the *amtB416-lacZ* fusion. GlnR-dependent regulation was examined by determining β-galactosidase levels in *tnrA* mutant cells containing the *glnRA17-lacZ* fusion. Cultures were grown in glucose minimal medium containing the indicated nitrogen sources. Glutamine is an excess nitrogen source, while glutamate is a limiting nitrogen source.

[b] NG, no growth.

TABLE 4. Specific activities of wild-type and mutant GS enzymes

GS enzyme	% sp act relative to wild-type level[a]	
	Mg^{2+}-dependent biosynthetic reaction	Transferase reaction
Wild type	100	100
V300A	76	18
G302A	80	58
Y303A	36	8
Y303L	87	46
Y303H	150	100
E304A	43	5
E304D	37	8
A305G	16	2
P306A	160	130

[a] The specific activities of wild-type GS for the Mg^{2+}-dependent biosynthetic and transferase reactions were 3.8 and 98 μmol/min/mg, respectively. All values are the averages of two or more determinations. The standard errors were less than 20%.

Q439R), these alterations were not located within the glutamate binding flap, and their characterization is not described in this communication.

To specifically evaluate the roles of all of the Glu304 flap residues, site-directed mutagenesis was used to generate additional amino acid substitutions in this protein loop. Residues Val300, Pro301, Gly302, and Pro306 were replaced with alanine. Tyr303 was mutated to phenylalanine, leucine, and alanine to provide a series of substitutions with decreasing side-chain sizes. Ala305 was replaced with glycine. All of the mutant *glnA* alleles were generated by site-directed mutagenesis and integrated into the chromosomal *B. subtilis glnA* locus.

When the growth properties of these mutants containing substitutions in the Glu304 flap were examined on plates containing glucose minimal medium, all of the strains exhibited normal growth with either glutamine or glutamate plus ammonium as the nitrogen source. Four of the *glnA* mutants (the Y303A, E304A, E304D, and A305G mutants) formed smaller colonies than the wild-type strain on glucose ammonium minimal medium plates. Because glutamate supplementation of the glucose ammonium minimal medium suppressed the growth defects seen with these four *glnA* mutants, the growth defects most likely result from the synthesis of GS proteins with partial enzymatic activity (see below). In addition, the strains encoding the E304A and E304D GS proteins were unable to grow on glucose minimal medium containing the limiting nitrogen source glutamate. This growth phenotype suggested that these mutant GS enzymes were defective in their ability to synthesize glutamine when cell growth is limited by ammonium availability (see below).

Regulation of TnrA and GlnR by the mutant GS proteins. The effect of the GS amino acid substitutions on gene expression by a TnrA-activated *amtB-lacZ* fusion and GlnR-repressed *glnRA-lacZ* were examined *in vivo* (Table 3). Two of the GS mutants, the P301A and Y303F mutants, had no significant effect on TnrA- or GlnR-mediated regulation, and these *glnA* mutants were not studied further. The P306A mutant had partially constitutive *glnRA-lacZ* expression but had no effect on *amtB-lacZ* regulation. The *in vivo* regulation of both TnrA and GlnR was partially defective in three of the mutants (the V300A, G302A, and Y303H mutants). High-level constitutive expression of the *amtB-lacZ* and *glnRA-lacZ* fusions was observed with the remaining five mutants (the Y303A, Y303L, E304A, E304D, and A305G mutants). These results indicate that the Glu304 flap has an important role in the regulation of TnrA and GlnR by FBI-GS.

Enzymatic properties of the mutant enzymes. To examine their biochemical properties, the nine mutant GS proteins defective in TnrA and GlnR regulation were overexpressed and purified to homogeneity. The specific activities for the Mg^{2+}-dependent biosynthetic reaction, which is the major route for glutamine synthesis *in vivo* (38, 46), were determined for the enzymes (Table 4). In these *in vitro* assays, the substrate ammonium was replaced with hydroxylamine. This substitution circumvents the glutamine feedback inhibition of enzymatic activity that is present in the ammonium-dependent assay, because the product of the hydroxylamine-dependent reaction is γ-glutamylhydroxamate. Four of the mutant GS enzymes (the Y303A, E304A, E304D, and A305G mutants) had biosynthetic specific activities that were only 16 to 43% of the values obtained with the wild-type enzyme (Table 4). As noted previously, the *glnA* mutants that contained these four amino acid substitutions also grew poorly on glucose plus ammonium minimal medium. Taken together, these results argue that the poor growth of these mutant strains was most likely due to the inability of the mutant enzymes to synthesize sufficient glutamine to allow growth at wild-type rates on this medium.

