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Temperature-Sensitive Glutamate Dehydrogenase Mutants of *Salmonella typhimurium*

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Mutants of *Salmonella typhimurium* defective in glutamate dehydrogenase activity were isolated in parent strains lacking glutamate synthase activity by localized mutagenesis or by a general mutagenesis combined with a cycloserine enrichment for glutamate auxotrophs. Two mutants with temperature-sensitive phenotypes had glutamate dehydrogenase activities that were more thermolabile than that of an isogenic control strain. Eight other mutants had less than 10% of the wild-type glutamate dehydrogenase activity. All the mutations were cotransducible with a Tn*10* element (*zcd-2*::Tn*10*) located at approximately 23 U on the *S. typhimurium* linkage map. These data strongly indicate that this region contains the structural gene (*gdhA*) for glutamate dehydrogenase.

In *Salmonella typhimurium* (10), as in other enteric microorganisms, e.g., *Escherichia coli* (2) and *Klebsiella aerogenes* (5, 6), there are two enzymes that can independently form glutamate; glutamate dehydrogenase (GDH) (EC 1.4.1.4) and glutamate synthase (EC 2.6.1.53). Since either activity produces sufficient glutamate when cells are grown with excess ammonia, the simultaneous loss of both is necessary to cause a glutamate requirement. However, for growth with either limiting ammonia or with slowly degraded nitrogen sources, such as arginine or proline, glutamate synthase activity is required. Thus, mutants lacking glutamate synthase have a discernible phenotype (Asm^-). Mutants lacking only GDH activity, however, are indistinguishable phenotypically from wild-type strains.

Mutants of *E. coli* (2, 20), *K. aerogenes* (1, 13) and *S. typhimurium* (10) lacking glutamate synthase or glutamate dehydrogenase activity or both have been isolated, but it is not known whether these mutations identify structural genes or regulatory genes. The control of GDH in *S. typhimurium* is of particular interest because there is substantial expression of this enzyme activity in cells grown in a variety of media, yet the enzyme appears to be unnecessary for growth (4, 10). No regulatory mutants have been characterized, and little is known about the physiological signal(s) or the mechanism(s) controlling its synthesis.

Glutamate dehydrogenase enzymes purified from *S. typhimurium* (9) and *E. coli* (21) have similar molecular weights. Data for the *E. coli* GDH indicate that it probably consists of six identical subunits (50,000 molecular weight), suggesting that there is only one gene encoding GDH. In *S. typhimurium*, mutations at either of two sites on the chromosome resulting in loss of GDH activity have been described. The mutation identified by Ortega and Aguilar is cotransducible with *metB* (19) and is located at 88 U on the *S. typhimurium* linkage map (22), whereas the *gdh-51* mutation obtained in our laboratory (10) is located near the *pyrC-purB* region (22 to 25 U). Although Ortega and Aguilar isolated the mutant as a glutamate auxotroph, they did not measure glutamate synthase activity, and it is unclear whether this mutation affects only GDH activity or both glutamate biosynthetic enzymes. Previous results indicate that the loss of GDH alone would not result in glutamate auxotrophy (10).

To identify the structural gene for GDH, we have isolated mutants of *S. typhimurium* with an altered thermolability for GDH activity. We describe the isolation and characterization of these and other auxotrophs and show that the mutations are contransducible with a Tn*10* insertion, *zcd-2*::Tn*10*, located at approximately 23 U on the linkage map (10). These data strongly suggest that it is this region that carries the structural gene for GDH.

