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Alteration of the *Bacillus subtilis* Glutamine Synthetase Results in Overproduction of the Enzyme

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A mutation leading to glutamine auxotrophy was located near a 5-fluorouracil resistance marker in the *citB-thyA* region of the *Bacillus subtilis* chromosome. This mutation resulted in a glutamine synthetase with altered kinetic and feedback properties. The specific activity of manganese-stimulated glutamine synthetase activity in crude extracts was 18-fold higher, and the magnesium-stimulated activity was about 30% that of the wild type. Quantitation of the enzyme by precipitation with antibody prepared against pure enzyme confirmed the presence of high enzyme levels in the mutant. This mutation is very closely linked (recombination index of 0.03) to another glutamine auxotroph containing enzyme with altered electrophoretic and heat sensitivity properties. Mutations in the structural gene for glutamine synthetase may result not only in altered catalytic and regulatory properties but also in altered production of the enzyme.

Ammonia assimilation in *Bacillus megaterium* (9) and probably *Bacillus subtilis* is dependent on the concerted activity of glutamine synthetase (GS) and glutamate synthase (17). The lack of glutamate dehydrogenase in these species (12) implies that the above reactions are pivotal for nitrogen metabolism. In these bacilli, as in *Klebsiella* (16), regulation of nitrogen metabolism may center on glutamine synthetase. The regulation could include not only feedback inhibition and catalytic properties of the enzyme but also the capacity of glutamine synthetase to control formation of various enzymes involved in nitrogen metabolism, including its own synthesis.

Another potentially interesting property of this enzyme in *Bacillus* centers on its possible role in regulating initiation of sporulation. There is evidence from studies with *B. megaterium* that glutamine and/or glutamine synthetase are involved in such regulation (10, 18).

To further study regulation of nitrogen metabolism and sporulation, a number of glutamine auxotrophs were isolated in *B. subtilis*. After transformation into isogenic backgrounds, mutations were mapped and characterized. In this report, some mutations in the structural gene are described that result not only in alterations of both the catalytic and feedback inhibition properties of glutamine synthetase but also in overproduction of the enzyme.

MATERIALS AND METHODS

Bacterial strains and cultivation. The strains of *B. subtilis* used in this study are listed in Table 1. Cells were grown in a minimal salts medium (19) supplemented with 0.2% (wt/vol) glucose and 50 μg of required amino acids per ml. For strains requiring glutamine, the media were supplemented with 0.2% glutamine (filter sterilized). Growth rates were determined by measuring absorbances at 610 nm in a Beckman DBG spectrophotometer.

Isolation of glutamine auxotrophs. Glutamine auxotrophs were isolated from strain JH642. Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was done according to Adelberg et al. (1). After mutagenesis, the cells were centrifuged at 10,000 rpm for 5 min in a Sorvall SS 34 rotor, washed, and then suspended in minimal media supplemented with 0.2% glutamine. After growth for 3 h, the cells were washed twice and suspended in minimal media supplemented with 0.2% glutamate. After incubation for 1 h, penicillin G was added to a final concentration of 500 U/ml and incubated for 2 h. A total of 2,000 U of penicillinase (EC 3.5.2.6) was then added and, after 5 min, the cells were centrifuged and washed once with minimal media. The cells were then diluted and plated on minimal media plus glutamine. Auxotrophs were recovered by scoring colonies after 24 h on minimal media replicas. Putative auxotrophs were streaked three times on plates containing minimal media plus glutamine and then tested for the glutamine requirement by plating on minimal media. A total of 50 auxotrophs were isolated during four independent screenings.

Preparation of crude extracts. Cells (150 ml) grown to the late log phase in minimal media supplemented with 0.2% glutamine were harvested as described above and washed twice with 30 ml of 50 mM imidazole hydrochloride (pH 7.0). The pellet was suspended in 5 ml of the same buffer and lysed by addition of 100 μg of lysozyme (EC 3.2.1.17) per

TABLE 1. *Strains used in this study*

Strain	Genotype	Source
JH641	*trpC2 phe-1 ilv-1*	Stock strain
JH642	*trpC2 phe-1*	Wild-type DNA × JH641
DRD-1	*trpC2 phe-1 glnA100*	Nitrosoguanidine mutagenesis of JH642
DRD-2	*trpC2 phe-1 glnA100 ilv-1*	DRD-1 DNA × JH641
DRD-3	*trpC2 phe-1 glnA103*	Nitrosoguanidine mutagenesis of JH642
DRD-4	*trpC2 phe-1 glnA103*	DRD-3 × JH641

ml and 10 μg of deoxyribonuclease (EC 3.1.4.5) per ml for 30 min at 37°C. Lysed cells were used directly as crude extract. Protein was determined by the method of Lowry et al. (15).

