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flrB, a Regulatory Locus Controlling Branched-Chain Amino Acid Biosynthesis in *Salmonella typhimurium*

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Salmonella typhimurium strain CV123 (*ara-9 gal-205 flrB1*), isolated as a mutant resistant to trifluoroleucine, has derepressed and constitutive levels of enzymes forming branched-chain amino acids. This strain grows more slowly than the parent at several temperatures, both in minimal medium and nutrient broth. It overproduces and excretes sizeable amounts of leucine, valine, and isoleucine in comparison with the parental strain. Both *leuS* (coding for leucyl-transfer ribonucleic acid [tRNA]synthetase) and *flrB* are linked to *lip* (min 20 to 25) by P1 transduction, whereas only *leuS* is linked to *lip* by P22 transduction. Strain CV123 containing an F' *lip*$^+$ episome from *Escherichia coli* has repressed levels of leucine-forming enzymes, indicating that *flrB*$^+$ is dominant to *flrB*. Leucyl-tRNA synthetase from strain CV123 appears to be identical to the leucyl-tRNA synthetase in the parent. No differences were detected between strain CV123 and the parent with respect to tRNA acceptor activity for a number of amino acids. Furthermore, there was no large difference between the two strains in the patterns of leucine tRNA isoaccepting species after fractionation on several different columns. Several other *flrB* strains exhibited temperature-sensitive excretion of leucine, i.e., they excreted leucine at 37 C but not 25 C. In one such strain, excretion at 37 C was correlated with derepression of some enzymes specified by *ilv* and *leu*. These latter results suggest that *flrB* codes for a protein.

Among mutants of *Salmonella typhimurium* resistant to 5',5',5'-trifluoro-DL-leucine are found strains in which the regulation of branched-chain amino acid biosynthesis is altered (10). These strains, identified initially as leucine excretors, have been divided into several groups on the basis of genetic and biochemical tests. Two groups (operator constitutive and feedback negative) had mutation sites linked to the leucine operon (3, 11). Another large group of mutants was found to have mutation sites in the *purE-gal* region of the genome (1). Among the latter were several *leuS* mutants having an altered leucyl-transfer ribonucleic acid (tRNA)-synthetase (L-leucine:tRNA ligase [adenosine monophosphate], EC 6.1.1.4) (2). We have discovered recently that another regulatory locus, designated *flrB*, also lies in the *purE-gal* region. This report deals with the isolation and characterization of *flrB* mutants.

MATERIALS AND METHODS

Strains, media, and culture techniques. All *Salmonella* strains used in this study (Table 1) were derivatives of *S. typhimurium* LT2 (LT2 and *ara-9 gal-205* are referred to as the wild type). A minimal salts solution (SSA) contained (per liter of distilled water): K_2HPO_4, 10.5 g; K_2HPO_4, 4.5 g; $(NH_4)_2SO_4$, 1.0 g; sodium citrate dihydrate, 0.47 g; and $MgSO_4$, 0.05 g. SSA supplemented with 0.2% glucose served as a liquid medium, and with the addition of 1.5% agar it served as a solid medium. A tryptone broth (L broth) contained: tryptone, 1%; yeast extract, 0.5%; and NaCl, 1% (pH 7.0). L broth supplemented with 1.5% agar and 10^{-2} M $CaCl_2$ was used as a solid medium for selection of phage FO-resistant colonies. SSA containing 0.5% glycerol, 0.1% 2-deoxy-D-galactose, and 1.5% agar was used for selection of 2-deoxy-D-galactose-resistant colonies. Plates for the assay of leucine excretion were prepared as described previously (10). Cells were grown aerobically in a rotary shaker, usually at 37 C.

Genetic methods. Transductions mediated by P22 phage were carried out with media and methods described by Margolin (20).

P1-mediated transductions were performed by the procedures of B. Stocker as communicated by C. Beck and E. Lederberg. Strains of *S. typhimurium* sensitive to P1 phage were isolated as follows. Colonies resistant to phage FO or to 2-deoxy-D-galactose were grown to log phase in L broth containing 10^{-2} M $CaCl_2$, and a portion was mixed with P1 phage (multiplicity of about 5) previously grown on a *S. typhimurium* host. After incubation for 50 min at

