JOURNAL OF BACTERIOLOGY, Apr. 1977, p. 4-10
Vol. 130, No. 1

Biochemical and Regulatory Properties of *Escherichia coli* K-12 *hisT* Mutants

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Received for publication 14 January 1977

Escherichia coli K-12 *hisT* mutants were isolated, and their properties were studied. These mutants are derepressed for the histidine operon, map close to the *purF* locus at about 49.5 min on the *E. coli* linkage map, and lack pseudouridylate synthetase activity. The defect in this enzyme leads to the absence of pseudouridines in the anticodon loop of several transfer ribonucleic acid species, as evidenced by the altered elution profile on reversed-phase chromatography and resistance to amino acid analogues. Finally, the *hisT* mutants studied have a reduced growth rate that appears to be linked to *hisT*, although it is not known whether it is due to the same mutation. The normal generation time can be restored by supplementing the medium with adenine, uracil, and isoleucine.

Regulation of the histidine operon has been thoroughly investigated in *Salmonella typhimurium* (9, 20). Genetic studies on *Escherichia coli* K-12 histidine auxotrophs (19, 21) have shown that the *his* operon is very similar, if not identical, to that of *Salmonella*. Histidine regulatory mutants of *S. typhimurium* have been found to belong to six different classes, and the physiological significance of almost every class has been elucidated. *hisO* mutants define an operator-promoter region contiguous with the structural genes of the operon (17, 29), whereas the other five classes (*hisR, hisS, hisT, hisU,* and *hisW*) all appear to be related to the structure, aminoacylation, or maturation of histidine transfer ribonucleic acid ($tRNA^{His}$). The involvement of histidyl-tRNA in regulation of the histidine operon was proposed on the basis of experiments showing that interference with the process of aminoacylation of $tRNA^{His}$ produces derepression of the histidine biosynthetic enzymes (31). Study of the *his* regulatory mutants has given this observation a firm biochemical basis (8, 9). The *hisT* class, deficient in a pseudouridylate synthetase (12, 14, 33), is of special interest. The $tRNA^{His}$ of *hisT* mutants lacks two pseudouridines in the anticodon loop (33). Nevertheless, the tRNA is absolutely normal in amount as well as in its acceptor activity, as judged by a variety of kinetic parameters measured in vitro with purified histidyl-tRNA synthetase (11). Furthermore, other tRNA species that have ψ in the anticodon loop also appear to have ψ replaced by U in *hisT* mutants (15, 33), as indicated by the altered mobility of these tRNA species on a reversed-phase chromatographic system. In some cases, the regulation of the respective biosynthetic systems (*his, ilv, leu, tyr,* and *lys*) is impaired, as judged by enzymatic assays (15) or resistance to amino acid analogues (16).

In spite of the extensive knowledge of histidine regulation in *S. typhimurium*, little is known about this system in *E. coli*. Therefore, we have isolated several classes of *E. coli* histidine regulatory mutants (C. B. Bruni, L. Sbordone, and F. Blasi, manuscript in preparation). Because of intrinsic interest in *hisT* mutants and of the advantage of having this mutation in the *E. coli* genetic background, we describe, in this paper, the isolation and characterization of these mutants.

MATERIALS AND METHODS

Bacterial strains, phages, and media. The genotypes of the bacterial strains used in this study are listed in Table 1. Phage P1CM*clr*100 (28) was used for transduction tests. Phages ϕ80*i*λcI857 and ϕ80*i*λcI857*dhis* (2) were used as a source of deoxyribonucleic acid (DNA) for RNA-DNA hybridization.

The liquid media used were minimal medium (medium E of Vogel and Bonner [35] supplemented with 0.5% glucose) and LB broth (25), which for P1 transduction was supplemented as described previously (28). Solid media contained 1.4% agar (Difco Laboratories) and were nutrient broth (0.8% Difco nutrient broth-0.4% NaCl), minimal medium (medium E of Vogel and Bonner [35] supplemented with 2% glucose), and medium A of Schaefler (30). When needed, histidine was supplemented at 0.1 mM; the other amino acids were supplemented at 0.5 mM.

Isolation of mutants. *his* regulatory mutants were isolated by the method of Chang et al. (13).