Assay for glutamine synthetase. Glutamine synthetase (EC 6.3.1.2) was assayed in crude extracts by a modification of the glutamyl transferase method of Elliott (8). The reaction mixture contained 50 mM imidazole hydrochloride (pH 7.0), 10 mM adenosine 5′-triphosphate (ATP), 100 mM glutamate, 40 mM hydroxylamine, and either 10 mM $MnCl_2$ or 40 mM $MgSO_4$ in a final volume of 1.8 ml. The reaction was initiated by addition of 0.2 ml of crude extract and terminated by addition of 1 ml of ferric chloride reagent (8). The optical density at 540 nm was read on a Beckman DBG spectrophotometer after removal of the precipitate by centrifuging for 5 min at 5,000 rpm in a Sorvall SS 34 rotor.

Purified GS was assayed by the biosynthetic method of Deuel et al. (6). The reaction mixture contained 50 mM imidazole hydrochloride (pH 7.0), 10 mM ATP, 100 mM glutamate, 50 mM NH_4Cl, and 40 mM $MgSO_4$ or 10 mM $MnCl_2$ in a final volume of 1 ml. For kinetic and feedback analysis, the biosynthetic assay was used.

Purification of GS. GS was purified as described by Deuel et al. (6). Enzyme isolated by this procedure was homogeneous as judged by the presence of only one band after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Electrophoresis. Electrophoresis was done according to the procedure of Laemmli and Favre (14) by using a slab gel apparatus. Before application to the gel, samples in 50 μl of 0.0626 M tris(hydroxymethyl)aminomethane (pH 6.8)–2.0% sodium dodecyl sulfate–10% glycerol–5% mercaptoethanol–0.001% bromophenol blue were placed in boiling water for 5 min. Slabs were stained for 6 h in 0.025% Coomassie brilliant blue dissolved in 25% isopropanol-10% acetic acid and destained in several changes of 10% acetic acid until clear.

Immunological methods. Two male New Zealand White rabbits (about 15 pounds [ca. 33 kg] each) were injected subcutaneously (once a week for 4 weeks) with 1 mg of purified GS emulsified in Freund complete adjuvant. Three weeks after the last injection the animals were bled from the marginal ear vein.

Serum was prepared, and the immunoglobulin fraction was obtained by precipitation with 50% (wt/vol) ammonium sulfate. Precipitated immunoglobulin was suspended in 0.1 M sodium phosphate buffer (pH 7.0) to the original volume and dialyzed 10 h against 200 volumes of the same buffer. Remaining precipitates were removed by centrifuging at 5,000 rpm.

Only a single band of precipitation was seen on Ouchterlony immunodiffusion plates when either purified GS or crude extract was tested against this antiserum. For immunoprecipitation curves, 5 to 100 μl of antisera was added to a fixed amount of enzyme. The volumes were brought to 0.2 ml with 50 mM imidazole (pH 7.0) and incubated at 27°C for 30 min and then at 4°C for several hours. The precipitates were removed by centrifuging at 4°C in a Sorvall SS 34 rotor at 10,000 rpm for 30 min, and 0.1 ml of the supernatant was assayed for residual GS activity. Preimmune serum showed no precipitation activity or inhibition of GS.

Heat inactivation. A total of 0.2 ml of crude extract was heated for up to 30 min at 70°C and then rapidly cooled in an ice bath to 4°C. GS activity was determined by the manganese-stimulated glutamyl hydroxamate assay.