TABLE 1. *Bacterial strains*

Strain	Genotype	Remarks	Source
Salmonella typhimurium LT2			
CV468	*ara-9 gal-205*	This strain and parent LT2 considered wild type	P. Margolin
CV123	*ara-9 gal-205 flrB1*	High constitutive levels of leucine, valine, and isoleucine-forming enzymes	
CV117	*ara-9 gal-205 leuS2*	Altered leucyl-tRNA synthetase resulting in high levels of leucine, valine, and isoleucine-forming enzymes	
CV357	*ara-9 gal-205 leuS3*	Teperature-sensitive leucyl-tRNA synthetase	
CV114, -115, -116, -127, -131, -132	*ara-9 gal-205 leuS*		
CV124, -125, -129, -130	*ara-9 gal-205 flrB*		
Hfr SU576	*purC7 strA*	Origin at 120 min, order of injection: *O-ara-gal*	H. O. Smith
SA342	*ara-9 lip-2*		K. Sanderson
	purE8		K. Sanderson
	glt-3		K. Sanderson
NE37	*uut-1 bio-25*		B. Magasanik
JL396	*galE*		C. Beck
NE324	*hut-162 pur-1262 thyA1047 thyR1050/F' gal⁺ hut⁺ bio⁺* (P376)		B. Magasanik
CV417	*ara-9 gal-205 flrB1*	Strain CV418 cured of its episome	
CV418	*ara-9 gal-205 flrB1/F' lac⁺ purE⁺ lip⁺*	Episome from ORF4 transferred to CV123	
CV423	*ara-9 gal-205 flrB1/F' lac⁺ purE⁺*	Episome from TR118 transferred to CV123	
CV424	*ara-9 gal-205 flrB1/F' gal⁺*	Episome from NE324 transferred to CV123	
CV425	*ara-9 gal-205 flrB1/F' gal⁺ lip⁺*	Episome from F152/KL253 transferred to CV123	
Escherichia coli			
ORF4	*F' lac⁺ purE⁺ lip⁺*	Requires serine or glycine for growth	R. Curtiss
TR118	*purE801 hisD27 trpA8 mtl/F'$_{18}$ lac⁺ purE⁺*		J. Roth
F152/KL253	*tyrA2 pyrD34 thi-1 his-68 trp-45 recA1 mtl-2, xyl-7 malA1 galK35 str-118 λ^R, λ⁻/F' gal⁺ lip⁺*		B. Low

37 C, a drop of each mixture was placed on a selective medium. P1-sensitive clones gave rise to transductants within the growth area after incubation at 37 C for 48 h. Such clones were purified and retested as recipients in P1-mediated crosses. From 100 survivors of FO phage or 2-deoxygalactose exposure, 2 to 20 P1-sensitive isolates can be expected.

Interrupted mating experiments were carried out by the procedure of Sanderson and Demerec (24). For plate matings, equal volumes of donor and recipient grown to stationary phase in nutrient broth were mixed, diluted, and plated on minimal agar plates containing arabinose.

Partial diploid strains were prepared as follows. Recipients were grown to stationary phase in minimal medium containing appropriate supplements. Donor strains were grown as follows. Strain TR118 was grown to stationary phase with aeration in supplemented minimal medium; strain NE324 was grown to stationary phase with aeration in nutrient broth; strain ORF4 was grown to stationary phase without shaking in L broth containing 0.1% glucose; strain F152/KL253 was grown to stationary phase in nutrient broth. They were then diluted 20-fold into minimal medium lacking citrate and containing galactose as carbon source, incubated for 24 h, centrifuged, and suspended in L broth containing 0.1% glucose, and incubated 1.5 h (all without aeration). Donor and

recipient cells were mixed in a ratio of 1:5, incubated for 1 h at 37 C without shaking, and plated on minimal agar plates containing 0.2% lactose or galactose as the sole carbon source. The number of recombinants was very low in most *Escherichia-Salmonella* crosses. In particular, heterologous crosses involving donors ORF4 and F152/KL253 yielded 10^6 times more recombinants with *Escherichia* recipients than with *Salmonella* recipients. To introduce the F152 episome into strain CV123, we used as a recipient a strain derived from strain CV123 (strain CV417) that had been cured of the ORF4 episome. Strains were cured in episomes by treatment with acridine orange, by the procedure of Miller (22), and subsequent screening of isolated colonies for Gal$^-$ isolates.

Analysis of amino acid excretion. Excretion of leucine was determined qualitatively by an auxanographic technique (10). Quantitative determination of amino acids in culture filtrates was carried out with an amino acid analyzer (21).