The transferase assay is a partial reverse reaction in which GS catalyzes the synthesis of γ-glutamylhydroxamate from glutamine and hydroxylamine (12). Five of the mutant enzymes (the V300A, Y303A, E304A, E304D, and A305G mutants) had levels of transferase activity that were less than 20% of the value for the wild-type enzyme (Table 4).

The steady-state kinetic constants for the physiologically relevant Mg^{2+}-dependent biosynthetic reaction were also deter-

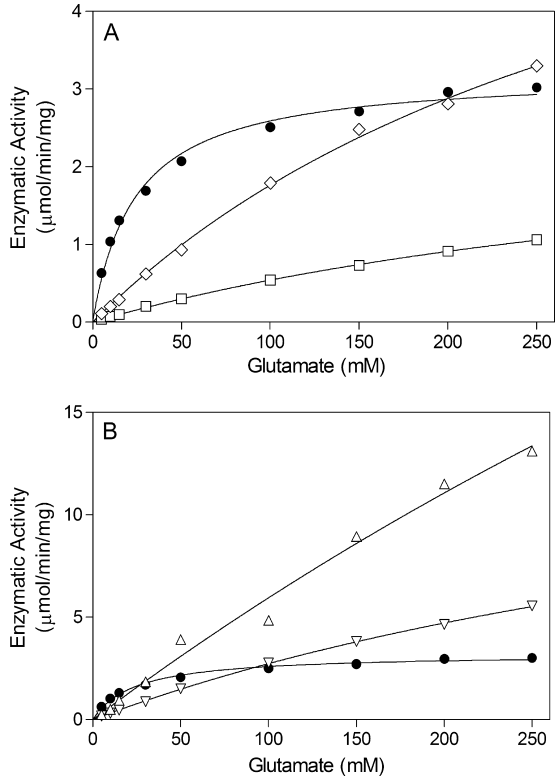

FIG. 3. Dependence of GS enzymatic activity on glutamate concentration for wild-type and mutant enzymes. The *y* axes for panels A and B are drawn to different scales. Symbols: ●, wild type; ◇, Y303A mutant; □, A305G mutant; △, P306A mutant; ▽, V300A mutant. The curve for the Y303L mutant (not shown) is similar to the curve for the V300A mutant.

mined. The V_{max} and K_m values for glutamate could not be determined for five of the enzymes (the V300A, Y303A, Y303L, A305G, and P306A enzymes), because the substrate saturation curves were essentially linear in the range of glutamate concentrations used in the assays (Fig. 3). Nonetheless, these data indicate that these six enzymes have higher glutamate K_m constants than wild-type GS. Compared to wild-type GS, the G302A and Y303H enzymes had only small differences (less than 4-fold) in their K_m values for glutamate and ATP (Table 5). The E304A and E304D enzymes were the only mutant proteins that had glutamate

TABLE 5. Enzymatic constants for wild-type and mutant GS proteins[a]

Enzyme	K_m (mM)				V_{max} (μmol/min/mg)
	Glutamate	ATP	Ammonium	Hydroxylamine	
Wild type	27 ± 2	2.4 ± 0.1	0.18 ± 0.03	0.83 ± 0.07	3.7 ± 0.2
G302A	84 ± 11	5.3 ± 1.1	ND[b]	ND	3.6 ± 0.2
Y303H	59 ± 5	9.4 ± 1.6	ND	ND	12 ± 1
E304A	3.4 ± 0.2	1.2 ± 0.1	32 ± 2	0.68 ± 0.09	1.3 ± 0.1
E304D	10 ± 1	11 ± 2	120 ± 10	0.74 ± 0.10	1.4 ± 0.1

[a] The kinetic constants were determined for the Mg^{2+}-dependent biosynthetic reaction. All assays were performed at least twice. The uncertainty is the standard error from the nonlinear regression analysis of the data.
[b] ND, not determined.

