THIALYSINE-RESISTANT MUTANT OF *SALMONELLA TYPHIMURIUM* WITH A LESION IN THE *thrA* GENE

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ABSTRACT

A mutant of *Salmonella typhimurium* was selected for its spontaneous resistance to the lysine analog, thialysine (S-2-aminoethyl cysteine). This strain, JB585, exhibits a number of pleiotropic properties including a partial growth requirement for threonine, resistance to thiaisoleucine and azaleucine, excretion of lysine and valine, and inhibition of growth by methionine. Genetic studies show that these properties are caused by a single mutation in the *thrA* gene which encodes the threonine-controlled aspartokinase-homoserine dehydrogenase activities. Enzyme assays demonstrated that the aspartokinase activity is unstable and the threonine-controlled homoserine dehydrogenase activity absent in extracts prepared from the mutant. These results explain the growth inhibition by methionine because the remaining homoserine dehydrogenase isoenzyme would be repressed by methionine, causing a limitation for threonine. The partial growth requirement for threonine during growth in glucose minimal medium may also, by producing an isoleucine limitation, cause derepression of the isoleucine-valine enzymes and provide an explanation for both the valine excretion, and azaleucine and thiaisoleucine resistance. The overproduction of lysine may confer the thialysine resistance.

THIALYSINE (S-2-aminoethyl cysteine) is structurally similar to the amino acid lysine and inhibits the growth of *Salmonella typhimurium* when added to glucose minimal medium. Lysine effectively overcomes this growth inhibition. Because amino acid analogs have proved useful in examining the regulation of other amino acid biosynthetic pathways (ROTH, ANTON and HARTMAN 1966; BRENCHLEY and WILLIAMS 1975), mutants of *S. typhimurium* resistant to thialysine were isolated to elucidate the controls for lysine biosynthesis. One of these strains, JB585, exhibits unusual pleiotropic properties, such as multiple resistance to amino acid analogs, sensitivity to methionine and a requirement for threonine. Since all the characteristics have some relationship to the biosynthesis of amino acids derived from aspartate, the regulatory aspects of these pathways were examined.

As illustrated in Figure 1, lysine, methionine, threonine and isoleucine are derived directly from aspartate; valine is indirectly derived because of the shared enzymes for isoleucine and valine synthesis. The gene-enzyme relationships in

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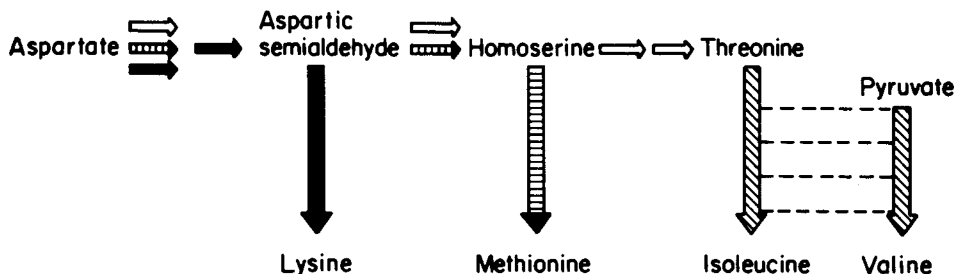


FIGURE 1.—Biosynthetic pathways for some amino acids derived from aspartate. Symbols: solid arrows, enzymes repressed by lysine; open arrows, enzymes repressed by threonine puls isoleucine; cross-marked arrows, enzymes repressed by methionine; diagonally-marked arrows, enzymes repressed by isoleucine, leucine and valine. Modified from THEZE *et al.* (1974).

this pathway have been examined in *Escherichia coli* K12 and three isoenzymes of aspartokinase exist for the first reaction converting aspartate to aspartylphosphate (THÈZE *et al.* 1974; UMBARGER 1969; and BOY and PATTE 1972). Two of these isoenzymes have a second activity, homoserine dehydrogenase, which is necessary for homoserine synthesis (Figure 1). Although the isoenzymes have the same biochemical function, they have distinct regulartory patterns. The aspartokinase-homoserine dehydrogenase I complex (AKI-HSDI) is repressed by threonine and isoleucine and inhibited by threonine, the aspartokinase-homoserine dehydrogenase II (AKII-HSDII) is repressed by methionine, and the aspartokinase III (AKIII), which lacks homoserine dehydrogenase activity, is repressed and inhibited by lysine (THÈZE and SAINT-GIRON 1974; COHEN 1969; THÈZE *et al.* 1974). The three genes encoding these activities are separated on the *E. coli* linkage map (THÈZE *et al* 1974). Although the gene-enzyme relationship has not been established for *S. typhimurium*, preliminary repression and inhibition studies suggest that the regulation is identical to that of *E. coli* and that *S. typhimurium* also has three aspartokinase isoenzymes (COLES and BRENCHLEY 1976).

Growth studies with the thialysine resistant strain, JB585, demonstrated that it has a partial threonine requirement and that methionine inhibits growth in glucose minimal medium. This phenotype suggested that the synthesis or regulation of the homoserine dehydrogenase enzyme was altered (Figure 1). Further characterization shows that the unusual, pleiotropic phenotype of strain JB585 is due to a mutation in the *thrA* gene affecting the aspartokinase and homoserine dehydrogenase activities.

