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Characterization of a Cold-Sensitive *hisW* Mutant of *Salmonella typhimurium*

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A cold-sensitive mutant of *Salmonella typhimurium* LT2 that grows at 37 C but not at 20 C has altered repression regulation in at least two amino acid biosynthetic pathways (histidine and isoleucine). The lesion conferring cold sensitivity that is linked with *hisW* is recessive. Assays for the acceptance of some amino acids by transfer ribonucleic acid (tRNA) reveal a decreased ability of the mutant tRNA to accept arginine, phenylalanine, and histidine. A mutation in a gene for tRNA maturation is a likely possibility for the mutation producing these effects on growth, regulation, and amino acid acceptance.

Temperature-sensitive mutants have been valuable for investigations of physiological functions that cannot be restored by nutritional supplements. Studies of heat-sensitive mutants have established that such lesions generally result in heat lability of the gene product either during or after synthesis. In contrast, auxotrophic cold sensitivity often results from slight configurational changes in allosteric proteins which precludes function at low temperature (2, 9, 16, 23). Thus mutants that cannot grow at low temperature (20 C) even on supplemented media seemed likely candidates to have altered regulatory mechanisms for the synthesis of various macromolecules, and a high proportion of such mutants has, in fact, proved to be defective in the assembly of ribosomes (13, 32).

This paper presents the characterization of a cold-sensitive mutant from the conditional lethal class that is non-ribosomal and affects the regulation of amino acid biosynthesis.

MATERIALS AND METHODS

Mutant isolation. A culture of *Salmonella typhimurium* LT2 (JL781) following mutagenesis with *N*-methyl, *N'*-nitro-*N*-nitrosoguanidine at a concentration of 100 μg/ml for 30 min by the procedure of Adelberg et al. (3), was filtered, washed, and grown in nutrient broth at 37 C for 8 h. The exponentially growing culture was then transferred to 20 C and 20,000 units of penicillin G per ml were added. After 20 h at 20 C, samples were filtered, plated on nutrient agar, and incubated at 37 C. Colonies appearing on these plates were tested for their ability to grow at 20 C. One mutant unable to form colonies at 20 C was isolated and designated JL250.

Strain JL578, a derivative of strain JL250, is unable to ferment arabinose (*ara-1*) and galactose (*gal-1*). The *ara-1* mutation was selected by picking colonies resistant to ribitol in the presence of L-arabinose according to the procedure of Katz (18). The *gal-1* mutation was selected without further mutagenesis by repeated penicillin counterselections in D-galactose minimal medium. Transducing phages grown on JL250 were unable to cure the cold sensitivity of JL578.

Media and strains. Minimal medium was medium 56 of Monod et al. (21) with 0.2% glucose as the carbon source except as indicated. Complex media were nutrient broth (NB) and nutrient agar (NA) from Difco Laboratories.

Strains used are described in Table 1, and the approximate map position (26) of relevant markers is shown in Fig. 1.

Genetic experiments. Interrupted matings were performed by the procedure of Goodgal et al. (12). Phage P22 *int*-4, a non-integrating mutant of phage P22 (30), was used for transductions which were performed by spreading 0.1 ml of donor phage (10^{10} phage/ml) and 0.1 ml of recipient bacteria (2×10^9 cells/ml) together on plates containing selective media.

Mutagenization of phage. A phage suspension was mutagenized by a modification of the procedures of Tessman (33) and Hong and Ames (17). Phage L4 was treated in 0.4 M hydroxylamine (pH 6.0) containing 10^{-3} M ethylenediaminetetraacetic acid (EDTA) for 8 h at 37 C. The reaction was stopped by diluting 100-fold into tryptone broth (13 g of tryptone plus 7 g of NaCl per liter) containing 10^{-3} M EDTA. These treated phage were used to transduce strain JL250 to growth on complex medium at 20 C, and the resulting transductants were screened for new nutritional requirements.