TABLE 6. Sensitivities of wild-type and mutant GS enzymes to inhibition

Enzyme	IC_{50} (mM)[a]			
	Glutamine	AMP	MetSox	Glycine
Wild type	2.4	0.5	0.13	31
V300A	7.0	1.4	0.12	ND[b]
G302A	11	1.7	0.28	ND
Y303A	13	1.3	0.61	ND
Y303L	19	1.5	0.10	ND
Y303H	4.1	1.0	0.10	ND
E304A	>140	>30	26	>800
E304D	79	1.7	20	>800
A305G	140	2.1	2.7	350
P306A	1.5	0.2	0.25	ND

[a] The IC_{50} is the inhibitor concentration that reduces enzymatic activity by 50%. The Mg^{2+}-dependent biosynthetic assay was used to measure the inhibition by glutamine, AMP, and MetSox. Glycine inhibition was determined with the Mn^{2+}-dependent biosynthetic assay. All values are the averages of at least two determinations. The standard errors were less than 10% for all values.
[b] ND, not determined.

K_m values which were significantly lower than that for wild-type GS (Table 5). Interestingly, the E304A substitution decreased the ATP K_m, while the E304D substitution had the opposite effect of increasing the ATP K_m (Table 5). The observation that amino acid substitutions in the glutamate binding flap can alter the ATP K_m suggests that there is an interdependent interaction between the glutamate and ATP binding sites.

Previous studies with *E. coli* GS showed that an E304A substitution increased the ammonium K_m value (1). The E304A and E304D mutant enyzmes of *B. subtilis* GS were also found to have significantly higher ammonium K_m values than the wild-type enzyme (Table 5). As noted previously, the *B. subtilis* mutants encoding the E304A and E304D mutant enzymes were unable to grow on glucose minimal medium containing the nitrogen source glutamate. Because ammonium availability is significantly restricted on this medium, the growth defect with these two mutants is most likely a direct consequence of the higher ammonium K_m values of the E304A and E304D mutant enzymes.

Curiously, when the substrate ammonium was replaced with hydroxylamine in the Mg^{2+}-dependent biosynthetic reaction of GS, the E304A and E304D enzymes were found to have hydroxylamine K_m values that were similar to that for wild-type GS (Table 5). The implications of this difference between the ammonium and hydroxylamine K_m values for these mutant enzymes are considered in the Discussion.

Sensitivities of the mutant enzymes to inhibitors. Three of the mutant enzymes (the E304A, E304D, and A305G mutants) were highly resistant to feedback inhibition by glutamine (Table 6). Four of the mutant enzymes (the V300A, G302A, Y303A, and Y303L mutants) had moderately higher levels (3- to 8-fold) of resistance to glutamine inhibition (Table 6). The two mutant enzymes with Glu304 substitutions, the E304A and E304D mutants, had very high levels of resistance to inhibition by MetSox (Table 6). Although A305G GS was not as resistant to MetSox inhibition as the E304A and E304D enzymes, the A305G mutant GS had 20-fold higher resistance to inhibition by MetSox than wild-type GS (Table 6).

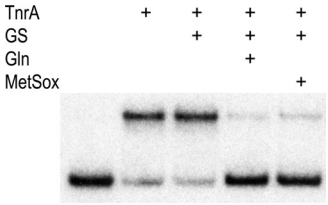

FIG. 4. Abilities of glutamine and MetSox to promote the GS-mediated inhibition of TnrA DNA binding. A gel mobility shift assay was used to examine the binding of TnrA to *amtB* promoter DNA. The binding reaction mixtures contained TnrA (100 nM), GS (1 μM), glutamine (20 mM), and MetSox (20 mM), as indicated above the autoradiograph. ATP is not present in these binding mixtures, and thus MetSox is not phosphorylated.

Glycine is a competitive inhibitor of *S*. Typhimurium GS that binds to the glutamate substrate site (32). The *B. subtilis* GS Mn^{2+}-dependent biosynthetic reaction is inhibited by glycine (11). The three GS mutants that had high-level resistance to glutamine (the E304A, E304D, and A305G mutants) were also found to also have increased resistance to glycine inhibition (Table 6). AMP inhibits the Mg^{2+}-dependent biosynthetic reaction of *B. subtilis* GS activity by binding to the ATP substrate site (11, 31). The E304A GS was the only mutant enzyme that had a significant increase in its resistance to AMP inhibition (Table 6). This result provides additional evidence for interdependence between the glutamate and ATP binding sites of GS.