Enzyme assays. The growth, harvesting, and disruption of cells (21) and the assays for α-isopropylmalate synthetase (8), β-isopropylmalate dehydrogenase (7), α-acetohydroxy acid synthase (27), and threonine deaminase (14) have been described previously. Cells were stored frozen prior to the assay for the first two enzymes. For the last two enzymes, fresh cells were employed. Protein was determined by the method of Lowry et al. (19), with bovine serum albumin as standard.

Isolation, fractionation, and assay of tRNA. Cells were grown in minimal medium at 37 C to late log or stationary phase. Bulk tRNA (150 mg/100 g [wet weight] of cells; absorbancy [A_{260}/A_{280}], about 1.95) was isolated by the procedure of Silbert et al. (26). Solutions of LiCl used to wash and elute diethylaminoethyl (DEAE)-cellulose columns contained 20 mM magnesium acetate and 10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5. tRNA preparations were stripped of amino acids by treatment with 1.8 M Tris-hydrochloride (pH 8.0) at 37 C for 45 min (25).

tRNA was chromatographed on reversed-phase columns either at pH 7 (28) or after charging with leucine at pH 4.5 (15). When columns were eluted at pH 7, the concentration of $tRNA^{Leu}$ in effluent fractions was determined by complete esterification with [^{14}C]leucine as described previously (21). Prior to chromatography at pH 4.5, tRNA was charged as follows. A 1-ml reaction mixture contained: 50 mM Tris-hydrochloride, pH 7.4; 0.5 mM ethylenediaminetetraacetate (EDTA), pH 7.0; 5 mM $MgCl_2$; 2.5 mM K_4-adenosine triphosphate (K_4ATP), pH 7.0; 0.2 mg of tRNA; 0.02 mM L-leucine (^{14}C, 342 mCi/mmol or ^{3}H, 3,000 mCi/mmol); and enzyme extract (an amount which gave complete charging of the tRNA). The extract was prepared by the method of Leis and Keller (17), except that 56 g of ammonium sulfate per 100 ml was used and the DEAE-cellulose column was eluted with 0.2 M KCl. Parallel reaction mixtures were prepared, one containing [^{14}C]leucine and tRNA from one strain and the other containing [^{3}H]leucine and tRNA from another strain. After incubation for 5 min at 37 C, the samples were chilled, mixed, and applied to a 3-ml DEAE-cellulose column equilibrated with 50 mM NaCl, 10 mM $MgCl_2$, and 10 mM sodium acetate, pH 4.5 (buffer). The column was rinsed with 15 ml of 0.25 M NaCl in buffer and eluted with 0.7 M NaCl in buffer until at least 90% of the radioactivity was collected. The sample was applied to a reversed-phase column (0.9 by 70 cm) (15) after the NaCl was diluted to 0.5 M. Fractions (1.25 ml) were collected at a flow rate of 60 ml/h (175 lb/in^2. To each fraction was added 0.025 ml of 0.4 M KOH, and after 30 min at room temperature (complete hydrolysis of leucine from tRNA) 0.025 ml of 0.4 M acetic acid was added, followed by 5 ml of Aquasol (New England Nuclear Corp.). ^{3}H and ^{14}C were determined by liquid scintillation counting.

For the amino acid acceptor assays described in Table 8, 1 ml of reaction mixture contained: 10 mM Tris-hydrochloride, pH 7.4; 0.1 mM EDTA, pH 7; 10 mM magnesium acetate; 2.5 mM K_4ATP, pH 7; and 0.05 mM ^{14}C-labeled amino acid, 10 μCi/μmol. A crude extract of *S. typhimurium*, freed from ribosomes by centrifugation at 160,000 $\times$ *g* for 30 min and from small molecules by passage through Sephadex G25 (equilibrated and eluted with 10 mM Tris-hydrochloride [pH 7.5] and 0.1 mM dithiothreitol), served as a source of aminoacyl-tRNA synthetases. Conditions for complete esterification of each amino acid were determined by incubating reaction mixtures for 10 min with increasing amounts of extract.

RESULTS

Isolation, growth rate, and pattern of amino acid excretion of strain CV123. Strain CV123 (*ara-9 gal-205 flrB1*) was selected from the parent (*ara-9 gal-205*) as a mutant resistant to trifluoroleucine after mutagenesis with 2-aminopurine (10). The mutation conferring resistance was given the allele designation *flrB1*. The growth rate of strain CV123 was measured in minimal medium and nutrient broth (Table 2). The mutant grew more slowly than the

TABLE 2. *Growth rate of strain CV123*

Strain	Temp (C)	Generation time (min)[a] on minimal medium	Temp (C)	Generation time (min)[a] in nutrient broth
Parent	25	89	30	22
CV123	25	127	30	30
Parent	37	47	37	17
CV123	37	57	37	23
Parent	42	60	42	23
CV123	42	300	42	33

[a] Time required for cells to double in number. Each value represents the average of at least two determinations. Generation times were determined as described by Mikulka et al. (21).

parent in both media at several different temperatures.