MATERIALS AND METHODS

Bacterial strains. The *S. typhimurium* LT2 strains are listed in Table 1. Three strains with low glutamate synthase activity (Asm^-), JB665 (*asm-102*) (10), JL785 (*asm-103*) and JB1462 (*asm-103*), were used to isolate mutants with altered GDH activity. Strain JL785, part of our laboratory collection, has an Asm^- phenotype and contains less than 6% of the glutamate synthase activity of an isogenic control strain. Strain JB1075 contains a transposable tetracycline resistance element, *zcd-2*::Tn*10*, which is 95% linked by KB1-mediated transduction with *gdh-51*

TABLE 1. *Genotype and origin of bacterial strains*

Strain	Genotype	Source (reference)
JB665	*galE hutR49 asm-102*	(10)
JB1075	*galE hutR49 asm-102 zcd-2*::Tn*10*	(10)
JL785	*glpD4 glpR1 mal asm-103*	Laboratory collection
JB1462	*glpD4 glpR1 mal asm-103 galE*	Spontaneous FO^r[a] mutant of JL785
JB1487	*glpD4 glpR1 mal asm-103 gdh-52 zcd-2*::Tn*10*	Localized mutagenesis with JL785 as recipient
JB1488	*glpD4 glpR1 mal asm-103 gdh-53 zcd-2*::Tn*10*	Localized mutagenesis with JL785 as recipient
JB1489	*glpD4 glpR1 mal asm-103 gdh-54 zcd-2*::Tn*10*	Localized mutagenesis with JL785 as recipient
JB1490	*glpD4 glpR1 mal asm-103 gdh-55 zcd-2*::Tn*10*	Localized mutagenesis with JL785 as recipient
JB1491	*glpD4 glpR1 mal asm-103 gdh-56 galE*	EMS[b] mutagenesis of JB1462
JB1493	*glpD4 glpR1 mal asm-103 gdh-58 galE*	EMS mutagenesis of JB1462
JB1496	*glpD4 glpR1 mal asm-103 gdh-61 galE*	EMS mutagenesis of JB1462
JB1582	*galE hutR49 asm-102 gdh-62 zcd-2*::Tn*10*	Localized mutagenesis with JB665 as recipient
JB1583	*galE hutR49 asm-102 gdh-63 zcd-2*::Tn*10*	Localized mutagenesis with JB665 as recipient
JB1584	*galE hutR49 asm-102 gdh-64 zcd-2*::Tn*10*	Localized mutagenesis with JB665 as recipient
JB1585	*galE hutR49 asm-102 gdh-65 zcd-2*::Tn*10*	Localized mutagenesis with JB665 as recipient
JB1586	*galE hutR49 asm-102 gdh-66 zcd-2*::Tn*10*	Localized mutagenesis with JB665 as recipient

[a] FO^r, resistance to FO phage.
[b] EMS, ethyl methane sulfonate.

(10). As the designation *zcd* indicates (8), the transposon is located at approximately 23 min on the *S. typhimurium* linkage map (10).

Media. The Luria broth and the glucose minimal media have been described previously (3). When glutamate auxotrophs were grown in minimal medium, it was supplemented with L-glutamate (4 mg/ml) or L-aspartate (1 mg/ml), or both. Tetracycline, when used, was added at 50 μg/ml. The inability to grow on medium containing glucose as the carbon source and arginine as the nitrogen source (glucose-arginine medium) was used to score the Asm$^-$ phenotype.

Mutagenesis and mutant isolation. Localized mutagenesis with P22HT*105/int* was by the procedure of Hong and Ames (14). A phage lysate was prepared on JB1075 (*zcd-2*::Tn*10*), mutagenized with hydroxylamine for 8 h, and used to transduce either JB665 or JL785 to Tet^r. The selected recombinants were then scored at 25, 37, and 43°C for glutamate auxotrophy. Ethyl methane sulfonate mutagenesis of JB1462 (a *galE* derivative of JL785) was performed as described by Miller (18), and cycloserine counterselection was used to enrich for glutamate auxotrophs.

Scoring mutants for temperature sensitivity. Strain JB665 (*asm-102*) is temperature sensitive for the Asm phenotype (grows slowly at 37°C, but not at 40°C on glucose-arginine) so that derivatives with *gdh* mutations could grow slowly without glutamate at 37°C, irrespective of the nature of the *gdh* mutation, owing to partial activity of glutamate synthase. Therefore, mutants lacking GDH activity were initially identified by scoring for a Glt$^-$ phenotype at 40°C. *gdh* mutations were scored for temperature sensitivity by transducing them into JB1462 (*asm-103*). Since the *asm-103* mutation is not temperature sensitive, the Glt phenotype in this background is an indication of the temperature-sensitive nature of the *gdh* mutation.

