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Mutations Affecting Glutamine Synthetase Activity in *Salmonella typhimurium*

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A positive selection procedure has been devised for isolating mutant strains of *Salmonella typhimurium* with altered glutamine synthetase activity. Mutants are derived from a histidine auxotroph by selecting for ability to grow on D-histidine as the sole histidine source. We hypothesize that the phenotype may be based on a regulatory increase in the activities of the D-histidine racemizing enzymes, but this has not been established. Spontaneous glutamine-requiring mutants isolated by the above selection procedure have two types of alterations in glutamine synthetase activity. Some have less than 10% of parent activity. Others have significant glutamine synthetase activity, but the enzyme have an altered response to divalent cations. Activity in mutants of the second type mimics that of highly adenylylated wild-type enzyme, which is believed to be inactive in vivo. Glutamine synthetase from one such mutant is more heat labile than wild-type enzyme, indicating that it is structurally altered. Mutations in all strains are probably in the glutamine synthetase structural gene (*glnA*). They are closely linked on the *Salmonella* chromosome and lie at about min 125. The mutants have normal glutamate dehydrogenase activity.

In bacteria, two routes for incorporation of ammonia into glutamate have been demonstrated. One is via glutamate dehydrogenase (EC 1.4.1.4, reaction 1). The other, recently demonstrated by Tempest et al. (14, 24), is via glutamine synthetase (EC 6.3.1.2) and glutamate synthase (EC 1.4.1.X) (reactions 2 and 3). The second pathway probably functions under conditions of ammonia limitation (4, 14). Incorporation of ammonia by this route requires high glutamine synthetase activity, since glutamine is the precursor of virtually all cellular nitrogen-containing compounds under these conditions (14).

$$\alpha\text{-ketoglutarate} + NH_3 + NADPH \rightarrow \text{glutamate} + NADP \quad (1)$$

$$\text{glutamate} + NH_3 + ATP \rightarrow \text{glutamine} + ADP + P_i \quad (2)$$

$$\alpha\text{-ketoglutarate} + \text{glutamine} + NADPH \rightarrow 2\ \text{glutamate} + NADP \quad (3)$$

$$\alpha\text{-ketoglutarate} + NH_3 + NADPH + ATP \rightarrow \text{glutamate} + NADP + ADP + P_i \quad (2 + 3)$$

One important mechanism for controlling the activity of glutamine synthetase in gram-negative bacteria is covalent modification by adenylylation (6, 21, 25). The elegant work of Stadtman, Ginsburg, and their co-workers has demonstrated that up to 12 adenylyl groups can be incorporated per molecule of *Escherichia coli* glutamine synthetase, one per subunit, and that this modification is reversible (reviewed in 8). The kinetic properties of the adenylylated enzyme are very different from those of the unmodified enzyme and indicate that the adenylylated form is the "inactive" or "less active" form (13, 21).

To gain further insight into the physiological functions of glutamine synthetase and of its covalent modification by adenylylation, we are studying mutant strains of *Salmonella typhimurium* with altered glutamine synthetase activity. In this report we describe a positive selection procedure for isolating such mutants and a preliminary characterization of several of them.

Magasanik et al. (12, 18) have characterized mutant strains of *Klebsiella aerogenes* with altered glutamine synthetase activity. They have proposed that in *Klebsiella* glutamine synthetase itself is a regulatory element controlling the levels of several enzymes involved in production of glutamate (4, 12, 18). Properties of the *Salmonella* mutants are contrasted with those of *Klebsiella* mutants.

(A preliminary report of this work has appeared [S. G. Kustu, Fed. Proc. **33**:1464 1974]).

MATERIALS AND METHODS

Chemicals. D-Histidine was obtained from Sigma Chemical Co. Fosfomycin was a gift from Merck and Co. Snake venom phosphodiesterase (SVP; EC 3.1.4.1; 1 mg/ml, 1.5 U/mg) was obtained from Boehringer Mannheim. Glutamine solutions (100 mM) were neutralized to pH 7 and were sterilized by filtration. They were stored at 4 C.

Media and growth of bacterial strains. Minimal medium was medium E of Vogel and Bonner (26) with 0.4 to 0.6% glucose added as carbon source. This medium contains (per liter) $MgSO_4 \cdot 7H_2O$, 0.2 g; citric acid H_2O, 2 g; K_2HPO_4, 10 g; $NaHNH_4PO_4 \cdot 4H_2O$, 3.5 g. Nutrient broth medium contained 8 g of nutrient broth (Difco) and 5 g of NaCl per liter. Bacterial growth was monitored turbidimetrically by measuring absorbance at 650 nm. For determination of growth rates, inoculum cultures were grown overnight in nutrient broth supplemented with 1 mM glutamine. They were diluted 1/100 or 1/50 into minimal medium containing the supplements specified.

Isolation of mutant strains. All strains constructed for this work were derived from *S. typhimurium* strain LT2 and are listed in Table 1. Spontaneous mutants requiring glutamine for optimal growth were derived from two parent strains, TA831 (*hisFΔ645*) and TA1646 (*hisFΔ645 dhuA1 hisJ5601*), by selecting for growth on D-histidine as the sole histidine source; 10^8 cells of each parent were spread on minimal agar plates containing 0.3 mM D-histidine. After 3 days of incubation at 37 C, small colonies were picked and purified on nutrient agar plates containing 1 mM glutamine. They were tested for ability to grow on minimal plates containing L-histidine or L-histidine plus glutamine. Strains requiring glutamine for optimal growth were saved for further study. Four independent glutamine-requiring strains were derived from strain TA831 and two were derived from strain TA1646. (Strain TA2193, one of the strains derived from strain TA1646, was isolated from a culture exposed to the frameshift mutagen ICR-372. However, strain TA2193 has a heat-sensitive glutamine requirement; in addition, it reverts to glutamine independence spontaneously and reversion is not increased by ICR, suggesting that it may be a spontaneous mutant.)