Culture filtrates from the parent and strain CV123 were analyzed in an amino acid analyzer (Table 3). Strain CV123 overproduced and excreted appreciable quantities of leucine, valine, and isoleucine in comparison with the parent. No differences between the two strains were observed for the other naturally occurring amino acids.

To test the possibility that the slow growth rate of strain CV123 was due to a second mutation unrelated to one affecting regulation, the following experiment was done. A plate mating was carried out between Hfr SU576 (*purC7*) and CV123 (*ara-9 gal-205 flrB1*), and selection was made for the ability to utilize arabinose as the sole carbon source. Recombinants were purified and tested for leucine excretion and growth rate. For 30 recombinants tested (10 of them were excretors), leucine excretion was associated with a slow growth rate and a lack of excretion was correlated with a normal growth rate. These results indicate that the two phenotypes are caused by either a single mutation or two closely linked mutations.

Levels of pathway-specific enzymes in strain CV123. The pathways leading to the branched-chain amino acids are shown in Fig. 1. The specific activities of two leucine-forming and three isoleucine and valine-forming enzymes were determined for strain CV123 grown in minimal medium and in the same medium supplemented with branched-chain amino acids (Table 4). All of these enzymes were highly derepressed in strain CV123 grown in minimal medium, and the addition of leucine, valine, and isoleucine did not cause significant repression. No fluoroleucine-resistant mutant analyzed in this laboratory has enzyme levels higher than those reported here for strain CV123.

TABLE 3. *Excretion of amino acids by strain CV123*

Strain	Cells/ml	Amino acid excreted per cell (10^{-12} μmol)[a]		
		Leucine	Valine	Isoleucine
Parent	3.0×10^9	0.4	10	0.8
Parent	3.0×10^9	0.8	2	1
CV123	2.5×10^9	49	463	18
CV123	3.3×10^9	21	403	11

[a] Determined by the procedure of Mikulka et al. (21). Histidine, lysine, arginine, threonine, proline, cysteine, methionine, tyrosine, phenylalanine, and tryptophan were undetected in culture filtrates from either strain. Less than 10^{-12} μmol alanine, aspartic acid, glycine, and serine per cell were observed in culture filtrates from both strains.

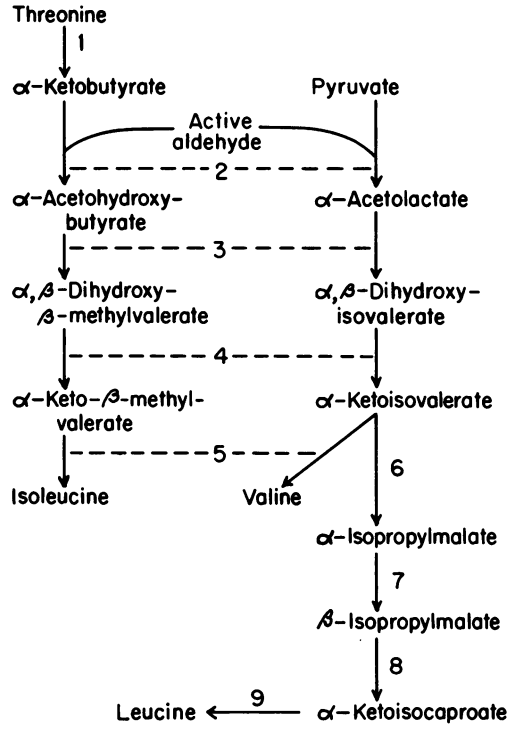

FIG. 1. *Pathways leading to the branched-chain amino acids.*

Map location of flrB. The approximate location of *flrB* was determined by interrupted mating experiments in crosses between strains CV123 (*ara-9 gal-205 flrB1*) and Hfr SU576 (*purC7*). Ara$^+$ Pur$^+$ recombinants were scored for galactose utilization and leucine excretion. In these experiments and in others involving strain CV117 (*ara-9 gal-205 leuS2*), no difference could be detected between the time of entry of *gal*$^+$ and *flr*$^+$ or *leuS*$^+$ (ca. 47 min, corresponding to a map position of ca. 22 min). These data suggested that *leuS* and *flrB* were close to each other and to *gal*.