Transformation and construction of isogenic strains. Transformation was done according to Anagnostopoulos and Spizizen (2). Strain JH641 (*phe trp ilv glnA*$^+$) was used as an isogenic background for the *glnA* mutations. The *glnA100* mutation was transformed into it by treating competent cells with 0.1 μg of deoxyribonucleic acid (DNA) from strain DRD-1 (*gln phe trp*). Treated cells were plated on minimal media supplemented with 50 μg each (per ml) of phenylalanine, tryptophan, isoleucine, and valine plus 0.2% glutamate as the nitrogen source. Strains bearing the *glnA100* mutation grow slowly on glutamate (see below) and form small colonies. Small colony transformants were picked, purified several times on complete media, and checked for the glutamine and isoleucine-valine requirement: DRD-2 (*gln trp phe ilv*). The *glnA103* mutation was transformed into strain JH641 by treating competent cells with 10 μg of DNA per ml from strain DRD-3 (*gln phe trp*). Cells were plated on minimal media supplemented with 50 μg each of phenylalanine and tryptophan per ml plus 0.2% glutamine. *ilv*$^+$ recombinants were replicated onto minimal medium plates without glutamine to detect *gln ilv*$^+$ double transformants. Approximately 1% of the *ilv*$^+$ recombinants also had the Gln phenotype (DRD-4).

Chemicals. Imidazole and ATP were obtained from Sigma Chemical Co.; acrylamide was obtained from Eastman Kodak Co.; *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine was from the Aldrich Chemical Co.; penicillin G was from E. R. Squibb & Sons, Inc.; penicillinase was from Calbiochem; Freund adjuvant (complete) was from Grand Island Biological Co.; lysozyme and deoxyribonuclease were obtained from Worthington Biochemicals Corp. Other chemicals were of reagent grade.

RESULTS

Growth characteristics of mutant *glnA100*. Of fifty glutamine auxotrophs isolated, at least four contained GS with altered catalytic properties in crude extracts. One of these, the

glnA100 mutation, was transformed into strain JH641 as described below and studied in detail. The resultant strain DRD-2 was first tested for its ability to grow on various nitrogen sources. There was no growth when ammonia or alanine was supplied as the sole nitrogen source. The mutant grew at a slow rate when glutamate, proline, or arginine (180 min mean doubling time in a glucose minimal medium supplemented with 0.2% [wt/vol] of various nitrogen sources) was provided, whereas the growth rate on glutamine was comparable to that of the wild type (50 min mean doubling time).

Kinetic and feedback inhibition properties of glutamine synthetase from strain DRD-2. Since there was appreciable activity of GS in extracts of strain DRD-2 (Table 2), substrate saturation curves were constructed in an effort to determine the the basis for the glutamine requirement. Purified enzyme from strain DRD-2 showed an apparent decrease in affinity for glutamate and ammonia in both the Mg^{2+}- and Mn^{2+}-stimulated activities (Fig. 1 and 2). The substrate saturation curve for ammonia (Fig. 1), by Mn^{2+} stimulation of the mutant enzyme, did not show inhibition of activity at high substrate levels characteristic of the prototrophic strain. GS from the mutant also did not exhibit substrate saturation for glutamate or ammonia when Mg^{2+} was the activating cation.

Results of feedback inhibition studies are summarized in Table 3. Glutamine, an inhibitor of the Mg^{2+}-stimulated activity (7), showed much less inhibition of enzyme from strain DRD-2 than from the wild type. Alanine and glycine, inhibitors of the Mn^{2+}-stimulated activity (7), showed no effect on the enzyme from strain DRD-2.

TABLE 2. *Specific activities of glutamine synthetase from wild type and mutant*[a]

Sp act	Activating cation	Sp act JH641	Sp act DRD-2
Crude extract[b] (glutamyl hydroxamate) assay	Mg^{2+}	0.011	0.0039
	Mn^{2+}	0.0089	0.156
Pure enzyme[b] (glutamyl hydroxamate) assay	Mg^{2+}	3.2	0.11
	Mn^{2+}	2.5	2.2
Pure enzyme[c] (biosynthetic assay)	Mg^{2+}	9.4	0.27
	Mn^{2+}	8.0	6.0

[a] Strains were grown in minimal media supplemented with 0.2% glutamine as the nitrogen source.

[b] Specific activity expressed as micromoles of glutamyl hydroxamate formed per minute per milligram of protein.

[c] Specific activity expressed as micromoles of P_i released per minute per milligram of protein.