Genetic analysis of mutant strains. Bacteriophage was P22 *int4* (22). Glutamine-requiring strains were transduced to histidine independence with phage grown on a wild-type strain on minimal plates supplemented with 1 mM glutamine. Phage-sensitive clones were isolated as described previously (20).

TABLE 1. *Bacterial strains*

Strain	Genotype	Parent	Comments
TA831[a]	*hisFΔ645*		
TA1772[a]	Wild-type (isogenic with TA831)	TA831	
TA2188	*gln-53 hisFΔ645*	TA831	
TA2190	*gln-54 hisFΔ645*	TA831	
TA2191	*gln-54*	TA2190	
SK9	*gln-51 hisFΔ645*	TA831	
SK10	*gln-52 hisFΔ645*	TA831	
TA1646[a]	*hisFΔ645 dhuA1 hisJ5601*		*dhuA*, *hisJ* mutations affect high-affinity histidine permease
TA2192	*gln-55 hisFΔ645 dhuA1 hisJ5601*	TA1646	
TA2193	*gln-56 hisFΔ645 dhuA1 hisJ5601*	TA1646	Heat-sensitive glutamine requirement
SK42	*gln-52 gln-62 hisFΔ645*	SK10	Heat-sensitive glutamine requirement
SK43	*gln-52 gln-63 hisFΔ645*	SK10	Heat-sensitive glutamine requirement
SA535[b]	*serA13* HfrK5		
SK7	*gln-54* HfrK5	TA2190	
SK8	*gln-54 cysA2502* HfrK5	SK7	
TA2185	*gln-59 nit9*	*nit9*	Heat-sensitive glutamine requirement
SK18	*gln-59 hisFΔ645*	TA2190	Heat-sensitive glutamine requirement
SK27	*gln-59* HfrK5	SK18	Heat-sensitive glutamine requirement
SK30	*gln-59 thy-1863* HfrK5	SK27	Heat-sensitive glutamine requirement
JL308[c]	*metA309 ara-9*		
SK6	*cya692 metA309 ara-9*	JL308	
SK14	*ilv-8 metA309 ara-9*	*SK6*	
SK39	*ilv-8 gln-59 ara-9*	SK14	Heat-sensitive glutamine requirement
SK41	*ilv-8 gln-59 rif⁻ ara-9*	SK39	Heat-sensitive glutamine requirement
JL400[c]	*purC7 his⁻* HfrA		
JL790[c]	*malB⁻ leu⁻*		
TA3301[d]	*cya408*		

[a] Obtained from G. F.-L. Ames.
[b] Obtained from B. N. Ames.
[c] Obtained from J. L. Ingraham.
[d] Obtained from M. Alper and B. N. Ames.

Glutamine-requiring strains were transduced to glutamine independence with phage grown on a wild-type strain or on other mutants (10^8 cells were mixed with 10^9 phage). To prevent heavy background growth of the recipient, recipient cells were centrifuged and suspended in glutamine-free medium. Similarly, phage were collected by centrifugation and suspended in buffer. Several glutamine-requiring strains were transduced to glutamine independence at 23 C with phage grown on strains TA2193, SK42, and SK43, strains with a heat-sensitive glutamine requirement. Transductants were scored for inheritance of the heat-sensitive phenotype.

To determine linkage of *gln*$^-$ mutations to possible cotransducing markers, phage grown on strain TA2185 (*gln-59 nit-9*) was used to transduce the following strains to prototrophy: *purAl*, *argA94*, and JL308 (*metA309 ara-9*). Crosses were done at 30 C in the presence of 1 mM glutamine and recombinants were scored for a glutamine requirement at 37 C. Similarly, this phage was used to transduce strain *rha-182* to growth on rhamnose and strain JL790 (*malB*$^-$ *leu*$^-$) to growth on maltose under the same conditions. (Strains carrying the *gln-59* mutation have a heat-sensitive glutamine requirement. The *gln-59* mutation is 80 to 90% linked to all other *gln* mutations described in this paper. It was originally isolated as a suppressor of *nit-9*, a mutation that results in loss of ability to utilize several nitrogen sources [J. Broach and S. Kustu, unpublished data].) To determine linkage of *gln*$^-$ mutations to *metE*, *ilv*, and *cya*, strain TA2191 (*gln-54*) was transduced to glutamine independence (in the presence of the appropriate supplements) with phage grown on strains *metE338*, *ilv-8*, and TA3301 (*cya408*), and recombinants were scored for inheritance of the donor phenotype. (The phenotype for strain TA3301 was failure to grow on citrate as carbon source.)

Construction of donor strains for conjugation mapping. The donor strain SK7 (*gln-54* HfrK5) was derived from strain TA2190 (*gln-54 hisFΔ645*) by mating it with strain SA535 (*serAl3* HfrK5) on minimal plates containing glutamine and screening for males among the histidine-independent recombinants. Males were identified by sensitivity to M13 phage (kindly provided by M. Bowes). Strain SK8 (*gln-54 cysA2502* HfrK5), which contains a counterselective mutation in *cysA*, was derived from strain SK7 by selecting for resistance to 0.25 mM chromate in the presence of 0.2 mM djenkolic acid (J. Wyche, personal communication). The donor strain SK27 (*gln-59* HfrK5) was derived from strain SK18 (*gln-59 hisFΔ-645*) by the procedure described above. [Strain SK18 was derived from strain TA2190 (*gln-54 hisFΔ645*) by transducing it to glutamine independence at 23 C with phage grown on strain TA2185 (*gln-59 nit-9*); a transductant with a heat-sensitive glutamine requirement was isolated.] Strain SK30 (*gln-59 thyl863* HfrK5), which contains a counterselective mutation in *thy*, was derived from strain SK27 by selecting for resistance to trimethoprim in the presence of thymine (17).