MATERIALS AND METHODS

Strains: Bacterial strains were derived from *S. typhimurium* LT2 and are listed in Table 1.

Media: Both the glucose minimal medium and Luria Broth (LB) complex medium were as described previously (BRENCHLEY 1973). For the genetic analysis of strain JB585 reported in Tables 3 and 4, the minimal medium described by STUTTARD (1972, 1973) was used. Amino acids and the analog supplements were added at concentrations given with each experiment.

Growth: Cultures were incubated overnight at 37° in minimal medium containing 0.02% glucose and the appropriate supplements. These cultures were used to inoculate fresh 0.4%

TABLE 1

Bacterial strains used in this investigation

Strain	Genotype	Relevant phenotype	Source
JL907	*galE hutR49*		J. L. Ingraham
JB585	*galE hutR49 thrA1009*	Threonine bradytroph	Thialysine resistant mutant of JL907
JL593	*thrB8*	Requires threonine	J. L. Ingraham
JL636	*lys-554 his-664 serA790*	Requires lysine	J. L. Ingraham
SD76	*trpR582 thrA11*	Requires threonine; excretes tryptophan	C. Stuttard
SD78	*trpR582 thrA16*	Requires threonine; excretes tryptophan	C. Stuttard
SD88	*trpR582 thrA1008*	Requires threonine; excretes tryptophan	C. Stuttard
SD83	*trpR582 thrB8*	Requires threonine; excretes tryptophan	C. Stuttard
SD30	*trpR582 thrC59*	Requires threonine; excretes tryptophan	C. Stuttard
SD84	*thr*Δ*BC12*	Requires threonine	C. Stuttard

glucose medium by diluting the cells to give an initial reading of between 5 and 10 with the Klett-Summerson Colorimeter (blue #42 filter). The cultures were incubated at 37° in a rotary-shaker waterbath and cells to be used for enzyme assays were chilled quickly and harvested at a reading of 100 ± 5 Klett units (approximately 9×10^8 cells/ml).

Amino acid analog sensitivity studies: The analogs were dissolved in minimal medium directly before use and agar plates containing thialysine or azaleucine were used within two days. The sensitivity of strains to analogs in agar medium was tested by spotting 36 hr old colonies from glucose minimal medium onto media containing the analogs. Growth patterns were recorded after 24, 48 and 72 hrs. Sensitivity in liquid cultures was tested by inoculating cells from a glucose-limited (0.02% glucose) culture into 0.4% glucose minimal medium containing the analog.

Amino acid excretion studies: Amino acids excreted into the medium by strain JB585 were studied by two methods. The first was cross-feeding of an auxotroph and the second was by direct analysis of culture filtrates. In the first method, cells from colonies of strain JB585 were stabbed into minimal agar covered with a layer of 0.75% agar (soft agar) containing the test auxotroph. After incubation, the areas surrounding the JB585 growth were examined for possible growth of the auxotroph caused by excretion of the required amino acid by strain JB585. The direct analysis of amino acids in Millipore-filtered culture media by paper chromatography was as described by Jones and Heathcote (1966).

Genetic crosses: Transductions were performed using KB1 *int-1* phage (Boro and Brenchley 1971; McIntire 1974). The recipient cells were infected with a multiplicity of about 10 by spreading the phage lysate and recipient cells directly on the selection plate.

Cell extracts: Cultures of cells at a Klett reading of 100 ± 5 units were chilled with ice and the cells harvested by centrifugation at 12,000 × g for 10 min at 4° and washed three times with cold 0.85% NaCl. Pelleted cells were resuspended in the buffer appropriate for each assay. Extracts were prepared by sonic oscillation for three 15-sec periods using the microprobe tip of a Bronwill Biosonik IV sonicator, at a setting of 50%. The extract was then centrifuged at 17,000 × g for 20 min at 4° to remove cell debris. The supernatant was maintained at 4° and enzyme activity measured within 4 hr. All enzyme assays were at 37°.

Protein determinations were by the method of Lowry *et al.* (1951).

Enzyme assays: The amount of total aspartokinase activity was estimated by the hydroxamate assay adapted from BISWAS, MAZUMDER and BISWAS (1968) and FILER, ROSENBERG and KINDLER (1973). The buffer used during sonic treatment contained 20 mM potassium phosphate (pH 6.8); 2 mM magnesium acetate, 2 mM EDTA and 0.1 mM dithiothreitol. The buffer was supplemented, as noted, with 0.5 mM L-threonine and 0.5 mM L-lysine. Enzyme activity is expressed as nmoles of aspartohydroxamic acid produced per mg protein per minute.

The homoserine dehydrogenase levels were measured by the method of ROWBURY, LAWRENCE and SMITH (1968). The buffer used for extract preparation contained: 10 mM Tris-HCl (pH 7.6), 400 mM KCl and 2 mM L-threonine where specified. Specific activity is expressed as the change in optical density (340 nm) per minute per mg protein $\times 10^3$ at 37°. Threonine deaminase [L-threonine hydrolyase (deaminating EC 4·2·1·16] levels were determined by the method of WASMUTH and UMBARGER (1973). The buffer used during sonic treatment contained: 250 mM potassium phosphate (pH 8.1), 0.1 mM disodium EDTA, 10 mM 2-mercaptoethanol and 0.1 mM L-isoleucine.