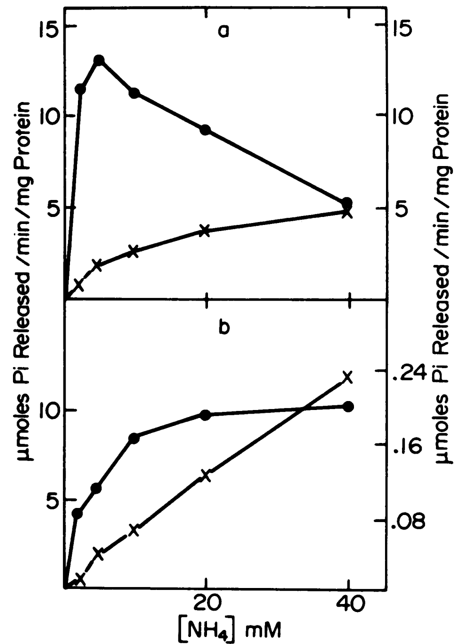

FIG. 1. *Substrate saturation curves of purified GS for ammonia with Mg^{2+} or Mn^{2+} as the activating cation. (a) Mn^{2+}-stimulated activity. (b) Mg^{2+}-stimulated activity. Left ordinate for strain JG641* (●); *right ordinate for strain DRD-2* (×).

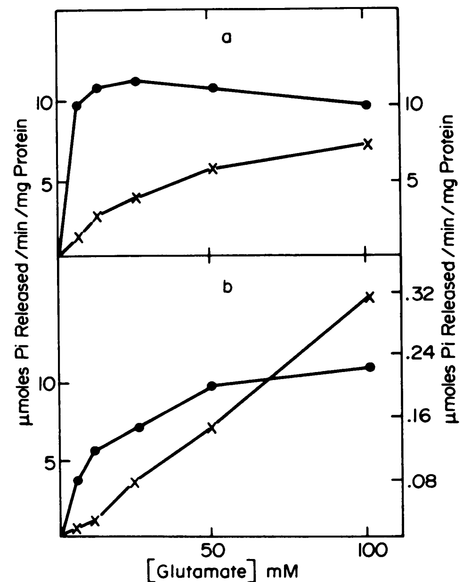

FIG. 2. *Substrate saturation curves of purified GS for glutamate with Mg^{2+} or Mn^{2+} as the activating cation. (a) Mn^{2+}-stimulated activity. (b) Mg^{2+}-stimulated activity. Left ordinate for strain JH641* (●); *right ordinate for strain DRD-2* (×).

TABLE 3. *Feedback inhibition of glutamine synthetase from mutant and wild type*

Inhibitor	Activating cation	Activity found in presence of inhibitor as % of uninhibited values[a]	
		JH641	DRD-2
Glutamine (1 mM)	Mg^{2+}	7	91
Alanine (5 mM)	Mn^{2+}	19	100
Glycine (5 mM)	Mn^{2+}	35	100

[a] Glutamine synthetase activity measured by the biosynthetic assay as described in the text, using purified enzymes.

Evidence for overproduction of glutamine synthetase in strain DRD-2. Crude extracts of strain DRD-2 showed an 18-fold increase in Mn^{2+}-stimulated specific activity and a 70% decrease in Mg^{2+}-stimulated activity when compared with those of the wild type (Table 2). Comparison of specific activities of purified enzymes from strain DRD-2 and the prototrophic strain (Table 2) indicates that the increased level of Mn^{2+} activity seen in crude extracts is probably a result of increased levels of enzyme rather than an increase in the Mn^{2+}-stimulated turnover number of the pure enzyme. These results were obtained by either the glutamyl hydroxamate or biosynthetic assay. The biosynthetic assay could not be used for measurement of enzyme activity in crude extracts due to high levels of nonspecific adenosine triphosphatase activity. Recovery of GS during purification was usually greater than 50% of the total activity in crude extracts for both strains.

Further evidence that DRD-2 has increased levels of enzyme was provided by immunoprecipitation of GS from extracts of the mutant and wild-type strains by using antiserum prepared against the pure enzyme (Fig. 3). The immunoprecipitation curves showed that a unit of Mn^{2+}-stimulated activity from the wild type was approximately equal to a unit of Mn^{2+}-stimulated activity from strain DRD-2 in capacity to be precipitated by anti-GS antibody. Similar results were obtained whether purified enzymes or crude extracts were used. This result confirms the specific activity measurements (Table 2) in showing that the increased level of Mn^{2+} activity in strain DRD-2 extracts reflects an increase in total enzyme.