TABLE 1. *Bacterial strains*

Strain	Genotype[a]	Source or reference
FB1	*hisGDCBHAFIE750 gnd rhaA*	P. Hartman
FB8	Wild-type *E. coli* K-12, UTH1038	22
FB26	*hisC463*	P. Hartman
FB104	*hisO75*	1
FB105	*hisT76*	This paper
FB106	*hisT77*	This paper
FB175	*ilvD105 argH1 xyl-7 supT3*λ^-	This paper
FB176	*ilvD105 argH1 xyl-7 supT3* λ^- *hisT76*	This paper
FB177	*hisT76* (β-gl^{+b})	This paper
FB178	*hisT77* (β-gl^{+b})	This paper
PS911	*ilvD105 argH1 purF71 xyl-7 supT3*λ^-	M. Levinthal
MI159	HfrH *ilvC608 thr-10 pyrA53 thi-1*λ	M. Iaccarino

[a] Genetic symbols according to Bachmann et al. (3).
[b] Able to grow on β-glucosides as the sole carbon source (30).

Overnight cultures of FB8 in minimal medium were mutagenized with diethyl sulfate (Fisher Scientific Co.; 20 μl/10^8 cells per ml) for 30 min at 37°C. Fractions from each mutagenesis were immediately spread on plates containing minimal medium supplemented with 20 mM aminotriazole (Aldrich Chemical Co.). In the center of the plates 20 μl of 0.2 M triazole-L-alanine (Sigma Chemical Co.) was applied on a sterile paper disk. Plates were incubated for 2 to 3 days at 37°C. Wrinkled colonies surviving the selection were isolated, purified on plates containing minimal medium and studied further.

Mutants able to grow on β-glucosides as the sole carbon source were isolated on A plates supplemented with 0.5% salicine, as described previously (30).

Transduction methods. Phage lysates were prepared on plates containing nutrient agar by the confluent lysis method (28) with P1CM*clr*100 at a multiplicity of infection of 5. Transductions were carried out as described elsewhere (28). Recipient strain PS911 is an adenine, valine, isoleucine, and arginine auxotroph (Table 1). PurF$^+$ transductants were isolated on plates containing minimal medium supplemented with isoleucine, valine, and arginine, with selection for adenine prototrophy (PurF$^+$), and the percentage of wrinkled colonies was scored. The wrinkled phenotype of transductants was verified again on minimal medium plates. Similarly, with strain FB26 as the recipient, histidine prototrophy was selected; with strain MI159, isoleucine and valine prototrophy was selected. With strain FB8, which is not able to grow on β-glucosides as the sole carbon source (like wild-type *E. coli* [30]), the β-gl$^+$ phenotype was selected by using A plates supplemented with 0.5% salicine.

Enzyme assays and preparation of cell extracts. The following histidine biosynthetic enzymes were assayed: histidinol phosphate phosphatase (L-histidinol phosphohydrolase; EC 3.1.3.15), the product of the *hisB* gene; and histidinol phosphate transaminase (imidazolylacetol phosphate:L-glutamate aminotransferase; EC 2.6.1.9), a product of the *hisC* gene. Cells were grown to mid-log phase in a rotary shaker in minimal medium at 37°C. After centrifugation at 4°C (20 min, 15,000 × *g*), cells were suspended in 2.5 ml of 50 mM tris(hydroxymethyl)aminomethane buffer (pH 7.5) and disrupted in a French pressure cell at 12,000 lb/in^2. The extract was centrifuged for 15 min at 30,000 × *g* at 4°C, desalted through a Sephadex G-50 coarse column (1 by 7 cm) equilibrated and eluted with 50 mM tris(hydroxymethyl)aminomethane buffer (pH 7.5), and assayed for protein and enzymatic activities. Protein concentrations were estimated by the method of Lowry et al. (23). An assay of histidine enzymes and the units of enzyme activity were described by Martin et al. (24).

Pseudouridylate synthetase activity was assayed by utilizing the tritium-exchange method and using as a substrate total ^{3}H-labeled tRNA isolated from the *S. typhimurium hisT1504* mutant as described elsewhere (14).

Measurement of histidine messenger RNA (mRNA^{His}) levels. Cells were grown in minimal medium with or without histidine. Logarithmically growing cells were labeled for 2 min with 25 μCi of [^{3}H]uridine (specific activity, ca. 50 Ci/mmol) per ml, and the RNA was extracted by the hot-phenol method (7). RNA-DNA hybridization in liquid was performed, as described previously, by using the separated strands of phages ϕ80*i*λ*c*I857 and ϕ80*i*λ*c*I857d*his* (1).