Construction of recipient strains for conjugation mapping. A set of recipient strains with single auxotrophic mutations was obtained from B. N. Ames. Multiply marked recipients were constructed. Strain SK6 (*cya692 metA309 ara-9*) was derived from strain JL308 (*metA309 ara-9*) by selecting for resistance to fosfomycin on MacConkey agar plates containing ribose and glycerol (M. Alper and B. N. Ames, personal communication). White colonies appearing on such plates have mutations in *cya* or *crp* (M. Alper and B. N. Ames, personal communication). Strain SK14 (*ilv-8 metA309 ara-9*) was derived from strain SK6 by transducing it to growth on ribose as carbon source with phage grown on strain *ilv-8* (in the presence of appropriate supplements). A phage-sensitive clone of a transductant that requires isoleucine and valine was isolated. Strain SK39 (*ilv-8 gln-59 ara-9*) was derived from strain SK14 by mating it with strain SK30 and selecting a *met*$^+$ *gln*$^-$ recombinant. Strain SK41 (*ilv-8 gln-59 rif*$^-$ *ara-9*) was derived from strain SK39 by selecting for resistance to rifampin (50 μg/ml on minimal glucose plates with appropriate supplements [9]).

Conjugation mapping: initial plate matings. Cultures of both the donor strain (SK8) and recipients (strains with single auxotrophic mutations) were grown overnight in nutrient broth at 37 C with shaking. Matings were performed directly on minimal agar plates supplemented with 0.5 mM glutamine by spreading 10^8 recipient cells with 10^6 to 10^8 donor cells. After 36 h, recombinants were transferred to nutrient agar plates supplemented with 0.5 mM glutamine and then scored for a glutamine requirement.

Three-factor cross and gradient of transmission experiments. A culture of the recipient strain SK41 was grown overnight in nutrient broth supplemented with 1 mM glutamine at 30 C with shaking. A culture of the donor strain JL400 was grown overnight in minimal medium containing appropriate supplements at 30 C without shaking. The donor strain was diluted 1/20 into nutrient broth and was grown with slow shaking to a cell density of about 10^8/ml. Matings were initiated as described by Beck and Ingraham (2), and their procedures were followed subsequently. Matings were carried out in nutrient broth supplemented with 1 mM glutamine. After interruption of mating, cells were immobilized in soft agar on minimal plates supplemented with 1 mM glutamine (gradient of transmission experiment) or on minimal plates containing arabinose as sole carbon source and supplemented with 1 mM glutamine, 0.3 mM isoleucine, 0.6 mM valine, 0.3 mM leucine, and 0.006 mM pantothenate (three-factor cross); plates were incubated at 30 C. Recombinants were transferred to nutrient agar plates supplemented with glutamine and were scored for a glutamine requirement at 37 C and for an isoleucine and valine requirement or resistance to rifampin at 30 C. Matings between strains SK14 and SK30 were carried out similarly.

Preparation of cell extracts. Cells were grown to early stationary phase in minimal medium supplemented with 0.3 mM L-histidine and 1 to 3 mM glutamine. Cultures were incubated with vigorous aeration at 37 C except as noted. To obtain adenylylated glutamine synthetase (10), strain TA831 was

grown to midexponential phase in a minimal medium (not medium E) containing citrate as carbon source and glutamate as sole nitrogen source. Cells were harvested by centrifugation, washed, and suspended in 1/10 volume of buffer. The buffer used to prepare extracts for glutamine synthetase assays and SVP treatment contained 10 mM $MgCl_2$ and 1 mM $MnCl_2$ in 30 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 8 (23). Cells were disrupted by sonic oscillation and extracts were clarified by centrifugation at 27,000 × *g* for 15 min.

Glutamine synthetase assays. Glutamine synthetase activity was measured using the γ-glutamyl transfer assay of Stadtman et al. (23) with the modification that the buffer in the reaction mixture was imidazole-hydrochloride (40 mM) and the final pH of the reaction mixture was 7.3. In the presence of 0.3 mM Mn^{2+} + 60 mM Mg^{2+}, the assay is a measure of the activity of unadenylylated subunits only. Without added Mg^{2+} the assay is a measure of total glutamine synthetase activity independent of adenylylation state (23). Thus the ratio of activities, $+Mg^{2+}/-Mg^{2+}$, provides an approximate measure of the degree of adenylylation of the enzyme; this ratio decreases as adenylylation increases. (The activity of wild-type *Salmonella* extracts from cells grown on minimal medium was reproducibly stimulated by addition of Mg^{2+}. Thus these extracts apparently had more "unadenylylated" activity than "total" activity. This was due to a 40 to 50% stimulation of the activity of unadenylylated wild-type enzyme in the presence of Mg^{2+} [S. Bancroft and S. Kustu, unpublished data]. Similar results were reported for wild-type *K. aerogenes* extracts [18].)

SVP treatment. Extract (0.5 ml) was treated with 0.05 ml of SVP at 37 C for 60 min and was then assayed for γ-glutamyl transfer activity. Extract (0.5 ml) to which 0.05 ml of water was added was incubated similarly and used as the control. SVP activity was checked with bis-*p*-nitrophenylphosphate as substrate and was between 1 and 1.5 U/ml.