Evidence that *glnA100* is a mutation in the structural gene for glutamine synthetase. Although the DRD-2 strain contains GS with altered catalytic and feedback inhibition properties, it was not entirely clear whether these changes were a result of a mutation in the structural gene or were due to another alteration, such as a lesion in an enzyme that might alter or process GS, i.e., analogous to adenylylation of the *E. coli* GS (13). The electrophoretic mobility in sodium dodecyl sulfate-polyacrylamide gels and the heat lability of GS from strain DRD-2 and wild type were found to be identical. Attempts to isolate temperature-sensitive revertants of strain DRD-2 were unsuccessful. Evidence that strain DRD-2 is altered in the structural gene for GS was obtained by demonstrating close linkage to another glutamine auxotroph that contains GS altered in electrophoretic mobility and heat stability.

A number of glutamine auxotrophs were screened for altered electrophoretic mobility of GS by precipitating the enzyme from crude extracts with antiserum and electrophoresing the precipitates on sodium dodecyl sulfate-polyacrylamide gels. Enzyme from one mutant, strain DRD-4, showed a slight increase in mobility relative to the wild type when electrophoresed alone or mixed with the wild-type enzyme (Fig. 4). GS from strain DRD-4 also showed increased lability at 70°C (Fig. 5). Parenthetically, this mutant also overproduced GS.

Transformation of strain DRD-2 by using DNA from strain DRD-4 showed that *glnA100* and *glnA103* were closely linked (Table 4). Since a recombination index of 0.1 is routinely obtained for markers in the same gene (4), the value obtained here (0.03) strongly suggests that they affect the same polypeptide.

Genetic mapping of *glnA100*. The *glnA100* marker was located on the *B. subtilis* chromosome by PBS-1-mediated transduction. Preliminary experiments indicated that this marker was linked to the *citB* locus, and further crosses were undertaken to locate the *glnA100* marker within this terminal region. The results of these crosses are presented in Fig. 6.

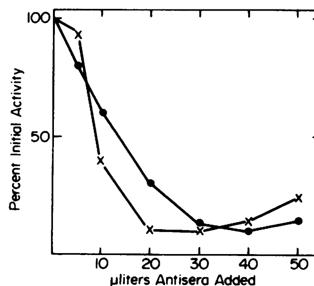

FIG. 3. *Immunoprecipitation of GS from crude extracts of strain JH641 (●) and strain DRD-2 (×). One hundred percent activity is the amount of enzyme that produces 1.5 μmol of glutamyl hydroxamate in 1 h using Mn^{2+} as the activating cation under standard assay conditions (see the text).*