Growth of cells and GDH assays. The procedures for growing cells and preparing crude extracts by sonic oscillation have been described previously (12). The GDH assays measured the rate of oxidation of NADPH at 37°C by the procedure of Meers et al. (17), with the modifications described previously (10). Proteins were determined by the method of Lowry et al. (15), with bovine serum albumin as the standard. Specific activity is reported as nanomoles of product formed per minute per milligram of protein. The results are averages of at least three individual experiments with duplicate assays for each. The whole-cell rapid assay for GDH activity followed the procedure of Pahel et al. (20), except that the pH of the buffer was 8.1.

Thermolability studies. Cultures were grown at a permissive temperature (30°C) in glucose minimal medium supplemented with glutamate, and extracts were prepared by sonic oscillation. Since Coulton and Kapoor (9) found the GDH of *S. typhimurium* to be stable to 55°C, we chose this temperature for heat lability studies. Samples (60 μl) of crude extract were heated for the designated time periods, rapidly chilled, and maintained at 4°C for at least 5 min before being assayed.

RESULTS AND DISCUSSION

Mutant isolation. We previously constructed a strain in which the transposon Tn*10* (*zcd-2*::Tn*10*) is 95% linked by KB1-mediated transduction to a mutation abolishing GDH activity (10). To isolate additional mutants with *gdh* lesions in this region, we used this transposon as a selective marker in a localized mutagenesis. Asm$^-$ strains JB665 (*asm-102*) and JL785 (*asm-103*) were transduced with mutagenized phage grown on the *gdh*$^+$ strain JB1075 that carries the *zcd-2*::Tn*10* element, and Tet^r recombinants were selected and scored. The above procedure selects mutants with lesions in a particular region of the *S. typhimurium* chromosome (approximately 23 U). Since mutations in other regions of the chromosome might cause the loss of GDH activity, we used a second method, ethyl

methyl sulfonate mutagenesis of strain JB1462 (*asm-103*), combined with a cycloserine enrichment to obtain glutamate auxotrophs. We screened the Glt$^-$ mutants by using a rapid assay for GDH activity to eliminate those that required glutamate owing to the loss of a tricarboxylic acid cycle enzyme. Four independent mutants obtained from JL785 and five from JB665 were isolated by using the localized mutagenesis procedure. Three independent mutants were obtained from the cycloserine counterselection.

Temperature sensitivity. The 12 mutants altered in GDH activity were scored for temperature sensitivity on solid medium at 25, 37, and 43°C. Three mutants, JB1488, JB1489, and JB1490, were Glt$^-$ at 43°C but Glt$^+$ at 25 and 37°C (Table 2). Supplementation with glutamate allowed growth at 43°C, showing that the requirement was specific. Precise temperature settings were crucial to score the phenotype accurately; temperatures below 43°C allowed growth without glutamate, and temperatures above interfered with the general growth of the mutants and the parent strain. Strain JB1487 was cold sensitive since it required glutamate at 25°C but grew slowly without glutamate at 37°C (Table 2). The remaining eight mutants showed the same Glt$^-$ phenotype at all three temperatures (data not shown).

Growth characteristics of the heat-sensitive mutants JB1488, JB1489, and JB1490 were studied further. At the permissive temperature (30°C), these three strains had growth rates comparable to that of the parent strain. When a logarithmic-phase culture of JB1490 in glucose minimal medium was shifted from 30 to 42°C at a cell density of 90 Klett units, growth was immediately arrested (data not shown). In similar experiments with strains JB1488 and JB1489, we were unable to demonstrate temperature sensitivity at 42°C in liquid medium, although these strains were strict glutamate auxotrophs on solid media.

GDH activity and heat lability. All eight non-temperature-sensitive mutants had greatly reduced GDH activities (Table 3). When grown at a permissive temperature (30°C), one of the heat-sensitive mutants, JB1490, had GDH activity comparable to that of the parent, whereas the other two, JB1488 and JB1489, had increased activity (Table 2). The glutamate synthase activity in each mutant was comparable to that of the parent (data not shown).