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TABLE 1. *Strains utilized in genetic investigations*

Strain	Genotype	Source
JB303	*hisW*$^+$	Spontaneous revertant of JL250
JL250	*hisW3333*	Mutagenesis of JL781
JL578	*ara-1, gal-1, hisW3333*	Derived from JL250
JL597	*HisOG203*	B. Ames
JL601	*aroD5, purF145*	B. Ames
JL602	*metE338*	B. Ames
JL603	*hisS1520*	B. Ames
JL604	*hisT1501*	B. Ames
JL605	*hisR1223*	B. Ames
JL606	*hisU1817*	B. Ames
JL607	*hisW1821*	B. Ames
JL608	*hisW1825*	B. Ames
JL667	*hisW1824*	B. Ames
SA535	HfrK5, *serA*	K. Sanderson
SA930	HfrB$_2$, *pro, aro*	K. Sanderson
SU576	HfrA, *purC*	K. Sanderson
TA1536	F32/*his-2461, aroD5, purF145*	G. Ames
TR35	FT80/*ser-821, aro-501 his-712*	G. Ames

Amino acid and uracil incorporation. Incorporation of labeled uracil (^{3}H-uracil at 1.0 μCi per 10 μg per ml of culture) and arginine (L-araginine-^{14}C at 0.05 μCi per 10 μg per ml of culture) into trichloroacetic acid insoluble material was used as an index of ribonucleic acid (RNA) and protein synthesis. Samples of the culture were added to an equal volume of 10% trichloroacetic acid containing 100 μg of the nonlabeled compound per ml. After at least 30 min at 0 C, the samples were filtered on membrane filters (0.45-μm pore size; Millipore Corp.) presoaked in the trichloroacetic acid solution, and washed with 20 ml of 5% trichloroacetic acid, followed by 10 ml of 1% acetic acid. The filters were placed in counting vials with 8 ml of Bray solution (6), and radioactivity was determined in a Packard liquid scintillation spectrometer.

Enzyme assays. Histidinol dehydrogenase (L-histidinol + 2 NAD = L-histidine + 2 NADH; EC 1.1.1.23) and histidinol phosphate phosphatase (L-histidinol phosphate + water = L-histidinol + orthophosphate; EC 3.1.3.15) were assayed according to the procedure of Ames et al. (4) except that 0.02 ml of histidinol phosphate was used instead of 0.22 ml. Ornithine carbamyltransferase (carbamoylphosphate + L-ornithine = orthophosphate + L-citrulline; EC 2.1.3.3) and aspartate carbamyltransferase (carbamoylphosphate + L-aspartate = orthophosphate + *N*-carbamoyl-L-aspartate; EC 2.1.3.2) were assayed by the methods of Abd-El-Al and Ingraham (1).

The procedure of Ning and Gest (22) was followed for measuring threonine deaminase (L-threonine + water = 2-oxobutyrate + NH_3 + water; EC 4.2.1.16) activity except that 10^{-4} M L-isoleucine was added to the standard buffer to stabilize the enzyme. Tryptophan synthetase (L-serine + indole = L-tryptophan + water; EC 4.2.1.20) was assayed by the procedure of Smith and Yanofsky (31) and α-isopropylmalate synthetase by the procedure of Burns et al. (8). Protein was determined by the method of Lowry et al. (20).

tRNA preparation. Bulk transfer RNA (tRNA) was prepared by the procedure of Silbert et al. (28) from cultures grown at 37 C, in glucose minimal medium. The OD_{260}/OD_{280} (OD, optical density) ratio was 2.

Aminoacyl-tRNA synthetase preparation and tRNA acylation. About 1 g of cells was suspended in 3 ml of cold buffer containing 5 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 20 mM NaCl, 0.5 mM EDTA, and 2 mM β-mercaptoethanol and sonically treated. The crude extract was centrifuged at 30,000 $\times$ *g* for 30 min and then put through a Sephadex G50 column (4-ml bed volume). The extract was either used fresh or stored at -10 C and used within a few days.

Acylation of tRNA was assayed by the procedure of Silbert et al. (28) except that Tris (pH 8.0) was substituted for potassium cacodylate (pH 7.0). The reactions were stopped after maximal acylation (15 min).

RESULTS

Growth characteristics of strain JL250. Of 25 cold-sensitive mutants isolated in this

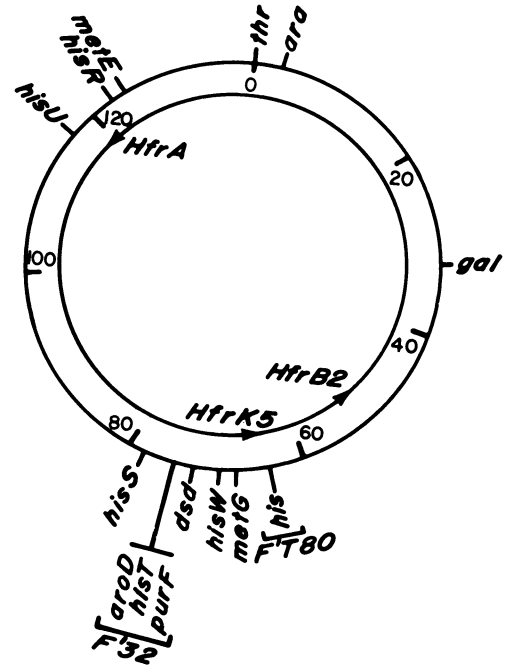

FIG. 1. *Salmonella linkage map showing the approximate location of relevant markers.*