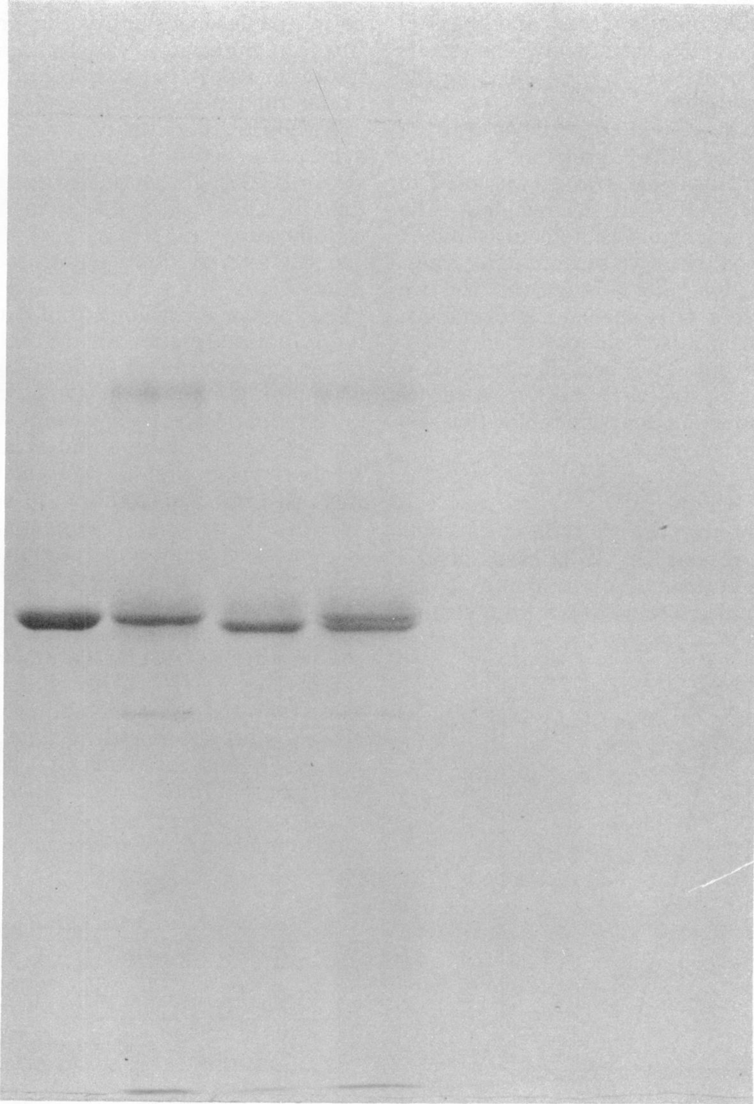

FIG. 4. *Sodium dodecyl sulfate-slab gel electrophoresis of purified GS and GS antibody precipitates. Sample preparation and electrophoresis as described in the text. From left to right, the slab contains: GS purified from the wild type; antibody precipitate from crude extract of wild type; antibody precipitate from crude extract of strain DRD-4; antibody precipitate from mixture of wild-type and strain DRD-4 extracts.*

Two-factor transduction results showed that the *glnA100* marker mapped between the *citB* (aconitase) locus and the *thyA* (thymidylate synthetase) locus. Since genetic manipulation with *thyA* markers is complicated (S. Zahler and T. Neubauer, personal communication), we employed the *fur-5* marker as a landmark for this region. The *fur-5* mutant was isolated as resistant to fluorouracil (40 μg/ml) in the presence of uracil (40 μg/ml) (J. Hoch and C. Chen, unpublished data). Linkage to *thyA* was tested by first constructing a strain, JH745, of the genotype *thyB fur-5*. DNA from strain JH745 was used to transform the 168 *thy* strain (*thyA thyB*) to *thyA*$^+$, and the presence of *fur-5* scored among the recombinants. Among 269 *thyA*$^+$ recombinants, 96 were cotransformed with the *fur-5* marker. Although *fur-5* is linked to *thyA* in transformation, no significant cotransfer of this marker with *glnA100* was ob-

served in transformation. On the other hand, there was a weak linkage of *thyA* and *glnA100* in transformation (15% cotransfer). The results suggest the order *citB*-*glnA*-*thyA*-*fur-5* for the markers in this region.

Anomalous behavior of *citB* alleles was observed when donor PBS-1, prepared on either *citB10*- or *citB17*-bearing strains, was used to transduce the *glnA100*-bearing recipient. The $glnA^+$ transductant clones that acquired a *citB* allele were mixed with two recombinant types, $glnA^+$ *citB* and $glnA^+$ $citB^+$. In general, the two recombinant types were in equal proportions, but the number of mixed clones varied widely from cross to cross. This behavior may be a peculiarity of *citB* strains or may be a consequence of transduction near the replication terminus of the chromosome.

DISCUSSION

The conclusion that the *glnA100* mutation is in the structural gene for GS is based on the following observations: (i) glutamine is required for normal growth; (ii) GS from strains bearing the *glnA100* mutation is altered in kinetic and feedback inhibition properties; and (iii) this mutation is closely linked to another mutation (*glnA103*) that contains GS altered in electrophoretic mobility and heat stability.

Although there is considerable glutamine synthetase activity remaining in extracts of strain DRD-2, glutamine is required for normal growth. This may be due to insufficient intracellular substrate levels for glutamine synthesis. The altered kinetic parameters of the mutant enzyme (Fig. 1 and 2) and the ability of glutamate or compounds that can be degraded to glutamate (proline or arginine) to partially restore growth (180 min doubling time versus 50 min on glutamine) support this possibility.