Lysyl-tRNA synthetase activity was measured using ^{3}H-lysine and tRNA prepared from *E. coli* (CHRISPEELS *et al.* 1968). The addition of cell extract initiated the reactions and the activity was stopped with 3 ml of cold 5% TCA containing 0.1 mg/ml L-lysine.

Chemicals: DL-4-azaleucine was obtained from Calbiochem, Inc., Los Angeles, CA. 4-Thiaisoleucine was a gift of DR. H. E. UMBARGER. All other amino acids and amino acid analogs were from Sigma Chemical Co., St. Louis, MO. L-lysine, 4,5-^{3}H was obtained from New England and Nuclear Corp. (Boston, MA). All other chemicals were reagent grade and commercially available.

RESULTS

Resistance to amino acid analogs.

Strain JB585 was selected without mutagenesis from JL907 as being resistant to 1 mg/ml thialysine. The data in Figure 2 show that the growth of the parent strain JL907 is inhibited by this concentration of thialysine and by another lysine analog, δ-hydroxylysine. The growth of strain JB585 is less affected by these analogs.

Thialysine-resistant mutants of *E. coli* have been reported which have altered levels of lysyl-tRNA synthetase activities (HIRSHFIELD and ZAMECNICK 1972; HIRSHFIELD, TOMFORD and ZAMECNICK 1972). The lysyl-tRNA synthetase activity from strain JB585 was therefore measured to determine whether an alteration in this enzyme could account for the thialysine resistance. The specific activities of the lysyl-tRNA synthetase from JL907 and JB585 were identical at 21 nmoles of product/hr/mg protein. Furthermore, there was no difference in the sensitivities of the two enzyme activities to thialysine inhibition *in vitro*.

Mutants of *S. typhimurium* described as being thialysine-resistant are the *hisT* strains which lack the enzyme necessary for modifying uridine to pseudouridine on several species of tRNA (SINGER *et al* 1972). One consequence of this mutation is the derepression of the histidine biosynthetic enzymes causing resistance to histidine analogs, such as 2-thiazolealanine and 1,2,4,triazolealanine, and a wrinkled colony morphology for cells grown on 2% glucose minimal agar (ROTH, ANTON and HARTMAN 1966). Strain JB585 was not resistant to these histidine analogs and did not form the wrinkled colonies. However, when JB585 was examined for its sensitivity to analogs for amino acids other than histidine, it was more resistant than JL907 to azaleucine and thiaisoleucine. Thus, strain

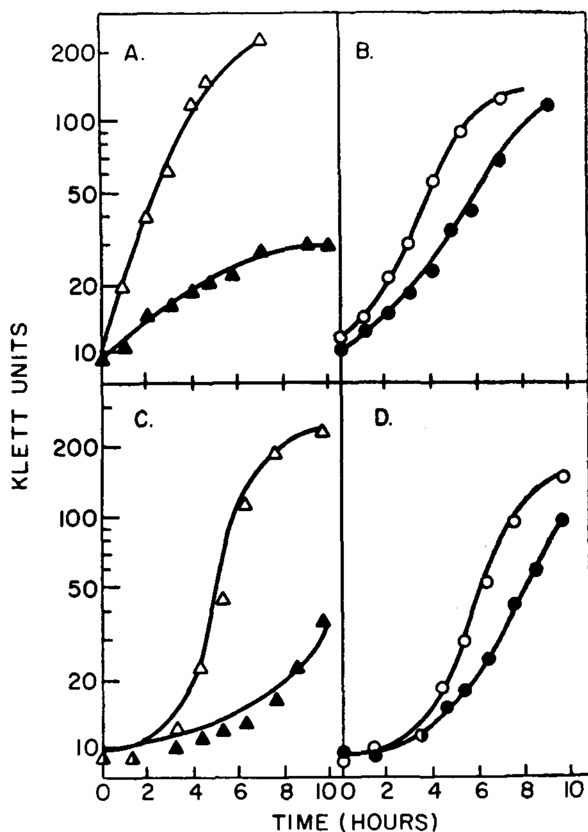


FIGURE 2.—Effect of thialysine and hydroxylysine addition to glucose minimal medium on growth of strains JL907 and JB585. A and B, thialysine addition at 1 mg/ml. C and D, δ-hydroxylysine addition at 5 mg/ml. Symbols: △, JL907, no analog; ▲, JL907, plus analog; ○, JB585, no analog; ●, JB585, plus analog.

JB585 is not similar to the previously reported thialysine-resistant mutants of either *E. coli* or *S. typhimurium.*

Pleiotropic growth properties.

Although strain JB585 is more resistant to thialysine inhibition than JL907, its generation time in glucose minimal medium is about 80 min compared to a generation time of 55–60 min for JL907. Growth of the strains in either complex LB medium or glucose minimal medium supplemented with 0.4% casamino acids was identical, suggesting that at least one amino acid was growth rate limiting. Various combinations of amino acids were added to determine if any restored the normal growth rate. Threonine or isoleucine, but not lysine, addition allowed growth of both strains at between 55 and 60 min per generation. This restoration of the normal growth rate by threonine suggests a defect in threonine, rather than lysine, biosynthesis in strain JB585. The effect of isoleucine could result from sparing the need for more threonine as a precursor of isoleucine (Figure 1).