Heat treatment. Crude extracts were prepared in buffer containing 10 mM $MgCl_2$ and 150 mM KCl in 10 mM imidazole-hydrochloride, pH 7. Samples (0.5 ml) were heated at 65 C for various times. They were then placed in ice and assayed for glutamine synthetase activity at 37 C. The heat lability of both wild-type and mutant enzymes is markedly dependent on the composition of the extraction buffer. The wild-type enzyme is completely stable to heating for 30 min at 65 C in the extraction buffer normally used for glutamine synthetase assays (see above); stability is due to the presence of 1 mM $MnCl_2$, which also stabilizes the *E. coli* enzyme (7).

Glutamate dehydrogenase assays. Glutamate dehydrogenase activity was determined by measuring oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) at 29 C. Reaction mixtures contained 0.25 mM NADPH, 5 mM α-ketoglutarate, 5 mM NH_4Cl, and 50 mM Tris-hydrochloride, pH 7.8.

RESULTS

Selection procedure and isolation of mutants. Strains with spontaneous mutations affecting glutamine synthetase activity were derived from histidine auxotrophs (containing *hisFΔ645*) by selecting for ability to grow on D-histidine as the sole histidine source (see Materials and Methods). These strains are distinguished from other D-histidine utilizers (1, 11) by the fact that they grow poorly on L-histidine as the sole histidine source and require glutamine for optimal growth. In Fig. 1, growth of one such mutant strain, TA2190 (*gln-54 hisFΔ645*), is compared with that of its parent strain, TA831 (*hisFΔ645*). On a minimal medium containing 0.3 mM L-histidine the mutant had a doubling time of 165 min, about three times longer than that of its parent (doubling time, 50 min). If the medium was supplemented with 1 mM glutamine, the mutant had a doubling time of 60 min, much closer to that of its parent. The doubling time of the mutant on minimal medium containing D-histidine as sole histidine source was the same as that on medium containing L-histidine. (The parent, TA831, was unable to grow on D-histidine.) Table 2 summarizes growth data for these strains and several other spontaneous mutants derived by the same selection procedure; some of the mutants did not show detectable growth on either L-histidine or D-histidine when tested in liquid medium.

Assay of glutamine synthetase activity. Glutamine synthetase activity was assayed in crude extracts of these mutants as described in Materials and Methods (Table 3). The mutants so far characterized are of two types. In some strains (SK10, TA2192, TA2193), total gluta-

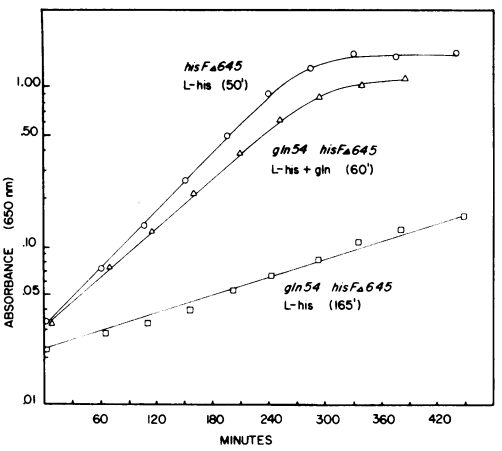


FIG. 1. *Growth of a strain with a mutation affecting glutamine synthetase, TA2190 (gln-54 hisFΔ645), and its parent, TA831 (hisFΔ645), on minimal medium containing 0.3 mM L-histidine or 0.3 mM L-histidine plus 1 mM glutamine. Doubling times are given in parentheses.*

TABLE 2. *Doubling times of strains with mutations affecting glutamine synthetase on minimal medium containing various supplements*[a]

Strain	Relevant genotype	Doubling time (min) on medium supplemented with:		
		L-Histidine (0.3 mM)	L-Histidine (0.3 mM) + glutamine (1 mM)	D-Histidine (0.3 mM)
TA831	*hisFΔ645*	50	49	—[b]
SK10	*gln-52 hisFΔ645*	—[c]	83	—
TA2192[d]	*gln-55 hisFΔ645*	—	68	—
TA2193[d]	*gln-56 hisFΔ645*	—	72	—
TA2190	*gln-54 hisFΔ645*	165	60	165
TA2191	*gln-54*	150	63	
TA2188	gln-53 *hisFΔ645*	165	79	150

[a] Medium and growth conditions are described in Materials and Methods. Growth temperature was 37 C.

[b] TA831 grows to an A_{650} of 0.1 to 0.2 on residual L-histidine from the inoculum; it reaches the same A_{650} in medium to which no histidine has been added. TA2190 and TA2188, mutants which grow on D-histidine, reach a final A_{650} of 1.4.

[c] Indicates no growth in liquid medium. On solid medium with the same supplement, the strain forms tiny colonies.

[d] The parent of this strain, TA1646, has the same doubling times as TA831.

mine synthetase activity was less than 10% of parent activity. In others strains (TA2190, TA2188, SK9), there was significant glutamine synthetase activity, but the ratio of activities in the presence of different divalent cations was drastically altered. The change in response to divalent cations suggested that adenylylation of the mutant enzymes was increased or that the glutamine synthetase subunit was altered in a way that mimicked an increase in adenylylation. Extracts of both types of mutants were mixed with parent extracts and assayed under the conditions indicated in Table 3. Activities were additive, indicating that changes in activity of mutant enzymes could not be explained by the presence of diffusible inhibitors or activators in the mutant extracts. Correlation of results in Tables 2 and 3 indicates that the mutants with low total glutamine synthetase activity are those that do not show detectable growth in liquid medium without added glutamine. (These mutants do form tiny colonies on solid medium without added glutamine.)

A spontaneous glutamine-independent revertant of strain TA2190 and a revertant of strain TA2192 had normal glutamine synthetase activity. These revertants were no longer able to grow on D-histidine, indicating that the change in glutamine synthetase activity and D-histidine growth are the result of a single mutation. Strains TA2190 and TA2192 were transduced to glutamine independence as described in Materials and Methods. Twenty glutamine-independent transductants of each mutant were tested for ability to grow on D-histidine and all had lost this ability, confirming the conclusion that altered glutamine synthetase activity and D-histidine growth are the result of a single mutation.