The isolation of a mutant having a temperature-sensitive leucyl-tRNA synthetase (21) made it possible to determine whether the mutation in strain CV123 was, in fact, a *leuS* allele. P22 phage grown on strain CV123 were mixed with cells of the temperature-sensitive strain CV357 (*ara-9 gal-205 leuS3*) and plated at the restrictive temperature (40 C). None of the recombinants growing at 40 C had the leucine excretion phenotype of the donor (Table 5). Thus, *flrB* and *leuS* are distinct loci which are not co-transducible by P22 phage. Further-

TABLE 4. *Levels of some enzymes involved in leucine, valine, and isoleucine biosynthesis*

Strain	Growth condition[a]	Sp act[b] of enzymes involved in the biosynthesis of:				
		Leucine		Isoleucine and valine		
		α-IPM synthase	β-IPM dehydrogenase	Threonine deaminase	Dihydroxyacid dehydrase	α-Acetohydroxy acid synthetase
Parent	M	1.0 (5)[c]	5.6 (5)[c]	15.6 (3)[c]	4.8 (1)[c]	1.9 (2)[c]
Parent	MIVL	0.26 (1)	1.9 (4)	6.4 (3)	2.6 (1)	0.79 (2)
CV123	M	12.5 (2)	78 (7)	56 (4)	20.2 (1)	47 (4)
CV123	MIVL	15.5 (4)	70 (7)	48 (4)	22.4 (1)	48 (4)
CV129	M		65.0 (2)	46.0 (3)		23.4 (3)
CV129	MIVL		62.0 (2)	28.4 (3)		20.0 (3)
CV124	M		45.5 (2)	32.6 (2)		10.7 (2)
CV124	MIVL		34.7 (2)	14.4 (2)		8.7 (2)

[a] Strains were grown in either minimal medium (M) or in supplemented minimal medium (MIVL) containing L-leucine and L-isoleucine (each at 50 μg/ml) and L-valine (100 μg/ml).
[b] Micromoles of product formed per hour per milligram of protein. IPM, Isopropylmalate.
[c] Numbers in parentheses refer to the number of determinations on separate cultures.

TABLE 5. *Transductions mediated by P22 phage*

Phage grown on strain	Recipient[a]	No. of transductants analyzed	Percentage having donor unselected marker[b]
CV123 (*flrB1*)	CV357 (*leuS3*)	1,531	<0.07
CV123 (*flrB1*)	*purE8*	642	<0.2
CV123 (*flrB1*)	*glt-3*	1,000	<0.1
CV123 (*flrB1*)	*bio-25*	599	<0.2
CV123 (*flrB1*)	*lip-2*	1,000	<0.1
LT2 (*flr*$^+$)	CV123 (*gal-205 flrB1*)	1,000	<0.1
CV117 (*leuS2*)	CV357 (*leuS3*)	863	98
CV117 (*leuS2*)	*purE8*	347	<0.3
CV117 (*leuS2*)	*glt-3*	800	<0.1
CV117 (*leuS2*)	*bio-25*	583	<0.2
CV117 (*leuS2*)	*lip-2*	1,000	57
LT2 (*flr*$^+$)	CV117 (*gal-205 leuS2*)	950	<0.1

[a] Transduction mixtures were plated on minimal agar, and selection was made for the wild-type allele of the recipient marker shown. For strain CV357, this was the ability to grow at 40 C and for strains CV117 and CV123, it was the ability to use galactose as a carbon source (citrate omitted from medium). Strain SA342 (*lip-2*) was grown, prior to transduction, in nutrient broth containing 4 mM sodium acetate and 4 mM sodium succinate, and plated on minimal agar lacking citrate and containing 50 mM sodium succinate.
[b] In all crosses, recombinants were scored for leucine excretion by an auxanographic test. *flrB1*, *leuS2*, Leucine excretion. *flr*$^+$, *leuS*$^+$, No leucine excretion.

more, *flrB* was not jointly transduced by P22 phage with other markers in the *gal* region (*purE*, *glt-3*, *bio-25*, *lip-2*, *gal*) (Table 5, Fig. 2).

Similar P22-mediated transduction crosses were carried out with phage grown on strain CV117 (*leuS2*). These crosses indicated (Table 5) that *leuS* is linked to *lip-2*, as is found in *E. coli* (18). A number of other fluoroleucine-resistant strains were tested as donors in crosses with strains *lip-2* and CV357 as recipients. In this way, the following strains were identified as probably having *leuS* mutations: CV114, CV115, CV116, CV127, CV131, and CV132.