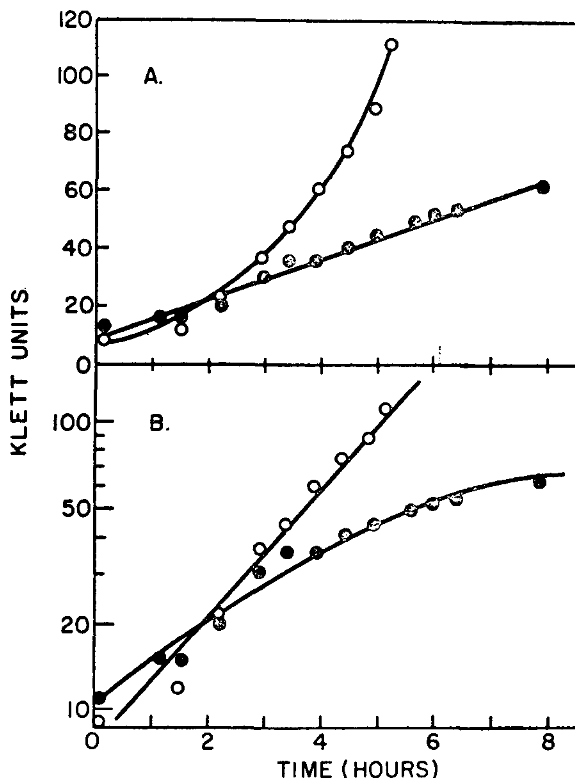


FIGURE 3.—Effect of methionine addition to minimal medium on the growth of strain JB585. A, results plotted on linear scale. B, results plotted on semilogarithmic scale. Symbols: ○, without methionine; ●, with methionine (100 μg/ml).

During these growth studies, it was observed that the addition of methionine to minimal medium reduced growth of the mutant from an exponential to a linear rate (Figure 3). This inhibition could be reversed by the addition of homoserine, threonine or isoleucine, indicating that methionine was preventing synthesis of homoserine as the precursor for threonine. As shown in Figure 1 homoserine can be produced by either the threonine- or methionine-controlled aspartokinase-homoserine dehydrogenase complex. Inhibition or loss of these activities results in a homoserine (or threonine and methionine) requirement.

Since JB585 has a growth requirement for threonine or isoleucine, it was possible that the resistance to the branched-chain amino acid analogs, azaleucine and thiaisoleucine, could be secondary effects caused by the limitation for isoleucine leading to derepression of the respective biosynthetic enzymes. The results in Table 2 show that the level of one of the isoleucine biosynthetic enzymes, threonine deaminase, is derepressed in mutant cells grown in minimal medium. If the isoleucine-valine enzymes are derepressed as predicted, then the mutant cells might be expected to produce excess valine from pyruvate. Indeed, paper chromatography of culture filtrates shows that the JB585 culture excretes large

TABLE 2

Threonine deaminase activity and valine excretion for strain JB585

Strain	Growth medium	Threonine deaminase specific activity*	Valine concentration† (μg/ml)
JL907	Minimal	31.6	< 1
JB585	Minimal	104.3	220
JB585	Minimal + Isoleucine‡	39.5	—
JB585	Minimal + Threonine	—§	22

* Expressed as nmoles of keto acid produced per minute per milligram protein.
† The valine concentration in the culture filtrate was determined by paper chromatography as described in MATERIALS AND METHODS. . .
‡ Isoleucine and threonine were added at 100 μg/ml each.
§ Not measured.

amounts of valine into the medium (Table 2). The elevated threonine deaminase levels, coupled with the valine excretion, validate the prediction that the isoleucine-valine biosynthetic enzymes would be derepressed in strain JB585. As expected, growth of the mutant with isoleucine both restores the normal growth rate and lowers the threonine deaminase to the level observed for JL907; the addition of threonine also restores normal growth and significantly reduces the valine excretion (Table 2).

We examined the possibility that the growth resistance of strain JB585 to the analogs azaleucine and thiaisoleucine could be caused by the large amounts of valine produced. We found that valine can partially overcome the sensitivity of the parent strain JL907 to these analogs. Thus, it appears that the resistance of the mutant to azaleucine and thiaisoleucine is caused by the derepression of the isoleucine-valine enzymes and the overproduction of valine. However, the thialysine resistance is not explained by valine excretion. An examination of culture filtrates failed to detect lysine (possibly because it is catabolized), but colonies of strain JB585 were able to crossfeed a lawn of a lysine auxotroph (JL636) on solid media. Since lysine effectively overcomes growth inhibition by thialysine, this overproduction of lysine by JB585 may account for its thialysine resistance.