study as being unable to grow on complex medium at 20 C, only strain JL250 stopped growing immediately upon shift to 20 C in minimal medium. On shifting cultures of the parent, strain JL781, growing in glucose minimal medium from 37 to 20 C, growth continues at a new slower rate, but cultures of strain JL250 stop completely (Fig. 2). Growth of strain JL250 resumes upon return to 37 C after a lag of only 5 to 10 min. The growth rate of the mutant at 37 C in glucose minimal medium is slightly slower (75–85 min/doubling) than that of its parent (55–60 min/doubling), but in NB medium is essentially the same (about 34 min/doubling).

The cold sensitivity is apparently not caused by alterations in specific permeability systems because cultures growing in glycerol or galactose minimal media also stop growing immediately on shift to 20 C; nor can the inability of JL250 to grow at 20 C be explained by a defect in the oxidative phosphorylation system since cultures growing anaerobically remain cold sensitive.

Protein and RNA synthesis. The incorporation of ^{14}C-arginine and ^{3}H-uracil into cold trichloroacetic acid-precipitable material by cultures of strain JL250 and its parent was followed at 37 C and after shift to 20 C. At 37 C the differential rate of uptake of uracil is the same in the two strains, but the rate of arginine uptake by the mutant is slightly slower (Fig. 3). Following a shift to 20 C, however, the differential rate of uracil incorporation by strain JL781 increases slightly while that of strain JL250 decreases immediately to a slow rate and eventually stops. The incorporation of arginine by strain JL250 stops after shift to 20 C.

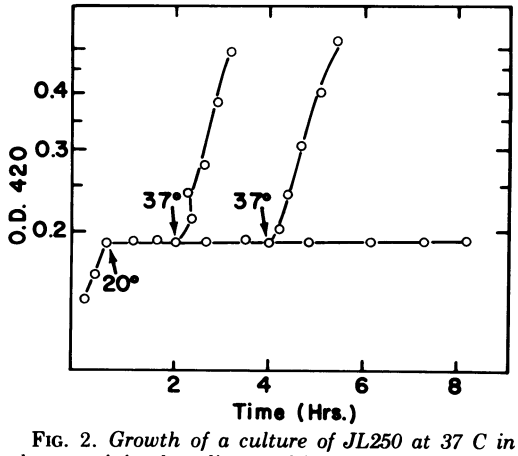

FIG. 2. *Growth of a culture of JL250 at 37 C in glucose minimal medium and following shift to 20 C. Arrows indicate times of temperature shifts.*

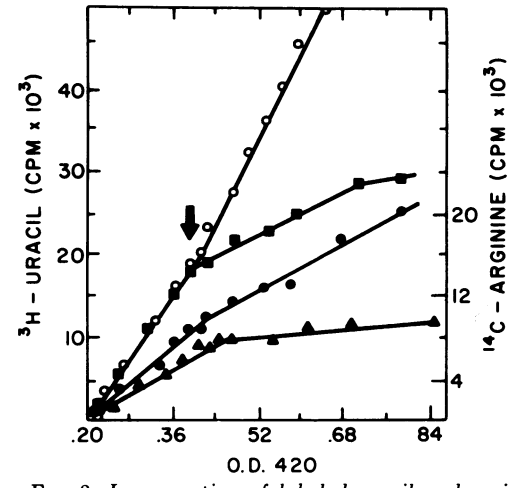

FIG. 3. *Incorporation of labeled uracil and arginine by cultures of JL781 and JL250 growing in glucose minimal medium. Cultures were shifted from 37 to 20 C at OD_{420} 0.4 as indicated by arrow. Symbols: ○ JL781-uracil ■ JL250-uracil ● JL781-arginine ▲ JL250-arginine.*

Thus the genetic lesion carried by strain JL250 appears to affect protein synthesis at 20 C, and since cold-sensitive mutants are frequently altered in ribosome synthesis (13, 32), in vitro evaluations of ribosome function were made by measuring the poly U-directed incorporation of ^{14}C-phenylalanine into cold trichloroacetic acid-precipitable material. Ribosomes prepared from cultures of either JL781 or JL250 grown at 37 C or shifted to 20 C gave the same relative activities when assayed at both 37 and 20 C (J. E. Brenchley, unpublished data).