Preparation and aminoacylation of tRNA. Strains FB175 and FB176 (smooth and wrinkled PurF$^+$ transductants, respectively, of strain PS911 with P1 grown on strain FB105) were grown on minimal medium, and the tRNA was extracted and aminoacylated as described elsewhere (10). FB175 tRNA was aminoacylated with [^{14}C]tyrosine, whereas for FB176 the tRNA was aminoacylated with [^{3}H]tyrosine. The reaction was stopped after 30 min at 37°C by the addition of 2 volumes of 1.0 M magnesium acetate and 15 volumes of 75% ethanol at −20°C. Aminoacylated tRNA was spun down, dried, and dissolved in RPC-5 buffer (27) containing 0.6 M sodium chloride.

Column chromatography. An RPC-5 column (1 by 60 cm) was packed and operated as described previously (27) at a pressure of 200 to 300 lb/in^2, giving a flow rate of 1.2 ml/min. Fractions of FB175 tRNA, aminoacylated with [^{14}C]tyrosine, and of

FB176 tRNA, aminoacylated with [^{3}H]tyrosine, were mixed in a 1:6 ratio and chromatographed. tRNA was eluted with a 1.0 to 2.0 M sodium chloride gradient. Sixty fractions (1.6 ml each) were collected, mixed with 3 ml of Insta-gel (Packard), and counted in a Packard liquid scintillation counter.

Analogue resistance test. Cells to be tested were grown in minimal medium or in LB broth. A 0.1-ml volume of a log-phase culture was mixed with 3 ml of top agar (0.6% agar in minimal medium) and layered on a minimal medium plate. After the top agar had solidified, a sterile paper disk was put in the center of the plate, and 20 μl of a 20-mg/ml 4-azaleucine (Calbiochem) solution was applied. Plates were incubated at 37°C, and the diameter of the zone of inhibition was measured after 36 h.

RESULTS AND DISCUSSION

Derepression of the histidine operon. Strains FB105 and FB106 were isolated on the basis of their wrinkled phenotype. In *S. typhimurium*, derepression of the *his* operon leads to a typical wrinkled colony morphology on minimal medium plates supplemented with 2% glucose (26, 29). To test whether the wrinkled colonies of *E. coli* were indeed derepressed for the *his* operon, histidine biosynthetic enzymes were assayed (Table 2). Levels of histidinol phosphate phosphatase (a product of the *hisB* gene) and histidinol phosphate transaminase (the product of the *hisC* gene) were found to be derepressed six- to sevenfold over the level in wild-type *E. coli* FB8.

Derepression of the histidine operon in *S. typhimurium* is due to an increase in the levels of *his* mRNA (22; T. Kasai and P. A. Hartman, unpublished data). We measured the levels of *his* mRNA by hybridization of [^{3}H]uridine-labeled RNA isolated from strains FB104, FB105, and FB8 to the R strand of the DNA from phage ϕ80*i*λ*c*I857*dhis* (which carries the wild-type *E. coli* K-12 *his* operon), subtracting the number of counts hybridized to the R strand of the parental phage ϕ80*i*λ*c*I857 (1) (Table 2). The level of *his* mRNA in strains FB104 and FB105 was eightfold higher than that of FB8 and was not influenced by the presence of histidine in the growth medium (data not shown). Therefore, the greater expression of the histidine operon is not due to any limitation in the supply of the amino acid.

Genetic and biochemical identification. *his* regulatory mutations on the *E. coli* K-12 chromosome were localized by P1 transduction (Table 3). Selected markers were chosen on the basis of known map positions of *S. typhimurium his* regulatory loci (9). Since *hisT* mutants of *S. typhimurium* are linked to *purF* by P22 transduction (29), the mutations in strains FB105 and FB106 which map close to the *purF* marker (Table 3) were thought to be *hisT* mutants as well. The constitutive mutation of strain FB104 (used as a control in these experiments) cotransduces 100% with *his* and is therefore assumed to be an *hisO*^{c} mutation. As an additional control, it was shown that the mutations *hisT76* (FB105 and FB177) and *hisT77* (FB106 and FB178) do not cotransduce with markers located close to other *his* regulatory loci (*his*, *ilv*, and *bgl*).