Linkage with the thrA *gene.*

The growth properties enumerated above demonstrate that strain JB585 is a threonine bradytroph. Transductions were therefore performed to determine if the lesion maps within any of the *thr* genes. Bacteriophage KB-1 *int-1* was grown on strain JB585 and used to transduce strains carrying either a *thrB8* or *thrC59* mutation to prototrophy. The transductants were scored for methionine sensitivity and thialysine resistance. The lesion causing the JB585 phenotype was 96% co-transducible with *thrB8* and 57% co-transducible with *thrC59* (Table 3). In addition, transductants were not produced with JB585 phage when a *thrA1008* strain was a recipient even though a few hundred transductants resulted when a similar phage preparation from JL907 was used. Based on the evidence that the lesion in strain JB585 causes a growth requirement for threo-

TABLE 3

Frequency of co-transduction of the mutation in JB585 with the thrB *and* thrC *mutations*

Recipient strain	Genotype of recipient	Selection plates	Number scored	Percent with JB585 phenotype
JL593	*thrB8*	Minimal	387	87
SD83	*trpB582 thrB8*	Homoserine*	100	96
SD30	*trpR582 thrC59*	Homoserine	95	57

* Because the donor, JB585, grows slowly on minimal medium it was possible that transductants having this phenotype would have a growth disadvantage when selected on minimal agar, hence, the co-transduction frequencies were checked for transductants selected on both minimal medium and medium containing 100 μg/ml homoserine.

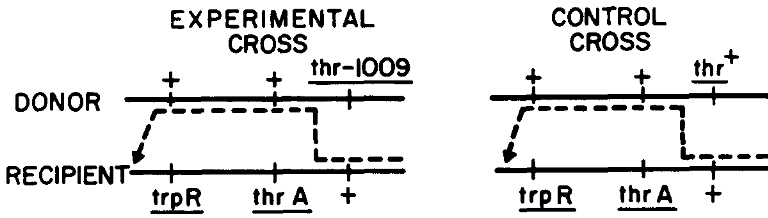
nine and is genetically linked to the *thr* genes, the mutation was designated *thr-1009*.

Use was made of a strain carrying a deletion covering all known sites in *thrB* and *thrC* to determine whether *thr-1009* was in this region (STUTTARD 1973). Phage from strain JB585 were crossed into SD84 (*thrBC12*) and transductants selected on minimal medium plus methionine to select against both the JB585 and SD84 phenotypes. The yield of wild-type recombinant colonies was comparable to that from a control cross of JB585 into SD83, which has a point mutation in *thrB8*. Thus *thr-1009* is located outside the region deleted in *thrBC12*.

A more specific mapping of the *thr-1009* lesion was obtained with a series of three factor crosses using *trpR582 thr⁻* double mutants as recipients (STUTTARD 1972 and 1973). Recombinants were selected on minimal medium containing methionine, and purified transductants were tested for the unselected *trpR* marker by scoring for their ability to allow growth of a *trpE95* auxotroph. The crosses are diagrammed in Figure 4 with the upper line representing the phage genome and the lower line the bacterial genome. The dotted line shows possible recombination patterns needed to yield a thr^+ genotype with either JB585 or the control, JL907, as the donor. In case 1 the donor *thr* marker is to the right of the recipient *thrA* mutation so two crossovers could give rise to $thrA^+$ $trpR^+$. In case 2, the experimental and control crosses would be expected to result in a different frequency of $trpR^+$ inheritance because four crossovers are required to give a thr^+ $trpR^+$ genotype with the *thr-1009* donor, but only two crossovers are required with the thr^+ control. The results in Table 4 compare the recombination frequency of the unselected $trpR^+$ gene for transductants obtained with JL907 and JB585. The percent $trpR^+$ recombinants for the various *thrA*, *thrB*, and *thrC* markers remains fairly constant between 24 and 30% with JL907 as the donor. The transductions of JB585 with the three *thrA* strains corresponds to the control with JL907. However, the frequency is reduced with the *thrB* and *thrC* strains suggesting that the *thr-1009* lesion lies between the *thrA1008* and *thrB8* mutations (Figure 5) and is most likely within the *thrA* gene, making it *thrA1009*.

Generally reciprocal three-factor crosses are used to confirm the gene order. Although the methionine sensitivity of strain JB585 allows clear scoring of trans-

CASE 1



CASE 2

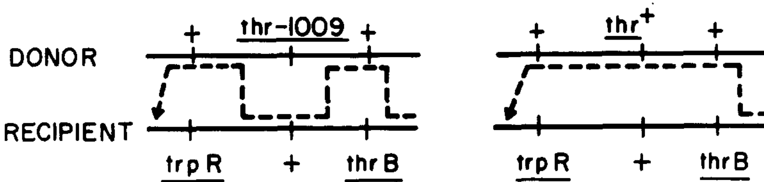


FIGURE 4.—Diagram of three-factor crosses. The upper line represents the phage genome and the lower line represents the bacterial genome. In case 1, two crossovers are required to give rise to *thrA*+ *trpR*+ transductants in both the experimental and control crosses, thus similar frequencies are expected for this gene order. In case 2, 4 crossovers are required to give rise to *thr*+ *trpR*+ in the experimental cross, whereas only two are required to give *thr*+ *trpR*+ in the control. The frequency of *trpR*+ will be less in the experimental cross for this gene order.

TABLE 4

Location of the thr-1009 *lesion by three factor crosses*

Recipient strain	Recipient *thr* genotype*	Donor JB585		Donor JL907	
		Number scored	% *trpR*+	Number scored	% *trpR*+
SD76	*thrA11*	50	28	50	30
SD78	*thrA16*	50	28	50	30
SD88	*thrA1008*	100	23	100	27
SD83	*thrB8*	124	11	100	28
SD30	*thrC59*	85	4	100	24

* All strains carry the *trpR582* mutation in addition to being threonine auxotrophs. Transductants were selected for *thr*+ in the presence of methionine to select against the *thr-1009* marker. The *trpR* mutants excrete tryptophan into the medium. The *trpR* phenotype is therefore scored by its ability to allow growth of a *trp*− mutant on solid medium.