Mapping of the lesion. To determine the location of this lesion an interrupted mating experiment using strain HfrA (origin at 120 min) as the donor and strain JL578 (an *ara-1*, *gal-1* derivative of strain JL250) as the recipient was performed to determine the approximate entry times of *ara*$^{+}$, *gal*$^{+}$, and the loss of cold sensitivity. Both *ara* and *gal* markers entered prior to the restoration of growth at 20 C and the map position of the cold-sensitive lesion appeared to lie between 60 and 90 min.

Mating experiments using two other Hfr's located the map position more precisely. Cold sensitivity is lost about 5 min after the beginning of mating with strain HfrK5 (Fig. 4), which transfers *metG* as an early marker, and after 13 min with strain HfrB2, which has an origin of transfer at about 57 min. These experiments place the mutation at about 70 min on the *Salmonella* chromosome.

No co-transduction could be detected between cold sensitivity and either *dsd* (D-serine deaminase) or *metG*. However, two histidine regulatory genes, *hisT* and *hisW*, lie in this region and since one phenotype of mutations in these genes is the formation of "wrinkled" colonies on plates containing high concentrations of glucose, strain JL250 was tested and found to produce highly wrinkled colonies on 2% glucose minimal agar at 37 C.

Other known histidine regulatory mutants, obtained from Bruce Ames, University of California at Berkeley, were then checked for possible cold sensitivity. All the *hisR*, *hisS*, *hisT*, *hisU*, and *hisW* strains so obtained grew well at 20 C. Thus it became possible to use these strains for transductional mapping. Phage were grown on various histidine regulatory mutants and used to transduce strain JL250 for growth at 20 C. The resulting transductants were purified at 20 C and streaked on 2% glucose minimal agar at 37 C to determine whether they were wrinkled. Table 2 shows the results of these transductions. Whenever *hisR*, *hisS*, *hisT*, *hisU* strains or strain LT2 are used as donors, only smooth transductants result; but with *hisW1825*, *hisW1824*, and *hisW1821* the majority of transductants are wrinkled. Thus the lesion in strain JL250 is linked to the *hisW* locus at approximately 70 min on the *Salmonella* map. There is some ambiguity in scoring the wrinkled colonies; hence we do not know whether the difference between the 99 and 85% co-transduction frequencies is significant.

Since no co-tranducing marker of the *hisW* locus is known, attempts were made to isolate one using the phage mutagenization procedure of Hong and Ames (17). A preparation of phage P22 *int*-4 was mutagenized with hydroxylamine and then used to transduce strain JL250 to growth at 20 C on complex medium. Thus transducing particles carrying the wild-type allele of the gene conferring cold sensitivity might be mutated elsewhere and result in some new defect for the transductants. Transductants were purified at 20 C and then replica plated onto a variety of media. One mutant unable to grow on minimal medium was isolated and found to require both pyridoxine (vitamin B6) and serine for growth. This double requirement could result from an altered 3-phosphoserine transaminase (10) or two closely linked, but separable, mutations. This mutant should allow further genetic mapping in this region of the chromosome and the determination of whether two (or more) separate genes confer the *hisW* phenotype.

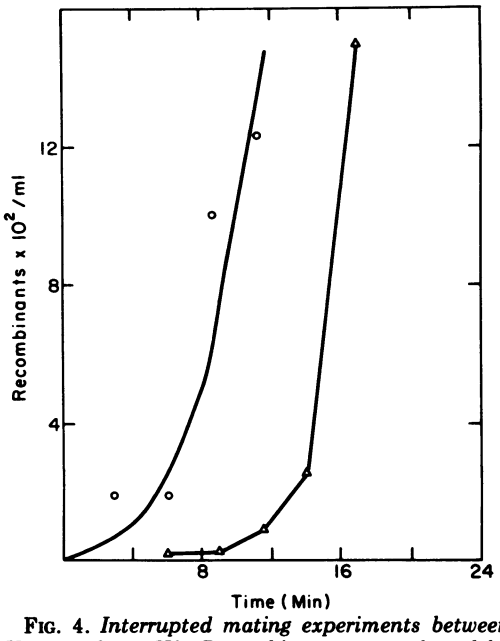

FIG. 4. *Interrupted mating experiments between JL250 and two Hfr. Recombinants were selected for growth at 20 C. Symbols: O, Hfr K5; Δ, Hfr B2.*

Nature of the mutation. Strain JL250 reverts from cold sensitivity at a frequency of about 2×10^{-8}. All revertants are altered with respect to wrinkling patterns and histidine biosynthetic enzyme levels. Some of the revertants have a smooth colony morphology and wild-type histidine biosynthetic enzyme levels (see Table 4 for enzyme assays for a smooth revertant strain JB303) while others are slightly wrinkled. The correlation of the altered derepression pattern with the loss of cold sensitivity (as well as the appearance of smooth colonies) indicates that a single mutation is responsible for both phenotypes. Many of the partial revertants have phenotypes similar to the *hisW* strains obtained from Bruce Ames' collection, i.e. they are able to grow at 20 C but are still wrinkled when grown on 2% glucose minimal agar.