The mutants described in Table 3 were transduced to histidine independence. As expected, alterations in glutamine synthetase activity persisted in histidine prototrophs (strain TA2191, Table 3).

Characterization of glutamine synthetases with altered response to divalent cations. Mutant glutamine synthetases with an altered response to divalent cations were further characterized to determine whether they were in a highly adenylylated form. Tronick et al. (25) have shown that adenylyl groups can be cleaved

TABLE 3. *Glutamine synthetase activity*[a]

Strain	Relevant genotype	Activity[b]		
		$-Mg^{2+}$	$+Mg^{2+}$	Ratio $+Mg^{2+}/-Mg^{2+}$
TA831	*hisFΔ645*	0.42	0.58	1.37
SK10	*gln-52 hisFΔ645*	0.004	0.004	
TA2192[c]	*gln-55 hisFΔ645*	0.01	0.01	
TA2193[c]	*gln-56 hisFΔ645*	0.01	0.01	
TA2190	*gln-54 hisFΔ645*	1.0	0.06	0.06
TA2191	*gln-54*	0.79	0.05	0.06
TA2188	*gln-53 hisFΔ645*	0.27	0.11	0.42
SK9	*gln-51 hisFΔ645*	0.18	0.01	0.05

[a] Activity was measured in crude extracts using the γ-glutamyl transfer assay described by Stadtman et al. (21) (see Materials and Methods). In the absence of Mg^{2+}, the assay is a measure of total glutamine synthetase activity independent of adenylylation state. In the presence of Mg^{2+}, the assay is a measure of the activity of unadenylylated subunits only. Thus the ratio of activities, $+Mg^{2+}/-Mg^{2+}$, provides an approximate measure of the degree of adenylylation of the enzyme. For wild-type *Salmonella* enzyme this ratio has varied from about 0.5 for (partially) adenylylated enzyme to 1.5 for unadenylylated enzyme (see data in Table 4).

[b] Expressed as micromoles of γ-glutamyl hydroxamate formed per minute per milligram of protein. Activity in blanks without adenosine diphosphate and arsenate was less than 10% of wild-type activity. No correction was made for the blank.

[c] Activity of the parent of this strain, TA1646, was the same as that of TA831.

from *Salmonella* glutamine synthetase by treating it with SVP. The cleavage can be monitored by assaying activity of glutamine synthetase in the presence of different divalent cations (assay conditions in Table 3); as adenylyl groups are cleaved, activity in the presence of Mg^{2+} increases. Treatment of mutant enzymes with SVP had no effect on their activity (Table 4). By contrast, treatment of adenylylated wild-type enzyme, prepared as described in Materials and Methods, increased its activity in the presence of Mg^{2+} (Table 4). Since phosphodiesterase treatment did not affect mutant enzymes and did affect adenylylated wild-type enzyme, we conclude that the mutant enzymes are probably composed of an altered glutamine synthetase subunit. (The *E. coli* glutamine synthetase is composed of 12 identical subunits [reviewed in 7].)

To show in an independent way that glutamine synthetases with an altered response to divalent cations are composed of an altered subunit, it was demonstrated that the enzyme from strain TA2190 (*gln54 hisFΔ645*) is more labile to heating at 65 C than either the adenylylated or unadenylylated form of the wild-type enzyme (Fig. 2). After heating at 65 C for 30 min, the mutant enzyme retained only 4% of its initial activity (assayed at 37 C) whereas the wild-type enzyme retained 45% (unadenylylated form) or 80% (adenylylated form) activity. Heat inactivation of a mixture of mutant and wild-type extracts was intermediate between that of the individual extracts.

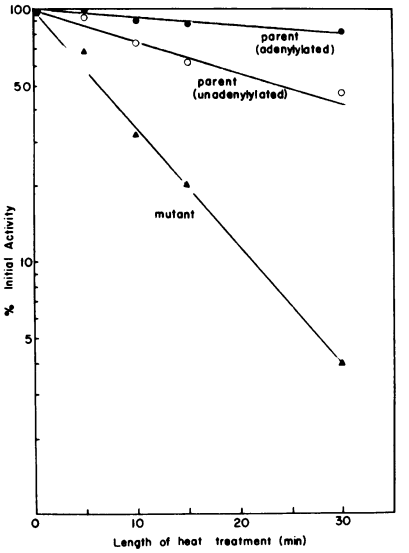


FIG. 2. *Heat lability of glutamine synthetase from a mutant strain TA2190 (gln-54 hisFΔ645) and its parent TA831 (hisFΔ645). Extracts were heated at 65 C and were assayed for activity at 37 C. Initial activities were 3.1 μmol/min per ml for unadenylylated wild-type enzyme, 1.8 μmol/min per ml for adenylylated wild-type enzyme, and 2.1 μmol/min per ml for mutant enzyme. (The ratio of activities +Mg^{2+}/-Mg^{2+} was 1.35 for unadenylylated wild-type enzyme and 0.41 for adenylylated wild-type enzyme.)*

TABLE 4. *Effect of SVP on the activity of wild-type and mutant glutamine synthetases*[a]

Strain	Activity (μmol/min per mg of protein)		
	$-Mg^{2+}$	$+Mg^{2+}$	Ratio $+Mg^{2+}/-Mg^{2+}$
TA831			
Before SVP	0.42	0.58	1.37
After SVP	0.40	0.56	1.42
TA831[c] citrate-glutamate grown			
Before SVP	1.6	1.4	0.89
After SVP	1.4	2.1	1.5
TA2190			
Before SVP	1.0	0.06	0.06
After SVP	1.0	0.07	0.07
TA2188			
Before SVP	0.27	0.11	0.42
After SVP	0.27	0.12	0.45

[a] γ-Glutamyl transfer activity was measured in crude extracts before and after treatment with SVP (21, 23) as described in Materials and Methods. See footnote *a* to Table 3 for the meaning of activities in the presence and absence of Mg^{2+}.