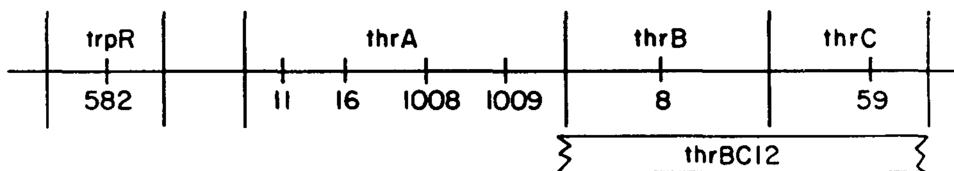


FIGURE 5.—Order of the genetic markers used in this study (STUTTARD 1973). The exact extent of the *thrBC12* deletion has not been determined.

ductant phenotypes, numerous revertants insensitive to methionine appeared whenever JB585 was used as a recipient, thus preventing the reciprocal crosses.

Analysis of various transductants demonstrated that the threonine requirement, thialysine resistance and methionine sensitivity were always inherited together. In order to provide further evidence that the pleiotropic phenotype of strain JB585 is due to a single mutation, methionine-insensitive revertants were isolated. One strain, JB586, appears to be a true revertant, having simultaneously lost all the characteristics of the mutant. It has a normal growth rate in either minimal medium or minimal medium with methionine and is thialysine, azaleucine, and thiaisoleucine sensitive.

Aspartokinase and homoserine dehydrogenase levels.

The *thrA* gene in *E. coli* K12 encodes the aspartokinase I-homoserine dehydrogenase I enzyme complex. These enzymes were measured to determine what effect the *thrA1009* lesion had on their activities. During initial assays for aspartokinase activity, the JB585 extract reproducibly contained less than 10% of the total specific activity observed for JL907, even though the extracts were prepared identically. Mixing the two extracts gave the expected activity suggesting that the low level for JB585 was not due to the presence of an inhibitor (data not shown).

This low level of aspartokinase activity was surprising because, as mentioned in the introduction, there may be three aspartokinase isoenzymes, and a single mutation would not be expected to cause the loss of all three activities. Furthermore, the loss of all aspartokinase function should result in a complete growth requirement for lysine and homoserine rather than a partial requirement for threonine. It was apparent therefore, that the cells have some aspatokinase activity *in vivo* for growth, and various buffers and sonicating conditions were examined in an attempt to maintain this activity *in vitro*. The results in Table 5 show that 34% of the control activity could be detected in extracts assayed immediately after sonication; additional activity could be detected by including threonine and lysine in the sonicating buffer suggesting that these stabilize the enzyme. The aspartokinase activity in the extracts from JL907 is also slightly higher with these additions in the extract buffer. Inhibition studies with threonine and lysine were done to try to identify which aspartokinase(s) compose the 34% activity for strain JB585. However, the results were complicated by the instability of this activity and the difficulty in obtaining reproducible data.

TABLE 5

Aspartokinase and homoserine dehydrogenase activity for strains JL907 and JB585*

Strain	Enzyme assayed†	Addition to sonicating buffer‡	Specific activity	% wild-type activity
JL907	AK	—	325	100
JB585	AK	—	112	34
JL907	AK	Thr + Lys	432	100
JB585	AK	Thr + Lys	218	50
JL907	HSD	—	27.0	100
JB585	HSD	—	1.8	6
JL907	HSD	Thr	77	100
JB585	HSD	Thr	9.5	12

* Specific activities as expressed in text.
† The assays are for total aspartokinase (AK) and homoesrine dehydrogenase (HSD) activities and are averages of triplicate samples for three different experiments.
‡ Additions to the buffer for sonic treatment are at 2 mM each; after diluiton into the reaction mixture the final concentration was from 20 μm to 60 μm which is too low to inhibit activity.

The homoserine dehydrogenase activities were also measured and extracts from strain JB585 contained only 6% of the wild-type total activity (Table 5). The addition of threonine to the buffer used during extract preparation was effective in increasing homoserine dehydrogenase activity for both the JL907 and JB585 extracts. The highest activity found for JB585 was 12% that of the control which is the level expected for the methionine-controlled homoserine dehydrogenase. Since this activity was not inhibited by threonine addition to the reaction mixture, but was reduced further to 3% when the mutant cells were grown in the presence of threonine and methionine, it represents the methionine-controlled homoserine dehydrogenase isoenzyme (CAFFERATA and FREUNDLICH 1969). These results show a complete loss of the threonine-controlled homoserine dehydrogenase activity and suggest normal levels of the methionine-controlled homoserine dehydrogenase in strain JB585.