The results of several experiments designed to test non-cold-sensitive revertants for their ability to suppress either ochre or amber nonsense mutations show no evidence of suppression; hence, it appears that *hisW3333* is not a nonsense mutation.

The mutation carried by strain JL250 is revertable by nitrosoguanidine and ethyl methanesulfonate but not by ICR-191 (a mutagen known to cause frameshift mutations). This reversion pattern makes it unlikely that strain JL250 has a frameshift mutation.

Dominance tests. The abrupt cessation of

TABLE 2. *Transductions of other histidine regulatory mutants into strain JL250*

Donor	Recipient	Selection	Colonies scored	No. wrinkled	Co-transduction (%)
hisR1223	*hisW3333*	Growth at 20 C	200	0	<0.5
hisS1520	*hisW3333*	Growth at 20 C	80	0	<1.2
hisT1501	*hisW3333*	Growth at 20 C	120	0	<0.8
hisU1817	*hisW3333*	Growth at 20 C	220	0	<0.4
hisW1825	*hisW3333*	Growth at 20 C	225	222	98.7
hisW1824	*hisW3333*	Growth at 20 C	160	136	85.0
hisW1821	*hisW3333*	Growth at 20 C	225	215	95.5
hisR1223	*metE338*	Met$^+$	160	9	5.0
hisS1520	*strB57*	Nic$^+$ Thi$^+$	160	18	11.2
hisT1501	*aroD purF*	Aro$^+$ Pur$^+$	70	63	90.0
hisU1817	*metE338*	Met$^+$	80	0	<1.2
hisW1825	*metE338*	Met$^+$	220	0	<0.4
hisW1824	*metE338*	Met$^+$	220	0	<0.4
hisW1821	*metE338*	Met$^+$	160	0	<0.6

growth in minimal medium at 20 C suggests that a functional component becomes inactive at 20 C. However, it is also possible that a toxic product could be produced that inhibits growth at 20 C; the latter possibility would lead to the prediction of at least partial dominance of the mutant allele. Accordingly, dominance tests with abortive transductants (14) and episomes were carried out with strain JL250.

When phage from non-cold-sensitive strains are used to transduce strain JL250, abortive transductants as well as normal transductants result, indicating that the wild-type allele is capable of restoring the normal function in the presence of the mutant allele.

The ability of episomes to restore growth at 20 C was also tested (Table 3). FT80 covers the histidine operon while F32 covers the *hisW-aroD-purF* region. FT80 was unable to restore growth at 20 C for strain JL578, but did for strain JL597 which is *hisOG203;* F32 did allow growth of strains JL578 and the *aroD5 purF145* strain JL601. As expected, it did not restore growth for strains JL602 (*metE338*) or JL597 (*hisOG203*). These results also confirm the map location of this mutation.

A derivative of strain JL578 was prepared. It carries the F32 episome. This strain is not cold sensitive, but still *ara-1 gal-1* is. It has the ability to transfer the episome and restore growth of an *aroD purF* recipient (Table 3). It forms smooth colonies on 2% glucose minimal agar at 37 C and segregates wrinkled colonies at a high frequency. A strain cured of the episome is again cold sensitive.

Enzyme assays. Wrinkled colonies on 2% glucose plates are often correlated with derepressed levels of the histidine biosynthetic enzymes (25). Enzyme assays of two enzymes, histidinol-P-phosphatase and histidinol dehydrogenase, show that strain JL250 does indeed have derepressed levels of these enzymes (Table 4). Furthermore, the addition of 100 μg of L-histidine/ml to the growth medium causes no significant repression of the enzymes.

TABLE 3. *Ability of F T80 and F 32 to restore growth in various recipients*

Recipient	Selection	Donors[a]		
		TR35 (F T80)	TA1536 (F 32)	JL578/ F 32
JL578	Growth at 20 C	−−	++	
JL601	Aro$^+$ Pur$^+$	−−	++	++[b]
JL602	Met$^+$	−−	−−	−−
JL597	His$^+$	++	−−	−−

[a] ++ Signifies growth, −− signifies no growth. Tests were performed by spreading a lawn of the recipient on the selective medium and placing a small volume of a full-grown donor culture on it. Reversion controls were included and showed that the observed growth was not due to revertants of either the donor or recipient.