[c] TA831 was grown on a minimal medium with citrate as carbon source and glutamate as sole nitrogen source. Glutamine synthetase is (partially) adenylylated under these growth conditions (10).

Characterization of glutamine synthetases from strains with a heat-sensitive glutamine requirement. Activity of glutamine synthetase from TA2193, a strain with a heat-sensitive glutamine requirement, was assayed at various temperatures to see whether catalytic function of the enzyme was heat sensitive. An extract prepared from cells grown at the permissive temperature (20 C) had normal glutamine synthetase activity at assay temperatures between 20 and 43 C. In addition, mutant and wild-type enzymes lost activity at the same rate when extracts were heated at 65 C. These results indicate that catalytic function of the mutant enzyme is not heat sensitive and suggest that heat sensitivity in vivo might be due to heat-sensitive synthesis of the enzyme.

In an attempt to correlate a heat-sensitive glutamine requirement in vivo with heat sensitivity of glutamine synthetase in vitro, glutamine-independent revertants of SK10, a mutant with low glutamine synthetase activity, were isolated at 23 C. These revertants were

tested for a glutamine requirement at 37 C, and the activity of glutamine synthetase from two revertants with a heat-sensitive glutamine requirement was studied. Extracts of one revertant, SK42 (prepared from cells grown at the permissive temperature), had normal glutamine synthetase activity at 23 and 37 C. However, the activity was more labile to heating at 65 C than that of the wild-type enzyme (data not shown). This suggests a structural alteration of glutamine synthetase in this revertant which can be correlated with its heat-sensitive glutamine requirement.

Extracts of a second revertant, SK43, had no detectable glutamine synthetase activity at either 23 or 37 C. In addition, glutamine synthetase activity could not be detected in whole cells of this revertant made permeable by toluene treatment. (Activity in toluene-treated cells of a wild-type strain is equivalent to that in an extract.) Since the second mutation introduced in this revertant was very closely linked on the chromosome to the original mutation (see below), it seems likely that the revertant had regained glutamine synthetase activity in the biosynthetic reaction. Failure to detect activity may have been due to the fact that the altered enzyme does not function in the γ-glutamyl transfer assay at any temperature.

Assay of glutamate dehydrogenase activity. Glutamate dehydrogenase activity was assayed in crude extracts of the glutamine-requiring mutants as described in Materials and Methods. The activity of this enzyme was normal in mutants having both types of alterations in glutamine synthetase activity (Table 5).

Genetic studies. To determine whether mutations causing the two types of changes in glutamine synthetase activity were closely linked on the *Salmonella* chromosome, transductional crosses between strains with different mutations were performed as described in Materials and Methods. The number of glutamine-independent transductants obtained by infecting mutant recipients with P22 phage grown on other mutants was much smaller than the number obtained by infecting them with phage grown on a wild-type strain (Table 6, column 3). Results are corrected for different transducing efficiencies of the phage preparations in column 5. They indicate that the mutations affecting glutamine synthetase activity are closely linked on the *Salmonella* chromosome. Mutations in strains TA2190, TA2192, and SK10 are 80 to 90% linked by transduction to the mutation in strain TA2193, a strain with a heat-sensitive glutamine requirement. Mutations in strains SK42 and SK43, revertants of strain SK10 with a heat-sensitive glutamine requirement, are closely linked by transduction to the original mutation in strain SK10; when phage grown on the revertants is used to transduce strain TA2190 to glutamine independence, 90% of the transductants inherit the heat-sensitive phenotype of the donor.

Berberich (3) reported that the glutamine synthetase structural gene lies between the *metE* and *ilv* genes in *E. coli*. These genes lie at

TABLE 5. *Glutamate dehydrogenase activity*

Strain	Relevant genotype	Glutamate dehydrogenase activity[a]
TA831	*hisFΔ6645*	0.33
SK10	*gln-52 hisFΔ645*	0.29
TA2192	*gln-55 hisFΔ645*	0.23
TA2193	*gln-56 hisFΔ645*	0.25
TA2190	*gln-54 hisFΔ645*	0.34
TA2188	*gln-53 hisFΔ645*	0.33
SK9	*gln-51 hisFΔ645*	0.21

[a] Expressed as micromoles of NADPH oxidized per minute per milligram of protein at 29 C.

TABLE 6. *Transductional crosses of strains with gln⁻ mutations*[a]

Recipient	Donor phage	No. of Gln⁺ transductants	No. of Pur⁺ transductants[b] (recipient *purFΔ145*)	Ratio Gln⁺ transductants/Pur⁺ transductants[b]
TA2191[c]	TA1772 (wild-type)	348	376	0.93
	TA2191	1[d]	1664	0.001
	TA2188	8	800	0.01
	TA2192	10	1300	0.01
	TA2193	12	388	0.03
TA2192[e]	TA1772	195	200	0.98
	TA2191	12	596	0.02
	TA2188	17	112	0.15
	TA2192	4[d]	294	0.01

[a] TA2191 and TA2192 were transduced to glutamine independence with P22 phage grown on a wild-type strain or on strains with *gln⁻* mutations (see text).

[b] All transducing phage preparations were used to transduce *purFΔ145* to purine independence, and the ratio of Gln⁺/Pur⁺ transductants was determined to correct for differences in transducing efficiency of different phage preparations.

[c] Crosses were done at 37 C.

[d] Probably revertants.