DISCUSSION

Other classes of thialysine-resistant mutants have been reported: *E. coli* mutants with an altered lysyl-tRNA synthetase (HIRSHFIELD and ZAMECNIK 1972; HIRSHFIELD *et al.* 1972), *Bacillus subtilis* and *Brevibacterium flavum* mutants with derepressed lysine sensitive aspartokinases (VOLD, SZULMAJSTER and CARBONE 1975; SANO and SHIIO 1970), and *S. typhimurium* mutants lacking the pseudouridine modification in tRNA (SINGER *et al.* 1972). The results obtained in this study indicate that the lesion in strain JB585 does not belong to any of the above classes. In fact, thialysine resistance for this mutant may be the indirect result of altered aspartokinase and homoserine dehydrogenase levels and lysine overproduction.

Data obtained from the physiological studies show that the mutant, JB585, is a threonine bradytroph. Growth limitation for threonine, and consequently isoleucine, derepress the isoleucine-valine biosynthetic enzymes resulting in ele-

vated levels of threonine deaminase, and excess valine production (Table 2). An effect of the valine excretion is the growth resistance of JB585 to azaleucine and thiaisoleucine. The proposal that these properties are the indirect result of the threonine limitation is supported by the reduction of valine excretion and normal threonine deaminase levels when threonine or isoleucine is added to the growth medium (Table 2).

The genetic data are consistent with the mutant being a threonine bradytroph. The lesion in strain JB585 is 96% co-transducible with *thrB8* and 57% co-transducible with *thrC59* (Table 3). Transduction with a mutant carrying a deletion in *thrB* and *thrC* genes showed that the lesion is outside the deleted area and the results of three factor crosses (Table 4) suggest the lesion lies between the *thrA1008* and *thrB8* mutations. This locates the *thrA1009* as the most distal mutation characterized in the *thrA* gene. Thèze *et al.* (1974) have reported that the proximal portion of the *thrA* gene of *E. coli* encodes the polypeptide responsible for the aspartokinase I activity whereas the distal region codes for the homoserine dehydrogenase I activity. Thus, a mutation in the *thrA* gene could affect one or both of these enzymes depending on its location. The *thrA1009* mutation appears to cause the complete loss of homoserine dehydrogenase I activity while making the aspartokinase I (and possibly the other aspartokinase activities) unstable. These results are consistent with the mutational site being in the distal region for homoserine dehydrogenase.

The relatively adequate growth of the strain in minimal medium suggests the cells have aspartokinase activity *in vivo* sufficient for biosynthesis. However, initial assays of aspartokinase activity, which demonstrated substantial levels for the parent strain, detected little activity in the mutant extracts. Including threonine and lysine in the buffer prior to sonication appears to stabilize some activity in extracts from the mutant, suggesting the aspartokinase is present in the mutant cells during growth but is unstable during extraction. Attempts to detect higher levels by using a whole cell assay were unsuccessful.

The surprising observation is that the total aspartokinase activity is affected. If *S. typhimurium* has three isoenzymes similar to those in *E. coli* K12, then a single mutation in the *thrA* gene would not be expected to alter all three activities. In particular, the lysine-controlled aspartokinase, which comprises about 40% of the total activity, should be detectable. One possibility to account for this observation is that *S. typhimurium* contains only one aspartokinase enzyme. Although the appropriate mutants are not available to test this directly, the following results suggest it is unlikely. First, the inhibition and repression patterns for the aspartokinase and homoserine dehydrogenase activities in *S. typhimurium* are identical with those reported for *E. coli* (Coles and Brenchley 1976). Also, the results of this investigation show that the threonine-controlled homoserine dehydrogenase activity can be lost while the methionine-repressible enzyme remains (Table 5). Thus, these data indicate that *S. typhimurium* contains the two homoserine dehydrogenase isoenzymes in the same proportion as *E. coli*.

Assuming that the isoenzymes exist in *S. typhimurium*, two other possibilities could account for the loss of aspartokinase activities. One is that the mutation

in strain JB585 causes instability of not only the threonine-controlled aspartokinase-homoserine dehydrogenase protein, but also the other two aspartokinase enzymes suggesting they exist in a large enzyme complex *in vivo*. Another explanation is that the threonine-controlled aspartokinase is fully active *in vivo* and large amounts of aspartylphosphate are produced and converted to aspartic semialdehyde. The complete loss of the threonine-regulated homoserine dehydrogenase, however, prevents its rapid conversion to homoserine. This could provide a physiological explanation for lysine excretion since this block between aspartic semialdehyde and homoserine might cause more aspartic semialdehyde to be converted to lysine. The overproduction of lysine could then repress the lysine-controlled aspartokinase and account for the absence of this activity in the mutant extracts. Lysine overproduction also provides an explanation for the thialysine resistance since lysine eliminates thialysine inhibition.

The pleiotropic properties of strain JB585 makes it a unique mutant resistant to lysine analogs and having a partial requirement for threonine. The *thrA1009* lesion causes the loss of the homoserine dehydrogenase I activity and affects the *in vitro* activities of all the aspartokinase isoenzymes, suggesting that the regulation of aspartokinase activity may be more complex in *S. typhimurium* than anticipated. Further biochemical and genetic analysis of mutants such as strain JB585 should be useful in determining the existence, regulation and function of these unusual isoenzymes.

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LITERATURE CITED

BISWAS, D. K., R. MAZUMDER and C. BISWAS, 1968 Regulation of synthesis of aspartokinase by methionine, threonine and lysine in *Escherichia coli* strain B. J. Biol. Chem. **243**: 3655–3660.