[b] Selection was made on galactose minimal medium to select against the donor strain JL578 (Gal$^-$).

Assays of histidinol dehydrogenase in cultures of strains JL250 and JL781 grown at 37, 30, and 25 C showed no temperature-dependent change in the level of derepression of this enzyme in cells grown in the presence or absence of histidine.

Since there was no evidence that derepression of the histidine biosynthetic enzymes causes cold sensitivity or that the lesion in JL250 is specific for histidine biosynthetic enzyme regulation, the levels of several other enzymes were examined. Strain JL250 has

TABLE 4. *Results of assays for histidinol phosphate phosphatase, histidinol dehydrogenase, ornithine carbamyltransferase and threonine deaminase*

Strain	Addition[a]	Enzyme assayed	Fold de-repression[b]
JL781(*hisW*$^+$)	None	Histidinol-P-phosphatase	1.3
JL781(*hisW*$^+$)	Histidine	Histidinol-P-phosphatase	1.0
JL250(*hisW3333*)	None	Histidinol-P-phosphatase	8.0
JL250(*hisW3333*)	Histidine	Histidinol-P-phosphatase	7.9
JL781(*hisW*$^+$)	None	Histidinol dehydrogenase	1.8
JL781(*hisW*$^+$)	Histidine	Histidinol dehydrogenase	1.0
JL250(*hisW3333*)	None	Histidinol dehydrogenase	3.7
JL250(*hisW3333*)	Histidine	Histidinol dehydrogenase	3.3
JL603(*hisS1520*)	None	Histidinol dehydrogenase	23.5
JL603(*hisS1520*)	Histidine	Histidinol dehydrogenase	0.3
JL608(*hisW1825*)	None	Histidinol dehydrogenase	5.5
JL608(*hisW1825*)	Histidine	Histidinol dehydrogenase	2.7
JB303(*hisW*$^+$)	None	Histidinol dehydrogenase	1.0
JB303(*hisW*$^+$)	Histidine	Histidinol dehydrogenase	1.1
JL781(*hisW*$^+$)	None	Ornithine carbamyltransferase	30.1
JL781(*hisW*$^+$)	Arginine	Ornithine carbamyltransferase	1.0
JL250(*hisW3333*)	None	Ornithine carbamyltransferase	24.2
JL250(*hisW3333*)	Arginine	Ornithine carbamyltransferase	0.9
JL781(*hisW*$^+$)	None	Threonine deaminase	2.9
JL781(*hisW*$^+$)	Leucine, valine, isoleucine	Threonine deaminase	1.0
JL250(*hisW3333*)	None	Threonine deaminase	0.78
JL250(*hisW3333*)	Leucine, valine, isoleucine	Threonine deaminase	0.26
JL781(*hisW*$^+$)	None	Threonine deaminase	2.7[c]
JL781(*hisW*$^+$)	Leucine, valine isoleucine	Threonine deaminase	0.97[c]
JL250(*hisW3333*)	None	Threonine deaminase	0.26[c]
JL250(*hisW3333*)	Leucine, valine, isoleucine	Threonine deaminase	0.13[c]

[a] Amino acid supplemented at 100 μg/ml.

[b] Fold depression compares the specific activities of the various enzymes to the activity obtained for JL781 cultures grown under repressing conditions. Cultures are grown at 37 C except where otherwise noted.

[c] Cells grown at 30 C.

lower levels of threonine deaminase, the first enzyme specific for isoleucine synthesis. The specific activity of this enzyme in JL250 is consistently lower than that in strain JL781, especially at 30 C where it is 10-fold less (Table 4).

Assays of α-isopropylmalate synthetase activity, the first enzyme of the leucine pathway, were done on cells grown at 37 and 30 C to determine if its regulation might be altered in a way similar to threonine deaminase. Although the values for strain JL250 were slightly less than those for strain JL781, the differences may not be significant. Other enzymes assayed and found to be present at normal levels are ornithine carbamyltransferase, aspartate carbamyltransferase and tryptophan synthetase. The levels of ornithine carbamyltransferase are included in Table 4 as an example of an enzyme with normal control.