Through recent advances in the laboratory of B. Stocker, it has become possible to transduce *S. typhimurium* with the *E. coli* K-12 phage, P1 (method communicated by C. Beck and E. Lederberg). This method, although requiring the isolation of P1-sensitive derivatives of both donor and recipient strains, offers great advantage because P1 is known to carry a larger piece of deoxyribonucleic acid than does P22 (23). The first cross in Table 6 demonstrates that the method of scoring the leucine excretion phenotype is valid—no excretors were observed among Lip$^+$ recombinants isolated after transduction with wild-type P1 phage. In the second cross, *leuS* and *lip-2* were found to be 74% co-transducible by P1 phage (compare with 57%

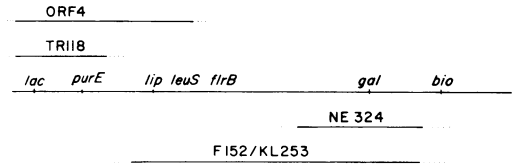

FIG. 2. *Map of the purE-gal region. The short lines represent episomes from E. coli (ORF4, TR118, F152/KL253) and S. typhimurium (NE324). glt-3 (not shown) is located somewhere near gal (1). As explained in the text, leuS and flrB are probably to the right of lip.*

TABLE 6. *Transductions mediated by P1 phage*

Phage grown on strain	Recipient[a]	No. of trans-transductants analyzed	Percentage having donor unselected marker[b]
Wild type (JL396)	*lip-2*	1,300	<0.08
CV117 (*leuS2*)	*lip-2*	749	74
CV123 (*flrB1*)	*lip-2*	1,050	10
CV124 (*flrB2*)	*lip-2*	550	10
CV125 (*flrB3*)	*lip-2*	1,000	15
CV129 (*flrB4*)	*lip-2*	1,000	14
CV130 (*flrB5*)	*lip-2*	200	12
Wild type (JL396)	CV357 (*leuS3*)	375	<0.3
CV123	CV357 (*leuS3*)	800	20
CV124	CV357 (*leuS3*)	850	22
CV125	CV357 (*leuS3*)	900	24
CV129	CV357 (*leuS3*)	550	20
CV117	*purE8*	885	<0.1
CV123	*purE8*	1,000	<0.1
CV129	*purE8*	900	<0.1
purE8	*lip-2*	1,400	<0.08
glt-3	*lip-2*	400	<0.3

[a] Recipient strains were grown in L broth containing 10^{-2} M $CaCl_2$, and transduction mixtures were plated on minimal agar. Selection was made for the wild-type allele of the recipient marker shown. For strain CV357, this was the ability to grow at 40 C. Transduction mixtures involving SA342 (*lip-2*) were plated on minimal agar lacking citrate and containing 50 mM sodium succinate.

[b] In all crosses except the last two, recombinants were scored for leucine excretion by an auxanographic test. *flrB*, *leuS2*, Leucine excretion. *flr*$^+$, *leuS*$^+$, No leucine excretion. In the last two crosses, *lip*$^+$ recombinants selected on plates containing adenine or glutamate were scored for growth on unsupplemented minimal agar plates.

co-transduction by P22 phage). The third cross in Table 6 establishes that *flrB* is also linked to *lip*$^+$ by P1 transduction, although at a lower frequency than *leuS* (10% versus 74%). Similar crosses employing P1 phage grown on other fluoroleucine-resistant strains identified strains CV124, CV125, CV129, and CV130 as probable *flrB* mutants (Table 6). These data exclude the map relationship *lip-flrB-leuS* and establish the relative order as *flrB-lip-leuS* or *lip-leuS-flrB*. The latter relative order is indicated from the second set of crosses in Table 6 (crosses 7 through 11): *flrB* is more closely linked to *leuS* (ca. 20%) than to *lip-2* (ca. 10%). The last five crosses in Table 6 indicate that neither *leuS* nor *flrB* is linked to *purE* and that neither *purE* nor *glt-3* are linked to *lip-2*. The absolute order cannot be established from these crosses and is either *purE-flrB-leuS-lip-gal* or *purE-lip-leuS-flrB-gal*.