BORO, H. and J. E. BRENCHLEY, 1971 A new generalized transducing phage for *Salmonella typhimurium* LT2. Virology **45**: 835–836.

BOY, E. and J. C. PATTE, 1972 Multivalent repression of aspartic semialdehyde dehydrogenase in *Escherichia coli* K12. J. Bacteriol. **112**: 84–92.

BRENCHLEY, J. E., 1973 Effect of methionine sulfoximine and methionine sulfone on glutamate synthesis in *Klebsiella aerogenes*. J. Bacteriol. **114**: 666–673.

BRENCHLEY, J. E. and L. S. WILLIAMS, 1975 Transfer RNA involvement in the regulation of enzyme synthesis. Ann. Rev. Microbiol. **29**: 251–274.

CAFFERATA, R. L. and M. FREUNDLICH, 1969 Evidence for a methionine-controlled homoserine dehydrogenase in *Salmonella typhimurium*. J. Bacteriol. **97**: 193–198.

CHRISPEELS, M. J., R. F. BOYD, L. S. WILLIAMS and F. C. NEIDHARDT, 1968 Modification of valyl-tRNA synthetase by bacteriophage in *Escherichia coli*. J. Mol. Biol. **31**: 463–475.

COHEN, G. N., 1969 The aspartokinases and homoserine dehydrogenases of *Escherichia coli*. pp. 183–231. In: *Current topics in cellular regulation*. Edited by B. L. HORECKER and E. R. STADTMAN. Academic Press, New York.

COLES, F. and J. BRENCHLEY, 1976 Inhibition of growth and aspartokinase activity of *Salmonella typhimurium* by thialysine. Biochim. Biophys. Acta **428**: 647–655.

FILER, D., E. ROSENBERG and S. H. KINDLER, 1973 Aspartokinase of *Myxococcus xanthus*: "Feedback stimulation" by required amino acids. J. Bacteriol. **115**: 23–28.

HIRSHFIELD, I. N., J. W. TOMFORD and P. C. ZAMECNIK, 1972 Thiosine-resistant mutants of *Escherichia coli* K12 with growth-medium-dependent-lysyl-tRNA synthetase activity. II. Evidence for an altered lysyl-tRNA synthetase. Biochim. Biophys Acta **259**: 344–356.

HIRSHFIELD, I. N. and P. C. ZAMECNIK, 1972 Thiosine-resistant mutants of *Escherichia coli* K12 with growth-medium-dependent lysyl-tRNA synthetase activity. I. Isolation and physiological characterization. Biochim. Biophys. Acta **259**: 330–343.

JONES, K. and J. G. HEATHCOTE, 1966 The rapid resolution of naturally occurring amino acids by thin-layer chromatograaphy. J. Chromatog. **24**: 106–111.

LOWRY, O., N. ROSEBROUGH, A. FARR and R. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

MCINTYRE, S. A., 1974 Transduction with integration-defective mutants of *Salmonella typhimurium* bacteriophage KB1. J. Bacteriol. **117**: 907–908.

ROTH, J. R., D. N. ANTON and P. HARTMAN, 1966 Histidine regulatory mutants in *Salmonella typhimurium*. I. Isolation and general properties. J. Mol. Biol. **22**: 305–323.

ROWBURY, R. J., D. A. LAWRENCE and D. A. SMITH, 1968 Regulation of the methionine-specific aspartokinase and homoserine hydrogenase of *Salmonella typhimurium*. J. Gen. Microbiol. **54**: 337–342.

SANO, K. and I. SHIIO, 1970 Microbial production of L-lysine. III. Production by mutants resistant to S-(2-aminoethyl)-L-cysteine. J. Gen. Appl. Microbiol. **16**: 373–391.

SINGER, C. E., G. R. SMITH, R. CORTESE and B. N. AMES, 1972 Mutant $tRNA^{His}$ ineffective in repression and lacking two pseudouridine modifications. Nature New Biol. **238**: 72–74.

STUTTARD, C., 1972 Location of *trpR* mutations in the *serB-thr* region of *Salmonella typhimurium*. J. Bacteriol. **111**: 368–374. ——, 1973 Genetic analysis of *thr* mutations in *Salmonella typhimurium*. J. Bacteriol. **116**: 1–11.

THÈZE, J., D. MARGARITA, G. N. COHEN, F. BORNE and J. C. PATTE, 1974 Mapping of the structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *Escherichia coli* K12. J. Bacteriol. **117**: 133–143.

THÈZE, J. and I. SAINT-GIRONS, 1974 Threonine locus of *Escherichia coli* K12: Genetic structure and evidence for an operon. J. Bacteriol. **118**: 990–998.

UMBARGER, H. E., 1969 Regulation of amino acid metabolism. Ann. Rev. Biochem. **38**: 323–370.

VOLD, B., S. SZULMAJSTER and A. CARBONE, 1975 Regulation of dihydropicolinate synthetase and aspartate kinase in *Bacillus subtilis*. J. Bacteriol. **121**: 970–974.

WASMUTH, J. J. and H. E. UMBARGER, 1973 Effect of isoleucine, valine or leucine starvation on the potential for formation of the branched-chain amino acid biosynthetic enzymes. J. Bactersiol. **116**: 548–561.

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