Derepression of biosynthetic pathways might be expected to overproduce certain end products. Accordingly, an analysis for basic amino acids was performed on cell-free supernatant fluids from strains JL250 and JL781 grown in minimal medium. Small amounts of histidine and lysine were detected in the sample from strain JL250 but not from strain JL781. The presence of the small quantity of histidine in the mutant supernatant agrees well with the observed derepression of the histidine biosynthetic enzymes. (Very little histidine is excreted by mutants derepressed for that pathway since feedback inhibition by histidine

prevents a large increase in the histidine production.) The excretion of lysine by the mutant suggests that the enzymes of that pathway may also be derepressed.

Strain JL250 is also more resistant than wild type to the amino acid analogue β-2-thienylalanine suggesting that the phenylalanine pathway may have altered regulation.

Amino acid acceptance by tRNA. Previous investigations (24, 27, 28) have demonstrated that when the aminoacylation of histidyl-tRNA is lowered, derepression of the histidine pathway results. Thus, the observed derepression for strain JL250 could be caused by a mutation preventing the normal acylation (charging) of histidine to its tRNA species. Consequently, in vitro aminoacylation assays were done comparing the amino acid acceptance by tRNA prepared from both strains JL781 and JL250.

Although the genetic data indicate that the mutation does not involve the histidyl-tRNA synthetase (the cold-sensitive lesion does not lie near *hisS*, the gene encoding the histidyl-synthetase) a direct assay of in vitro synthetase activity was done to eliminate this possibility. No difference was found between the mutant and wild-type synthetase preparations in their abilities to charge histidyl-tRNA.

Aminoacylation assays using seven different labeled amino acids were carried out to determine if any tRNA species from strain JL250 varied in acceptor activity. Table 5 gives the results of these assays. The activity for phenylalanine and arginine are about 0.8 and 0.7 of wild type at 37 C and 0.8 and 0.6 at 20 C. The values for proline (and perhaps serine and valine), however, appear higher than wild type. The value for histidine at 37 C appears normal.

TABLE 5 *Amino acid acceptance by tRNA prepared from JL781 and JL250 cultures*

Amino acid	Relative activity[a]	
	37 C[b]	20 C
Histidine	0.94	0.70
Leucine	1.19	0.90
Arginine	0.70	0.60
Serine	1.15	1.25
Phenylalanine	0.80	0.80
Tyrosine	1.05	1.00
Proline	1.30	1.20
Valine	1.15	0.90

[a] Values represent the ratio of JL250 activity/JL781 activity. Each reaction mixture contained 0.2 mg of tRNA/50-μliter volume.

[b] Assay temperature.

This result agrees with the observation by Brenner and Ames (7) that in vitro activity for tRNA prepared from *hisW* mutants is normal at 37 C. The level at 20 C, however, is lower than that of the wild-type preparation.

The decreased acylation of arginine in the mutant tRNA preparation is interesting since at least one enzyme of the arginine pathway, ornithine carbamyltransferase, has normal levels and regulation (Table 4). These results do not eliminate the possibility that arginyl-tRNA is involved in regulation, but they are consistent with some previous evidence (15) suggesting that lowering the amount of $tRNA^{Arg}$ does not cause derepression of the biosynthetic enzymes.

Kinetic experiments for the acylation of phenylalanine were performed at 37 and 20 C. The results show no apparent difference between the kinetics of phenylalanine attachment to wild-type or mutant tRNA, but the total quantity of $tRNA^{Phe}$ formed is less for the mutant.

DISCUSSION

Genetic and physiological characterization of the cold-sensitive mutant strain JL250 has demonstrated that a number of effects result from this lesion. This mutant cannot grow at 20 C; it produces derepressed levels of the histidine biosynthetic enzymes, lowered threonine deaminase activity, and altered amino acid acceptance by several tRNA species. The results of the reversion and transduction studies provide evidence that the cold sensitivity and the derepression of the histidine enzymes result from a single mutation. The lack of reversion by ICR-191 and the inability of the revertants to suppress nonsense mutations indicate that the *hisW3333* lesion is neither a frameshift nor nonsense mutation. Thus it does not appear that the observed pleiotropic phenotype is due to double mutations nor to polarity effects resulting from nonsense or frameshift mutations.

The possibility that these effects are a consequence of a general temperature-sensitive alteration of some step in the translation process is eliminated by the fact that the enzyme activities of ornithine carbamyltransferase, aspartate carbamyltransferase, tryptophan synthetase and α-isopropylmalate synthetase are present at the normal levels; the lesion is expressed by selective effects on protein synthesis. The conclusion that ribosomes are not altered is supported by the finding that the mutant ribosomes function normally when as-

sayed for polyU-directed phenylalanine incorporation.