Diploid analysis. In Fig. 2 are represented the episomes that were useful in this study. The presence of bacterial genes on these episomes was verified by crosses with appropriate recipients. For example, strain TR118 can transfer *lac*$^+$ and *purE*$^+$ (but not *lip*$^+$ and *gal*$^+$) to recipients, and the resulting recombinants are partial diploids as evidenced by curing with acridine orange. These episomes were then transferred to a number of *S. typhimurium* recipients to test for dominance of *leuS* and *flrB*. In each cross, selection was made for lactose utilization or galactose utilization. Recombinants were classified as partial diploids if they satisfied the following criteria: (i) they had the relevant recipient markers, (ii) they were *Salmonella* strains as evidenced by Simmons citrate agar tests and sensitivity to P22 phage, (iii) they served as donors of *lac*$^+$ or *gal*$^+$ genes to appropriate recipients, and (iv) they lost the ability to utilize lactose or galactose when grown in the presence of acridine orange. Haploid and partial diploid strains were assayed for the specific activity of β-isopropylmalate dehydrogenase (Table 7). In cases where the partial diploids were unstable, cells were grown into log phase and stored at 4 C while diagnostic tests were made. If fewer than 90% of the cells in the culture could utilize lactose or galactose, the culture was discarded. Episomes from strains ORF4, TR118, and NE324 had little effect upon the levels of one of the enzymes in leucine biosynthesis. The presence of the episome from strain F152/KL253, however, caused repression of this enzyme to near wild-type levels. We conclude that *flrB*$^+$ is dominant to *flrB* and that the product of an *flrB*$^+$ gene from *E. coli* (F152/KL253 is an *E. coli* strain) functions in a *S. typhimurium* background. These data also suggest that the absolute gene order is *purE lip flrB gal*. However, this conclusion must be considered tentative because in other studies (J. Jones, unpublished data), the episome from strain ORF4 apparently carries *leuS*$^+$ but does not totally complement a *leuS* strain.

Leucyl-tRNA synthetase from strain CV123. Some mutations affecting the structure of leucyl-tRNA synthetase are known to result in derepressed levels of enzymes functioning in branched-chain amino acid biosynthesis (2). For this reason, it was important to determine whether the phenotype of strain CV123 (derepressed enzyme levels) was in any way connected with leucyl-tRNA synthetase. Accordingly, a number of properties of the latter enzymes were examined, both in crude extracts and 100-fold-purified preparations (21) from the parent and strain CV123. No differences between the two strains were obtained for any of the following parameters: K_m values for leucine, ATP, and four $tRNA^{Leu}$ species (21); pH op-

TABLE 7. *Specific activity of β-isopropylmalate dehydrogenase in haploid and partial diploid strains*

Strain	Episome from strain	Genotype	Sp act of β-isopropylmalate dehydrogenase[a]
CV123		*ara-9 gal-205 flrB1*	43, 75
CV423	TR118	*ara-9 gal-205 flrB1*/F' *lac*$^+$ *purE*$^+$	79
CV123		*ara-9 gal-205 flrB1*	30
CV424	NE324	*ara-9 gal-205 flrB1*/F' *gal*$^+$	32
CV123		*ara-9 gal-205 flrB1*	26
CV418	ORF4	*ara-9 gal-205 flrB1*/F' *lac*$^+$*purE*$^+$*lip*$^+$	33
CV417[b]		*ara-9 gal-205 flrB1*	52, 70, 74
CV425	F152/KL253	*ara-9 gal-205 flrB1*/F' *gal*$^+$ *lip*$^+$	8.4, 10, 9.2
ara-9 gal-205			5.9

[a] Cells were harvested in late log phase from minimal medium. Specific activity equals micromoles per milligram of protein per hour.

[b] This strain was prepared by curing strain CV418 of its episome.

timum; activation energy; and rate of inactivation at 49.5 C. The conditions of these experiments and the values for the parameters are given in reference 21. We conclude that the altered regulatory patterns observed for strain CV123 do not result from any alteration in leucyl-tRNA synthetase.

tRNA from strain CV123. Mutations affecting both the amount and structure of $tRNA^{His}$ are known to result in derepressed levels of histidine-forming enzymes (6). It was important therefore to determine whether the altered regulatory properties of strain CV123 had a similar basis. tRNA was isolated from the parent and strain CV123, and amino acid acceptor activity was determined for a number of amino acids (Table 8). Acceptor activity for leucine was not reduced in tRNA isolated from strain CV123 compared to tRNA isolated from the parent. Furthermore, although there were differences in acceptor activity for some of the amino acids tested, these differences were not large and in both directions. We do not consider them significant.