Proof that FB105 and FB106 are indeed *hisT* mutants comes from the assay of pseudouridylate synthetase in extracts of these cells. The phenotype of *hisT S. typhimurium* mutants is, in fact, due to the absence of this enzyme, which catalyzes the conversion of certain uridylic acid residues to pseudouridylic acid residues in the anticodon loop of several tRNA's (14, 33). [5-^{3}H]uridine-labeled *hisT* total tRNA is a substrate for this enzyme. Incubation of this tRNA with pseudouridylate synthetase releases ^{3}H into the incubation mixture. A measure of tritium release is the basis for the enzymatic assay (14). Incubation of cell extracts from strain FB8 (wild-type *E. coli* K-12 isogenic

TABLE 2. *Levels of histidine biosynthetic enzymes and of his mRNA in hisT mutants*

Strain	Histidinol phosphate transaminase[a]	Derepression (fold)	Histidinol phosphate phosphatase[a]	Derepression (fold)	*his* mRNA[b] (% of total)	Derepression (fold)
FB8	2.5	1.0	5.9	1.0	0.15 ± 0.04	1.0
FB104	22.2	8.8	32.5	5.5	0.95 ± 0.38	6.3
FB105	16.9	6.7	31.5	5.3	1.33 ± 0.59	8.9
FB106	17.5	6.9	28.4	4.8	ND	

[a] Data are expressed as units of enzyme activity per milligram of protein. Enzymatic units were described elsewhere (24).

[b] The data reported are the averages of at least five experiments. *his* mRNA was measured as the difference in [^{3}H]RNA counts hybridized to the R strand of ϕ80*i*λ*c*I857*dhis* minus the counts hybridized to the R strand of ϕ80*i*λ*c*I857 (1). Data are expressed as the percentages of input RNA as determined by trichloroacetic acid precipitation. Typical hybridization experiments, with an input of about 2×10^6 cpm, gave about 3,000 cpm hybridized for FB8 RNA and 20,000 or more cpm for FB104 or FB105. ND, Not done.

TABLE 3. *Cotransduction of hisT mutations to hisC, ilvC, bgl⁺, and purF markers*[a]

Donor	Recipient	Selected marker	Colonies scored	No. wrinkled	No. smooth	Cotransduction (%)
FB104	FB26	His⁺	435	435	0	>99.77
FB105			500	0	500	<0.20
FB106			385	0	385	<0.26
FB105	MI159	Ilv⁺	518	0	518	<0.19
FB106			425	0	425	<0.24
FB177	FB8	*bgl*⁺	290	0	290	<0.34
FB178			350	0	350	<0.29
FB104	PS911	PurF⁺	409	0	409	<0.25
FB105			354	144	210	40.67
FB106			637	131	506	20.56

[a] Phage P1CM*clr*100 was grown on the donor strains. The lysates obtained were used for transduction into the recipient strains indicated in the second column. Transduction methods are described in Materials and Methods and in reference 27.

with strains FB105 and FB106) with ^{3}H-labeled *hisT* tRNA did, in fact, lead to tritium release (Fig. 1). On the other hand, extracts of strain FB105 did not show any pseudouridylate synthetase activity. Analogous results were obtained with strain FB106 (data not shown).

Pleiotropy of the *hisT* mutants. In *S. typhimurium*, the *hisT* gene product is dispensable for the life of the cell, since amber and frameshift mutants have been isolated (12). These mutants display a slightly reduced growth rate in minimal medium which is not due to derepression of the histidine operon, but is presumably due to some other pleiotropic effect of this mutation. The *E. coli hisT* mutants we isolated showed a more-pronounced reduction in their growth rate (Table 4). Strains FB105 and FB106 had approximately a twofold reduction of growth rate in minimal medium. This difference was still apparent in rich medium (LB broth). The isogenic *hisT* transductant FB176 also had a longer generation time (data not shown). These data suggest that the slow growth rates of strains FB105 and FB106 are not due to mutations different from *hisT*. To determine whether a requirement for any specific compound(s) was responsible for the prolonged generation time of *hisT* mutants, we measured the growth rates of these strains in minimal medium supplemented with a variety of solutions containing groups of amino acids, purines, pyrimidines, and vitamins, arranged so as to make possible the identification of the growth-stimulating substance. From this preliminary study, it became apparent that uracil, adenine, isoleucine, methionine, thiamine, and pyridoxine stimulated the growth rates of strains FB105 and FB106, although none to the full extent. Thiamine, pyridoxine, and methionine stimulated both parental strain FB8 and *hisT* mutants; uracil, adenine, and isoleucine