To determine whether the GDH activity of the heat-sensitive mutants was thermolabile, extracts were heated at 55°C and then assayed. For two mutants, heat treatment resulted in a more rapid inactivation of enzyme than that exhibited by the control strain (Fig. 1). The GDH activities of JB1488 and JB1490 were in-

TABLE 3. *GDH activities of Glt$^-$ mutants*

Strain	Phenotype	GDH sp act[a] (nmol min^{-1} mg^{-1})
JB1462 (*asm-103*)	Glt$^+$	406
JB1491 (*asm-103 gdh-56*)	Glt$^-$	41
JB1493 (*asm-103 gdh-58*)	Glt$^-$	5
JB1496 (*asm-103 gdh-61*)	Glt$^-$	4
JB665 (*asm-102*)	Glt$^+$	627
JB1582 (*asm-102 gdh-62*)	Glt$^-$	6
JB1583 (*asm-102 gdh-63*)	Glt$^-$	0
JB1584 (*asm-102 gdh-64*)	Glt$^-$	3
JB1585 (*asm-102 gdh-65*)	Glt$^-$	4
JB1586 (*asm-102 gdh-66*)	Glt$^-$	1

[a] All cultures were grown at 37°C in supplemented glucose minimal medium. The medium for strain JB1462 and its derivatives was supplemented with 0.4% glutamate and 0.1% aspartate; that for strain JB665 and its derivatives was supplemented with 0.4% glutamate.

TABLE 2. *Phenotypes and GDH activities of temperature-sensitive Glt$^-$ mutants*

Strain	Phenotype	Growth on GM[a]			GDH sp act[b] (nmol min^{-1} mg^{-1})		
		25°C	37°C	43°C	Without K$^+$[c]	With K$^+$	Effect of K$^+$
JL785	Glt$^+$	+	+	+	586	1,041	2-Fold stimulation
JB1487	cs[d] Glt$^-$	−	(+)	(+)	204	ND[e]	
JB1488	hs[f] Glt$^-$	+	+	−	1,106	151	7-Fold inhibition
JB1489	hs Glt$^-$	+	+	−	2,29	123	18-Fold inhibition
JB1490	hs Glt$^-$	+	+	−	692	1,130	2-Fold stimulation

[a] +, Growth within 24 h; (+), growth within 36 h; −, no growth within 48 h. GM, Glucose minimal medium.
[b] Cultures were grown at 30°C in GM supplemented with 0.4% glutamate.
[c] 500 mM KCl added to the reaction mixture.
[d] cs, Cold sensitive.
[e] ND, Not determined.
[f] hs, Heat sensitive.

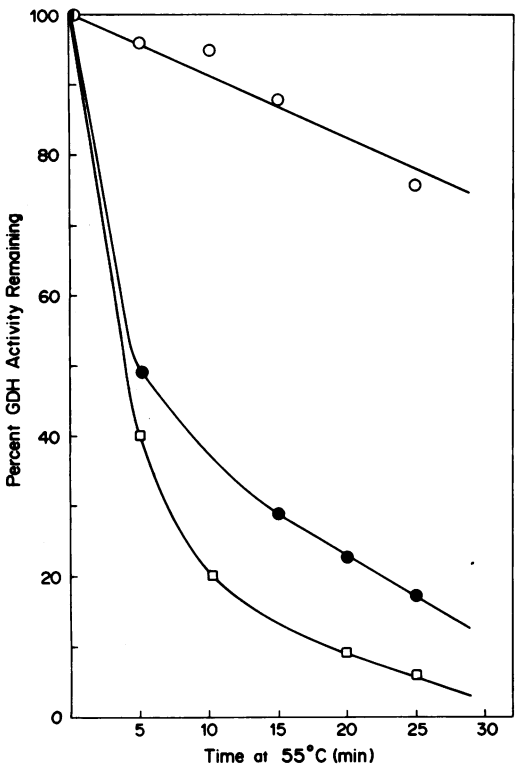

FIG. 1. *Thermolability of GDH measured in crude extract as described in the text. Symbols: ○, JL785 (wild type); ●, JB1488; □, JB1490.*