***In vitro* regulation of TnrA by GS and MetSox.** Although glutamine and MetSox both bind to the glutamate substrate site of GS, the Glu304 residue of *S*. Typhimurium GS hydrogen bonds with MetSox but does not interact with glutamine (16, 30, 46). *In vitro* experiments have shown that glutamine greatly enhances the ability of *B. subtilis* GS to inhibit the DNA-binding activity of TnrA (47). This result suggests that feedback-inhibited *B. subtilis* GS has a different conformation than uninhibited GS. To determine whether glutamine and MetSox induce similar conformational changes in *B. subtilis* GS, the abilities of these two compounds to stimulate the GS-mediated inhibition of TnrA DNA binding were examined *in vitro*. In these experiments, the binding of TnrA to the *amtB* promoter was monitored with a DNA gel mobility shift assay. As shown in Fig. 4, GS by itself does not alter the binding of TnrA to DNA. In contrast, when either glutamine or MetSox is present, GS is able to inhibit the DNA-binding activity of TnrA (Fig. 4). These results argue that the glutamine- and MetSox-bound forms of *B. subtilis* GS have similar conformations.

DISCUSSION

The observation that the amino acid residues in the Glu304 flap from bacterial GSI proteins are highly conserved implies that the Glu304 flap has a common function in these enzymes. Since GS structural studies showed that this flap acts as a cover for the glutamate entrance to the active site, this flexible loop is likely to be involved in ligand binding to the active site (13). This idea is supported by our observations that amino acid substitutions in Glu304 flap residues of *B. subtilis* GS alter the glutamate K_m values (Table 5; Fig. 3). These results argue that the residues in the Glu304 flap have been optimized during evolution for their role in GS enzymatic activity.

The E304A mutant enzymes of both *B. subtilis* and *E. coli* GS have higher ammonium K_m values than their corresponding wild-type enzymes (Table 5) (1). These observations suggest that the Glu304 residues of *B. subtilis* and *E. coli* GS have the same catalytic role in the synthesis of glutamine. Crystallographic studies have shown that when the glutamate binding flap closes over the active site of GS, the Glu304 carboxylate side chain forms part of the ammonium substrate binding site (20, 21, 29). Thus, it is reasonable to assume that the E304A substitutions increase the ammonium K_m values by directly reducing the mutant enzymes' affinities for ammonium. Although the E304A and E304D substitutions increase the ammonium K_m values, they do not alter the hydroxylamine K_m values (Table 5). This difference in effect on the substrate K_m values is most likely a result of the different pK_a values for these two substrates. While ammonia has a pK_a value of 9.3, hydroxylamine has a significantly lower pK_a value of 6.0 (6). Under the assay conditions used in these experiments, ammonium has a positive charge and needs a negatively charged pocket for optimal binding. In contrast, hydroxylamine is uncharged and thus the negatively charged side chain of Glu304 is not required for binding. In this scenario, removal of an amino acid side chain that contributes to the binding of a charged ammonium ion is expected to increase the observed ammonium K_m value but to have no effect on the hydroxylamine K_m value. Indeed, this behavior was obtained with the E304A and E304D GS substitutions (Table 5).

The *B. subtilis* Glu304 residue is required for glutamine feedback inhibition of GS (Table 6). The novelty of this observation is that although the corresponding glutamate residue is also present in *E. coli* GS, the *E. coli* enzyme is not feedback inhibited by glutamine (43). Therefore, the Glu304 residue is required but is not sufficient for glutamine inhibition, and additional protein residues must also contribute to the stable binding of glutamine to *B. subtilis* GS. Indeed, we have shown previously that amino acid substitutions in several *B. subtilis* GS active site residues can give rise to a feedback-resistant phenotype (16, 18, 46). Taken together, these results argue that multiple residues act together with Glu304 to stabilize feedback inhibitor binding to the active site. Nonetheless, it is noteworthy that *B. subtilis* GS has evolved so that the conserved Glu304 residue has retained its role in catalysis while acquiring an additional function in glutamine feedback inhibition.