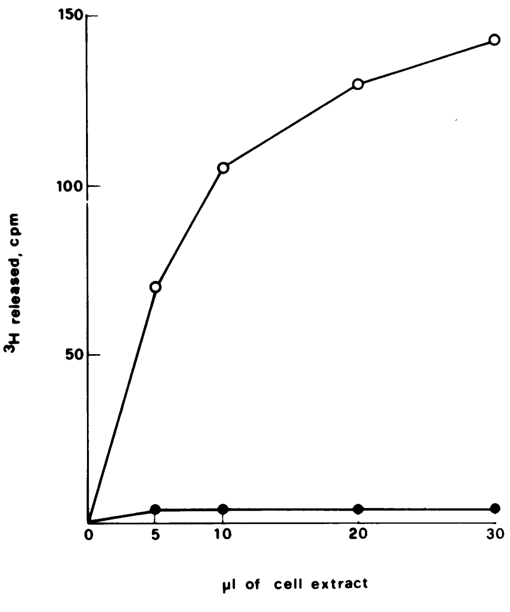

FIG. 1. *Pseudouridylate synthetase activity in the wild type and a hisT mutant of E. coli K-12. Symbols:* ○, *FB8 extract;* ●, *FB105 extract. The incubation mixture contained 40 pmol of tritiated hisT1504 tRNA (1,200 cpm/pmol), 100 mM glycine-sodium hydroxide buffer (pH 9.0), 20 mM* $MgCl_2$*, and different amounts of cell extract from strain FB8 (15.4 mg of protein per ml) and strain FB105 (12.2 mg of protein per ml), as indicated on the abscissa. The cell extracts and* ^{3}*H-labeled tRNA were prepared as described by Cortese et al. (14).*

only stimulated the growth of the *hisT* strains. Although each of these compounds was stimulatory, the greatest stimulation of growth rate was obtained when all six were added together. We therefore measured the generation time of strains FB105 and FB106 in LB broth and in minimal medium, both supplemented with the above-mentioned compounds (Table 4). These supplements restored the growth rates of strains FB105 and FB106 to essentially the level of parental strain FB8, both in minimal medium and in LB broth. These results indicate that the *hisT* mutation per se is responsible for the slow growth rate and that this can be overcome by the simultaneous addition of several compounds (adenine, uracil, and isoleucine). Further investigation is required to understand the basis of this pleiotropic behavior and its correlation with the multiple regulatory defects of the *hisT* mutation.

The modified base pseudouridine (ψ) is present in the anticodon loop of several species of tRNA's (i.e., tyrosine [4], histidine [32], leucine [6], glutamine [18], phenylalanine [5], and methionine [16]). Since aminoacyl-tRNA's seem to be involved in the regulation of several of the corresponding pathways, one would have expected *hisT* mutants to show alterations in the regulatory properties of several amino acid biosynthetic systems, analogous to what happens with the *his* operon. Actually, a pleiotropic effect was observed in *S. typhimurium* well before the genetic lesion was identified. *hisT* mutants, in fact, were found to excrete valine (12). $tRNA^{His}$ from *hisT* mutants was found to be retarded on reversed-phase chromatography column no. 3 (33) as compared with $tRNA^{His}$ from the wild type. Other tRNA's, having ψ in the anticodon loop, also displayed an altered chromatographic mobility ($tRNA^{Tyr}$ $tRNA^{Leu}$), whereas tRNA's that do not have ψ in the anticodon loop behaved like the wild-type tRNA on the same column (i.e., $tRNA^{Val}$) (15, 33). It was concluded, therefore, that the altered chromatographic behavior of tRNA from *hisT* mutants is correlated with the U $\rightarrow \psi$ substitution in the anticodon loop.

To determine whether the pseudouridylate synthetase of *E. coli* acts in the U $\rightarrow \psi$ conversion in more than one tRNA species, we studied the chromatographic behavior of tyrosyl-$tRNA^{Tyr}$ extracted from a *hisT* mutant. For this purpose, strains isogenic except for the *hisT* mutation were constructed by isolating a smooth (FB175) and a wrinkled (FB176) $PurF^+$ transductant of PS911 with P1 grown on strain FB105. tRNA extracted from these cells was aminoacylated with [^{14}C]tyrosine (FB175) and [^{3}H]tyrosine (FB176). A mixture of these tRNA's was chromatographed on an RPC-5 column (Fig. 2). Whereas wild-type $tRNA^{Tyr}$ showed only one peak, *hisT* $tRNA^{Tyr}$ showed two peaks, one coinciding with the wild type and the other being clearly retarded. The occurrence of a retarded $tRNA^{Tyr}$ peak is in agreement with the absence of ψ in the anticodon loop. However, the occurrence of two peaks may signify either that only one of the two $tRNA^{Tyr}$ species of *E. coli* is pseudouridylated by the *hisT*-coded enzyme, or that the enzymatic activity in vivo in the mutant is not totally absent but rather only decreased. The latter possibility seems more likely, since both species of $tRNA^{Tyr}$ from wild-type *E. coli* K-12 have a ψ residue in the anticodon loop (4).