activated (83 and 94% loss of activity, respectively) within 25 min, whereas there was only a 24% loss of wild-type enzyme activity. The results of mixing experiments demonstrated that the addition of extracts from the mutants did not cause increased heat lability of the wild-type activity (data not shown), excluding the possibility that the mutant extracts contained a temperature-dependent inhibitor of GDH activity. Three heat-sensitive Glt$^-$ transductants obtained from a backcross of JB1490 had GDH activities with the same increased thermolability as their parent, JB1490 (data not shown). The GDH activity of the third heat-sensitive mutant, JB1489, showed no altered thermolability relative to the control, JL785.

Effect of 500 mM K$^+$ on GDH activity. We examined the effect of 500 mM K$^+$ on GDH activity since Measures reported (16) that the activity of this enzyme from several bacterial species is stimulated by a high concentration of K$^+$ ions. The wild-type parent JL785 and one of the mutants, JB1490, showed a twofold stimulation of activity, whereas JB1488 and JB1489 showed substantial inhibition (Table 2).

These data together with the thermolability data indicated that the lesions in all three mutants are within the structural gene for GDH. This conclusion is based on the observation that two heat-sensitive mutants (JB1488 and JB1490) had GDH activity that was more thermolabile that the parent strain. In addition, the GDH activity of one, JB1488, was inhibited, rather than stimulated, by 500 mM K$^+$. A third heat-sensitive mutant, JB1489, although not showing any altered thermolability, also had a GDH activity substantially inhibited by K$^+$ (18-fold). Although rigorous proof requires purification and characterization of these altered enzymes, we suggest that these mutations identify *gdhA*, the structural gene for GDH in *S. typhimurium*.

Mapping of the gdh mutations. By using P22-mediated transduction, all of the mutants obtained by localized mutagenesis were found to have *gdh* lesions tightly linked (93 to 97%) to the *zcd-2*::Tn*10* transposon located at about 23 U (data not shown). The three mutants obtained by the counterselection procedure, which was nonselective with respect to the chromosomal location of their lesions, also had mutations located in this region (86 to 97% linked) (data not shown). We are determining the number of genes in addition to *gdhA* in this region by constructing episomes to use in complementation tests with the *gdh* mutants.

There is uncertainty about the precise locations of the *zcd-2*::Tn*10* element and the *gdhA* locus, owing to a lack of identified genes in the 22- to 33-U region of the *S. typhimurium* linkage map (22). In *E. coli*, mutations abolishing GDH activity are linked by P1-mediated transduction with both *purB* (3%) at 25 U and *trpA* (2%) at 27 U (20). Although the *gdhA* locus in *S. typhimurium* is in this same area, it is not linked with either of these markers by P1-mediated transduction (10). A comparison with the *E. coli* map position is further complicated owing to an extended inversion of this region of the *S. typhimurium* genome relative to that of *E. coli*. Although the exact ends of the inversion are unclear, it encompasses approximately the portion of 25.5 to 36.5 U (22). Recently, 37% contransduction of *gdhA* and *pncA* by P22-mediated transduction has been found (J. W. Foster and S. Dendinger, personal communication and unpublished data). Based on conjugation data, Foster et al. mapped *pncA* at approximately 27 U on the linkage map (11).

Other genes involved in nitrogen metabolism may be clustered in this region. Nit$^-$ mutants described by Broach et al. are unable to use a variety of nitrogen sources (7) and carry lesions

linked to the *zcd-2*::Tn*10* element (10). The biochemical basis for the Nit$^-$ phenotype is unknown. Although these mutants have an Asm$^-$ phenotype, glutamate synthase is not affected (7). Mutants that have an aspartate requirement under certain conditions were isolated from our localized mutagenesis procedure and have lesions in this region also. Further genetic characterization of the *gdh* mutants relative to others with lesions in this region will determine whether there is a functional basis for the location of these genes.

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