[e] Crosses were done at 23 C since there is less background growth of the recipient at this temperature.

min 122 in *Salmonella*. With P22 phage, the *gln-54* mutation is not cotransducible with *metE* (0/175) or *ilv* (0/100). In addition it is not cotransducible with *cya* (0/300), which lies about halfway between *metE* and *ilv* is cotransducible with both of them (*cya* is 7% cotransducible with *metE* and 3.5% cotransducible with *ilv*). These results indicate that the *gln-54* mutation (and hence the other *gln* mutations discussed here) do not lie between *metE* and *ilv* on the *Salmonella* chromosome.

The approximate location of the *gln*$^-$ mutations on the *Salmonella* chromosome was determined by conjugation mapping. A donor carrying the *gln-54* mutation was prepared as described in Materials and Methods and was mated with a series of recipients carrying single auxotrophic mutations spaced at about 15-min intervals around the chromsome. The percentage of recombinants requiring glutamine for optimal growth was scored (Fig. 3). There was a peak of co-recombination with *argA* at 128 min, indicating that the *gln*$^-$ mutations lie in this region of the chromosome.

To locate the *gln*$^-$ mutations more precisely, a three-factor cross was performed by mating strain JL400 (*purC7 his*$^-$ HfrA) with strain SK41 (*ilv-8 gln-59 rif*$^-$ *ara-9*) for 40 min in liquid culture and selecting recombinants (*ara*$^+$) for the distal marker. Those *ara*$^+$ recombinants, which were also *rif*$^+$, were scored for inheritance of proximal markers (*gln*$^+$ and *ilv*$^+$). The results of the cross (Table 7) indicated that *gln-59* lies between *ilv* (122 min) and *rif* (128 min). The location of the *gln-59* mutation was estimated by a gradient of transmission experiment in which the same strains were mated and recombinants (*ilv*$^+$) for the proximal marker were selected. These recombinants were scored for co-inheritance of distal markers (*gln*$^+$ and *rif*$^+$). By plotting the logarithm of the number of *ilv*$^+$ (341) and *rif*$^+$ (38) recombinants as a function of map distance and locating the number of *gln*$^+$ (122) recombinants on this plot, it was estimated that *gln* lies at 125 min. (Of the 38 *ilv*$^+$ *rif*$^+$ recombinants obtained in this cross, 37 were also *gln*$^+$, as expected if *gln* is the middle marker.) Similar results were obtained from a mating of strain SK30 (*gln-59 thy-1863* HfrK5) with strain SK14 (*ilv-8 metA309 ara-9*).

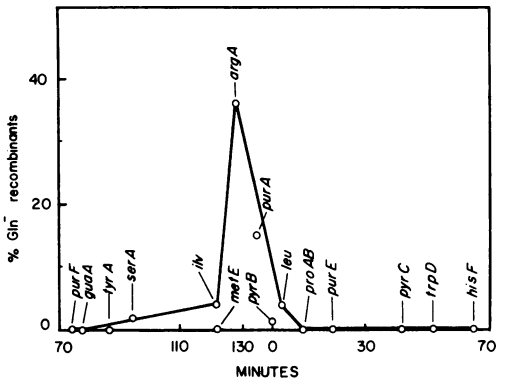


FIG. 3. *Mapping glnA by conjugation. SK8 (gln-54 cysA2502 HfrK5) was mated with strains containing single auxotrophic mutations as indicated. Matings were done directly on minimal agar plates containing glutamine (0.5mM), and 100 recombinants from each cross were tested for co-inheritance of the gln*$^-$ *mutation. The origin of transfer for HfrK5 is around 70 min and purF is donated early. The peak of co-inheritance is unusually sharp, probably because the glutamine concentration in plates used for matings was limiting and this caused selective pressure against inheritance of the gln*$^-$ *mutation.*

TABLE 7. *Mapping of glnA relative to neighboring markers*

Cross	Recipient	Donor Hfr	Selected marker	No. of recombinants scored	Unselected markers	% Co-inheritance
1	SK41	JL400[a]	*ara*$^+$	143	*rif*$^+$ *gln*$^+$ *ilv*$^+$	19.5
					rif$^+$ *gln*$^-$ *ilv*$^+$	0.7
					rif$^+$ *gln*$^+$ *ilv*$^-$	8.4
					rif$^+$ *gln*$^-$ *ilv*$^-$	71
2	SK41	JL400	*ilv*$^+$	38	*rif*$^+$ *gln*$^+$	97
					rif$^+$ *gln*$^-$	2.6

[a] Matings were carried out as described in Materials and Methods. JL400 contains HfrA and donates *ilv* as an early marker. The gene order indicated by the crosses is

Hfr origin - - *ilv*$^+$ *gln*$^+$ *rif*$^+$ *ara*$^+$ donor

recipient

ilv$^-$ *gln*$^-$ *rif*$^-$ *ara*$^-$
(122 min) (128 min) (3 min)

There was no (0/200) P22-mediated cotransduction of *gln-59* with the following markers: *purA* (135 min), *malB* (130 min), *metA* and *argA* (128 min), and *rha* (127 min).

DISCUSSION

Mutant strains of *S. typhimurium* with altered glutamine synthetase activity were derived from a histidine auxotroph by selecting positively for growth on D-histidine as the sole histidine source. Spontaneous glutamine-requiring mutants isolated by this selection procedure have two types of alterations in glutamine synthetase activity: some have less than 10% of parent activity, whereas others have activity with an altered response to divalent cations. The following evidence indicates that both types of mutations are in the structural gene for the glutamine synthetase subunit (*glnA*).

(i) A mutant enzyme (from strain TA2190) with altered response to divalent cations is more labile to heat than either the adenylylated or unadenylylated form of the wild-type enzyme (Fig. 2). This indicates that the mutant enzyme is composed of an altered glutamine synthetase subunit.