Another pleiotropic effect of the *hisT* mutation in *S. typhimurium* is resistance to certain

TABLE 4. *Generation times of hisT mutants in several media*

Strain	Generation time (min) in:			
	Minimal	LB broth	Supplemented minimal[a]	Supplemented LB broth[a]
FB8	65	35	55	36
FB104	65	36	ND	ND
FB105	127	53	70	39
FB106	132	53	59	43

[a] Minimal medium and LB broth (see Materials and Methods) were supplemented with isoleucine and methionine (0.5 mM each), thiamine and pyridoxine (10 µg/ml each), uracil (80 µg/ml), and adenine (0.4 mM). Cells were grown at 37°C. ND, Not done.

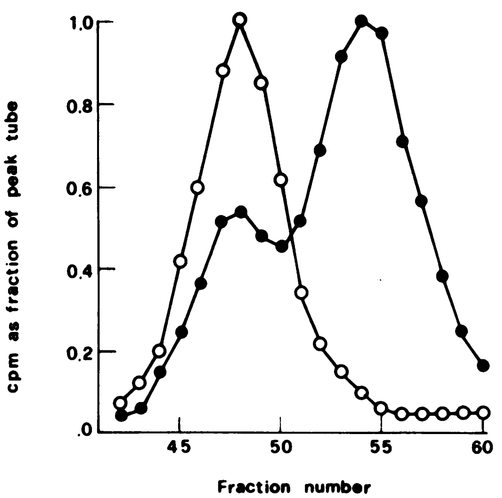

FIG. 2. *Comparison of the elution profiles on a RPC-5 column of tyrosyl-tRNA^Tyr from a pair of isogenic strains differing only in the hisT mutation. Symbols:* ○, *FB175 (hisT*$^+$*), [*^{14}*C]tyrosyl-tRNA*^{Tyr}*;* ●, *FB176(hisT), [*^{3}*H]tyrosyl-tRNA*^{Tyr}*. The applied sample contained 15,000 cpm of tRNA from strain FB175, charged with [*^{14}*C]tyrosine, and 90,000 cpm of tRNA from strain FB176, charged with [*^{3}*H]tyrosine. Peak tubes of* ^{14}*C and* ^{3}*H had 2,500 and 15,000 cpm, respectively.*

TABLE 5. *Resistance of E. coli hisT mutants to 4-azaleucine*[a]

Strain	Area of inhibition (mm)	
	With shift-down	Without shift-down[b]
FB8	25	22
FB104	23	23
FB105	<7	23
FB106	<7	24

[a] For experimental details see the text.

[b] Cells were grown in minimal medium rather than in LB broth.

amino acid analogues such as trifluoroleucine, 3-aminotyrosine, and thialysine (15) in addition to the histidine analogue triazole-L-alanine (29). The basis for this resistance is the increased level of the enzymes for the biosynthesis of histidine (29), isoleucine, leucine, and valine (15). However, resistance to thialysine and 3-aminotyrosine does not appear to be due to measurably increased levels of lysine and tyrosine biosynthetic enzymes (R. Cortese, Ph.D. thesis, University of California, Berkeley, 1973). We investigated the resistance of strains FB105 and FB106 to the leucine analogue 4-azaleucine, since trifluoroleucine is a poor inhibitor of *E. coli* K-12 (34). Analogous to *S. typhimurium*, *E. coli hisT* mutants were resistant to 4-azaleucine in a shift-down experiment (Table 5), i.e., when cells were grown in LB broth and then plated on minimal plates. The *hisO*^{c} strain, FB104, displayed a normal sensitivity to the analogue.

hisT mutants of *E. coli* K-12 are very similar to those of *S. typhimurium* with respect to derepression of the histidine operon, map position, biochemical defect, and pleiotropy. Isolation of these mutants will allow further investigation on the mechanism of gene expression of *E. coli his* operon. Moreover, because the regulation of many amino acid biosynthetic pathways, the biosynthesis and maturation of tRNA's, and the control of cell growth can be studied more easily in *E. coli* than in *S. typhimurium*, the availability of *E. coli* K-12 *hisT* mutants should prove to be quite useful.

ACKNOWLEDGMENT

We are grateful to Robert F. Goldberger and M. S. Ciampi for helpful discussions, and to P. Hartman, M. Iaccarino, and M. Levinthal for providing useful strains.

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