Although the cold-sensitive lesion does not affect the levels of all enzymes, neither is it specific for the histidine pathway because threonine deaminase activities in the mutant are reduced. Some inherent instability of this enzyme could account for these results, but it seems unlikely that the enzyme would be more unstable in cells grown at 37 C than at 30 C or that a single mutation could cause derepression of the histidine enzymes plus an instability in threonine deaminase. The slightly slower growth rate of the mutant does not explain the lowered levels either since its parent JL781 grows more slowly at 30 C than at 37 C, but the specific activities of the enzyme remain the same.

The altered enzyme regulation and amino acid acceptance by some tRNA suggests that strain JL250 has some tRNA species with conformations which alter their normal functions in regulation and protein synthesis. The involvement of histidyl-tRNA^{His} in the regulation of the histidine biosynthetic enzymes has been demonstrated by using the analog, α-methylhistidine (27), and by the analysis of a number of regulatory mutants (5, 11, 25), (*hisO*, *hisR*, *hisS*, *hisT*, *hisU* and *hisW* strains).

Lewis and Ames (19) have recently determined the amounts of charged and uncharged histidyl-tRNA present in vivo for these mutants and have found a direct correlation between the amount of charged tRNA^{His} in vivo and repression of the histidine biosynthetic enzymes. They also examined the amount of histidyl-tRNA^{His} for this cold-sensitive mutant and found a reduction of 45% when the quantity of histidyl-tRNA^{His} was compared with the total amount of nucleic acid extracted. Furthermore, Brenner and Ames (7) found altered acceptance of other amino acids, including lysine, by tRNA prepared from strain JL250. Their finding of reduced lysine acceptance by the mutant tRNA and our observation that the mutant excretes lysine suggest that this pathway's regulation has been altered also.

These distinct pleiotropic effects of the strain JL250 mutation make it unlikely that it involves the structural gene for the histidyl-tRNA. This conclusion is supported by other evidence indicating that the *hisW* region does not encode the histidyl-tRNA (7). Thus, it appears that the function associated with the *hisW* region involves a general effect with several tRNA species altering their normal functions in regulation and protein synthesis. A likely candidate for this lesion is the loss of an enzyme involved in the maturation of the tRNA molecules. It has been demonstrated that subsequent to the transcription of the precursor RNA a number of alterations occur before it becomes a completed, functional tRNA molecule. Included in these alterations are a number of base modifications to form pseudouridine, 4-thiouridine, 5,6-dihydrouridine, and a number of different methylated bases. The absence of an enzyme responsible for modifying a specific base(s) in a number of tRNA species could very well account for the results found with this mutant. In fact, it has been shown recently that the histidyl-tRNA of another histidine regulatory mutant, *hisT1504*, contains two uridine residues which are normally modified to pseudouridine in the parent strain's tRNA (29). This demonstrates that a change in the maturation of the tRNA can alter its ability to function normally in the repression mechanism.

The characterization of amber mutants for the *hisT* function shows that it is not essential for cell growth under any conditions tested thus far. However, our characterization of the *hisW3333* mutant demonstrates that its function is necessary for cell growth. The temperature-sensitive effect in this mutant could be caused by a cold-sensitive enzyme which functions partially at 37 C, but not at all at 20 C, or by the complete loss of an enzyme function even at 37 C with the resulting cold sensitivity caused by altered conformations of some tRNA species preventing protein synthesis at 20 C. The abrupt cessation of growth suggests the latter possibility since the altered tRNA would be immediately nonfunctional, whereas with the first possibility the tRNA made at 37 C should function at 20 C until it is diluted out during growth. However, since we know so little about the regulation and function of these enzymes it is still possible that an altered maturation enzyme could interfere directly with protein synthesis in some way.

The precise mechanism causing the lowered threonine deaminase levels is unknown. If a tRNA maturation process has been altered, the structures of several tRNA species might be changed such that either the nonacylated tRNA for isoleucine, valine, or leucine continue to cause repression or tRNA acylated with different amino acids now structurally resemble the normal repressing species enough to cause repression. In vivo determinations of the percentage of different acylated tRNA species as was reported by Lewis and Ames (19) for tRNA^{His} would probably be more effective in answering questions concerning repression

mechanisms than further in vitro amino-acylation assays. The further characterization of this cold-sensitive mutant should prove useful in the understanding of the function and regulation of maturation enzymes as well as being useful in physical studies on tRNA conformations and structure. It is also likely that other previously described mutations involving amino acid regulation may affect tRNA maturation.

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