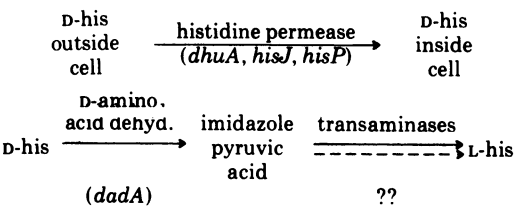
(ii) Mutant enzymes with altered response to divalent cations have activity like that of highly adenylylated ("inactive") wild-type enzyme. However, they are not sensitive to treatment with SVP, which de-adenylylates wild-type enzyme and converts it to a "more active" form (see Table 4). This suggests that the mutant enzymes are composed of an altered glutamine synthetase subunit that mimics the activity of adenylylated wild-type subunits.

(iii) A spontaneous revertant (strain SK42) of a mutant with low glutamine synthetase activity has a heat-sensitive glutamine requirement and heat-labile glutamine synthetase activity (data not shown).

(iv) Mutations in all strains characterized are closely linked on the *Salmonella* chromosome, which is consistent with their being in the same gene. This gene (*glnA*) lies at about min 125 on the *Salmonella* chromosome. (TA2193, a strain with a heat-sensitive glutamine requirement, may have a mutation in a regulatory gene which is closely linked to the structural gene, *glnA* [see results].)

The selection procedure described is specific for strains with decreased or altered glutamine synthetase activity, in contrast to those completely lacking activity, because glutamine is not provided in the selection plates. Thus, most mutants so far characterized probably have missense mutations in the glutamine synthetase structural gene. Altered glutamine synthetase enzyme from such mutants should be useful in further correlating the structure of this complex enzyme with its biosynthetic and possible regulatory functions. Cimino et al. (5) have demonstrated that chemical modification of glutamine synthetase by acetylation or nitration produces effects analogous to those of enzymatic modification by adenylylation. In one type of mutant described here, alteration of the glutamine synthetase subunit apparently has similar effects, insofar as it causes a change in divalent ion specificity of the enzyme in the γ-glutamyl transfer assay and a glutamine requirement of the organism in vivo.

The basis for D-histidine utilization by strains with mutations affecting glutamine synthetase has not been established. Present knowledge of the pathway of D-histidine utilization is summarized in the following reaction sequence.



There is genetic and biochemical evidence that D-histidine enters the cell through the high-affinity histidine permease (1, 11) and that it is converted to imidazole pyruvic acid by a D-amino acid dehydrogenase with broad substrate specificity (*dadA* gene product) (27). Several transaminases catalyze conversion of imidazole pyruvic acid to L-histidine in vitro (T. Klopotowski, personal communication), but there is no genetic evidence to establish which of them catalyze this reaction in vivo. Both the transport system and the racemizing enzymes are synthesized constitutively but not at high enough levels to enable a histidine auxotroph to grow on D-histidine as sole histidine source (11). Consistent with this, a previously characterized class of D-histidine utilizers (*dhuA*) has elevated transport activity (about 10-fold) (1, 11). Strains with a *dhuA* mutation have a normal 50-min doubling time on minimal medium containing either L-histidine or D-histidine as sole histidine source.

We hypothesize that mutations affecting glutamine synthetase activity may result in a regulatory increase in the levels of the D-histidine racemizing enzymes. The levels of enzymes such as D-amino acid dehydrogenase may be elevated when the glutamine pool is low because

they catalyze release of ammonia that can then be utilized for glutamine synthesis. One observation suggests that D-histidine growth is mediated by a low-glutamine pool and not by a change in the structure of glutamine synthetase. Feeding glutamine to strains with *glnA* mutations inhibits their growth on D-histidine (data not shown); that is, feeding glutamine phenotypically reverses the effect of the mutation. (Glutamine does not inhibit growth of a strain with a *dhuA* mutation on D-histidine.) We are presently checking levels of D-histidine racemizing enzymes in mutant cultures grown in the absence of glutamine to see if these levels are elevated.

Magasanik and his co-workers (12) have investigated the effects of glutamine synthetase on regulation of other enzymes involved in glutamate production in *K. aerogenes*. Glutamine synthetase is elevated under conditions of ammonia limitation, consistent with its role in glutamate synthesis in conjunction with glutamate synthase. Under conditions of ammonia limitation, glutamate dehydrogenase is repressed. Brenchley et al. (4) and Magasanik et al. (12) postulate that glutamine synthetase is directly responsible for repression of glutamate dehydrogenase. Mutants lacking glutamine synthetase activity have elevated levels of glutamate dehydrogenase, which is elevated physiologically in response to the availability of excess ammonia. By contrast, we find that *Salmonella* mutants with low glutamine synthetase activity have normal glutamate dehydrogenase activity (Table 5). This is consistent with the observation of Miller and Stadtman (15) that the level of glutamate dehydrogenase in *E. coli* does not vary with the ammonia concentration of the growth medium.

Magasanik et al. (12) and Prival et al. (18) proposed that glutamine synthetase itself is a positive regulatory element for synthesis of enzymes that produce glutamate from other amino acids. Thus, under conditions of ammonia limitation, enzymes such as histidase and proline oxidase can be induced in the presence of glucose (16, 19). Mutants lacking glutamine synthetase activity cannot induce these enzymes in the presence of glucose (18). We believe that *Salmonella* mutants with low glutamine synthetase activity may have elevated levels of the D-histidine racemizing enzymes. Whatever the effect that allows these mutants to utilize D-histidine as a histidine source, it appears to be reversed by feeding them glutamine. Thus, glutamine rather than glutamine synthetase may mediate the presumed regulatory effect that allows D-histidine utilization in *Salmonella*.

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