A comparison of Mn^{2+}-stimulated specific activities from wild-type and strain DRD-2 extracts suggests that the mutant has an 18-fold increase in GS. Since sulfhydryl modification of GS from *B. subtilis* results in a decrease in Mg^{2+} activity and an increase in Mn^{2+} activity (5), it was initially thought these results might reflect a catalytic alteration resulting from the mutation. Overproduction of the enzyme was confirmed, however, by immunological precipitation (Fig. 3). It is likely that a single mutation alters the structure and production of the enzyme since the marker is in an isogenic background. It was also found that prototrophic revertants contained normal amounts and feedback properties of GS. Among a total of 20 glutamine auxotrophs screened to date (includ-

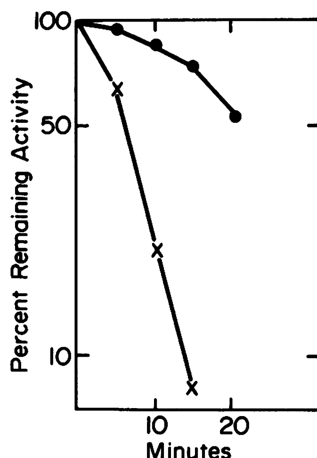

FIG. 5. *Heat inactivation of GS in crude extracts of strain JH641 (●) and strain DRD-4 (×). Crude extracts containing an amount of enzyme that produces 2.0 μmol of glutamyl hydroxamate in 1 h using Mn^{2+} as the activating cation were placed at 70°C, and samples were removed at the indicated times for assay.*

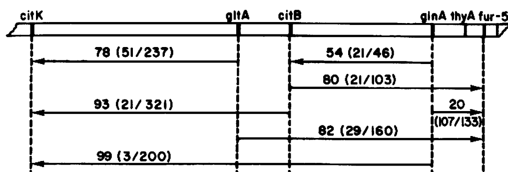

FIG. 6. *Genetic map of the terminal region of the B. subtilis chromosome constructed by PBS-1 transduction. The head of the arrow points to the donor (selected) marker. The numbers are percentage of recombination [(1 − cotransfer of donor and recipient markers) × 100]. The numbers in parentheses are the number of recombinants that acquired the donor marker divided by the total recombinants tested.*

TABLE 4. *Linkage of glutamine auxotrophs by transformation*[a]

Recipient strain	Donor strain	Transformants/ml		gln^+/ilv^+	RI[b]
		gln^+	ilv^+		
DRD-2	JH642	5.3×10^5	7.7×10^5	0.69	0.03
DRD-2	DRD-4	6.3×10^3	2.8×10^5	0.02	

[a] See text for details.

[b] RI, Recombination index as defined in reference 3.

ing strains DRD-2 and DRD-4), 4 contained 9 to 18 times the parental Mn^{2+} activity and 30 to 50% of the Mg^{2+} activity. It is unlikely, therefore, that this phenotype is due to rare double mutations.

Magasanik and co-workers have reported that mutations affecting GS from *Klebsiella aerogenes* may alter regulation of enzymes important in nitrogen metabolism (16). In the case of histidase it has been shown in vitro that GS may activate transcription of the histidase genes (20). More recently, Foor et al. (11) have reported that GS production appears to be regulated by GS and that transcription of the structural gene is probably dependent on the adenylylated state of the enzyme. Although the *glnA100* mutation described here does affect the production of GS, we have not yet examined the effect of the mutation on enzymes that catabolize certain amino acids to provide glutamate or ammonia.

In contrast to *K. aerogenes*, GS from *B. subtilis* is not covalently modified by adenylylation (7). However, Deuel has demonstrated catalytic changes analogous to adenylylation when GS is treated in vitro with sulfhydryl-modifying reagents (5). The Mg^{2+} and Mn^{2+} activities are distinguished by their catalytic properties, sensitivity to feedback inhibitors (7), and response to limited alkylation (5). The two activities also appear to be differentially affected in the *glnA100* mutation since the Mg^{2+} activity is almost entirely lost, whereas the Mn^{2+} activity is nearly normal when assaying pure preparations at saturating substrate concentrations.

Initially, we had hoped to isolate GS mutants deficient in the ability to sporulate. Studies with *B. megaterium* (10) suggest that glutamine or some compound derived from glutamine may be a catabolite repressor of sporulation. It has also been proposed that GS may be responsible for initiation of sporulation since most GS mutants from *B. megaterium* are unable to sporulate (18). All of the 50 glutamine auxotrophs isolated in this study sporulate normally in a nutrient sporulation medium supplemented with glutamine. It should be noted, however, that all of the mutants contain some GS activity, albeit catalytically altered. Although our results suggest that active GS may not be a necessary element for sporulation in *B. subtilis*, they do not preclude the possibility that the enzyme may be involved in regulating sporulation under certain conditions (i.e., nitrogen starvation) or that glutamine may be a catabolite repressor of sporulation.

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