Several observations support the idea that glutamine and MetSox have equivalent interactions with *B. subtilis* GS. First, both of these compounds are inhibitors of GS that bind to the enzyme active site (30, 46). Second, amino acid substitutions of the Glu304 residue confer resistance to both of these inhibitors (Table 6). In addition, the observation that both glutamine and MetSox are able to promote the interaction between GS and TnrA (Fig. 4) suggests that glutamine and MetSox promote similar conformational changes in *B. subtilis* GS. The Glu304 side chain of *B. subtilis* GS may directly form a hydrogen bond with glutamine in a manner that is similar to the interaction observed between the *S*. Typhimurium Glu304 residue and MetSox (Fig. 2) (20, 30). Because the Glu304 residue of *S*. Typhimurium GS interacts with the sulfonimide nitrogen (N^{ε})

of MetSox, it is tempting to speculate that the Glu304 residue of *B. subtilis* GS interacts with the analogous amide nitrogen (N^{ε}) of glutamine. One shortcoming of this proposal is that glycine does not have a side chain that can interact with Glu304 and thus this model does not explain why glycine is able to promote the interaction between GS and TnrA (47) or why the E304A and E304D substitutions confer high-level resistance to glycine inhibition (Table 6). An alternative proposal consistent with all of our experimental observations is that the Glu304 side chain of *B. subtilis* GS interacts with the α-amino nitrogen of glutamine, glycine, and MetSox. A definitive explanation for the role of Glu304 in glutamine feedback inhibition of *B. subtilis* GS will require structural analysis of the feedback-inhibited enzyme.

The A305G mutant GS is similar to the enzymes with Glu304 substitutions in that it is resistant to inhibition by glutamine, MetSox, and glycine (Table 6). Since glycine residues in polypeptide chains have more conformational freedom than other amino acids, the replacement of Ala305 with glycine is expected to increase the local peptide backbone flexibility. This effect presumably alters the stability of the glutamate binding flap in A305G GS, and thus the A305G substitution most likely confers resistance to the inhibitors by indirectly affecting the adjacent Glu304 residue.

In addition to its catalytic functions, the Glu304 flap of *B. subtilis* GS plays a critical role in the regulation of TnrA and GlnR. Amino acid substitutions in five of the Glu304 flap residues resulted in mutant GS proteins unable to regulate the activity of these two transcription factors (Table 3). It is not known whether the Glu304 flap of FBI-GS directly interacts with TnrA and GlnR or whether the Glu304 flap participates in a GS conformational change that is required for the interaction of FBI-GS with TnrA and GlnR. This uncertainty means that it is unknown whether the Glu304 flap substitutions have a direct or indirect effect on the interaction of FBI-GS with TnrA and GlnR. Nonetheless, these data indicate that during evolution of the *B. subtilis* GS enzyme the Glu304 flap acquired a regulatory function while retaining its sequence conservation and role in glutamine synthesis.

Studies with animal model systems have shown that GS is required for the virulence of *M. tuberculosis*, *S.* Typhimurium, and *Streptococcus pneumoniae* (25, 28, 41). In addition, MetSox has been shown to inhibit the growth of *M. tuberculosis* in human mononuclear phagocytes, the bacterium's primary host cells, and in a guinea pig model of pulmonary tuberculosis (23, 24). While these studies demonstrate the potential of using GS as a drug target in antimicrobial therapy, specific mutants of GS can give rise to high-level MetSox resistance. For instance, the *B. subtilis* E304A and E304D mutant enzymes had levels of resistance to MetSox inhibition that were 200- and 150-fold higher, respectively, than that for wild-type GS (Table 6). This property is unique in that none of the previously isolated glutamine feedback-resistant *B. subtilis* GS enzymes had such high levels of resistance to MetSox inhibition (16, 18, 46). Studies with *Anabaena azollae* GS also showed that a mutant enzyme with a D54E amino acid substitution has high-level resistance to MetSox inhibition (10). Moreover, this Asp54 mutant had a growth defect in ammonium-limited medium similar to that seen for the *B. subtilis* Glu304 mutants (10). Although the biochemical mechanism for MetSox resistance of these mutant GS proteins is not known, it is interesting that the side chains of Asp54 and Glu304 are both part of a negatively charged pocket where ammonium binds to GS (1, 13). It is commonly observed that the acquisition of antibiotic resistance confers a reduction in fitness that is manifested as a decreased growth rate (2). Indeed, MetSox-resistant bacteria encoding mutant GS proteins with amino acid substitutions in the Asp54 or Glu304 residue have a conditional reduction in fitness in that these mutants have reduced growth rates on ammonium-limited medium (10). It is unclear whether this fitness reduction would affect the viability of pathogenic bacteria in their native hosts and thus preclude the emergence of MetSox-resistant strains.

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