Bulk tRNA contains a number of isoaccepting $tRNA^{Leu}$ species, and an alteration in a single species might not be detected in the experiments described above. For this reason, tRNA from the parent and mutant was fractionated separately on reversed-phase columns (28) at pH 7.0, and leucine acceptor activity was measured on individual fractions. The patterns of isoaccepting $tRNA^{Leu}$ peaks were similar for the two strains. A more sensitive test was performed by charging tRNA from the parent and mutant with [^{14}C]leucine and [^{3}H]leucine, respectively, mixing the two preparations, and chromatographing the mixture on reversed-phase columns at pH 4.5 (15; Fig. 3). Although there were occasional aberrations observed in the $^{14}C/^3H$ ratios of some peaks (eg., second peak from the right in Fig. 3), no consistent differences were observed in $tRNA^{Leu}$ patterns for the two strains with this procedure. The shoulder on peak 1 in Fig. 3 was consistently observed and, in fact, could be completely resolved by chromatography on a longer column. Thus, there may be six species of $tRNA^{Leu}$ in *S. typhimurium*.

E. B. Keller has also fractionated tRNA from these strains on benzoylated-diethylaminoethyl

TABLE 8. *Amino acid acceptor activity of tRNA from the parent and strain CV123*

Amino acid	Expt	Acceptor activity of tRNA from[a]		Normalized ratio[b]
		Parent	CV123	
Leucine	1	1,021	1,358	
Phenylalanine		1,015	1,128	0.84
Histidine		468	462	0.74
Threonine		134	142	0.80
Methionine		299	433	1.09
Leucine	2	1,216	1,428	
Arginine		1,000	1,064	0.91
Aspartate		630	804	1.09
Glutamate		504	579	0.98
Serine		944	860	0.78
Tyrosine		934	1,250	1.14
Glycine		1,079	1,451	1.15
Alanine		1,026	931	0.77
Valine		1,256	1,693	1.15

[a] Counts per minute bound to 0.2 mg of tRNA.

[b] $[(cpm_L/cpm_X)_{parent}]/[(cpm_L/cpm_X)_{CV123}]$, where cpm denotes counts per minute, *L* denotes leucine, and *X* denotes the amino acid being compared to leucine.

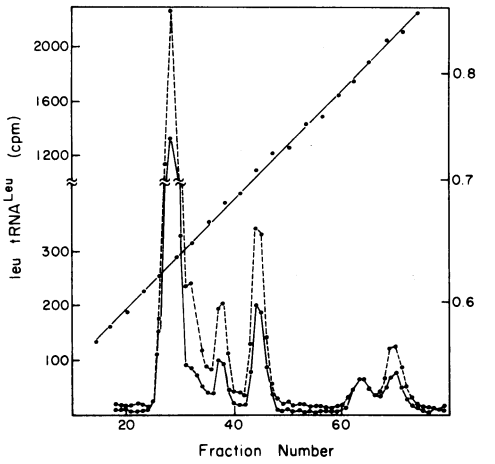

FIG. 3. *Co-chromatography of leucyl tRNA^{Leu} from strain CV123 (●——●) and parental strain ara-9 gal-205 (●- - -●) on a reversed-phase column.*

cellulose and has observed no significant differences in the patterns of leucine acceptor activity (personal communication). In summary, we have no evidence that the mutation in strain CV123 affects the amount or structure of tRNA^{Leu}.

Levels of pathway-specific enzymes in other flrB strains. Most of the work reported here was done with strain CV123. The data from transduction studies (Table 6), although not definitive, suggest that strains CV124, CV125, CV129, and CV130 also have mutations in *flrB*, and in this paper we assume that this is the case. The levels of some pathway-specific enzymes for strains CV124 and CV129 are shown in Table 4. They are generally lower than the corresponding values for strain CV123. Furthermore, threonine deaminase levels in these strains are partially repressed, though not to wild-type levels.

Does flrB specify a protein? Two types of data are often cited as indicating that a locus specifies a protein: (i) suppression of a phenotype by nonsense suppressors and (ii) identification of a strain having a temperature-sensitive phenotype. Having the most extreme phenotype (highest enzyme levels), strain CV123 (*ara-9 flrB1*) was singled out for tests of nonsense suppression. One *leu* ochre mutation and two *leu* amber mutations were transduced into strain CV123, selection being made for arabinose utilization. Prototrophic revertants carrying amber or ochre suppressors were then isolated and identified by the procedure of Berkowitz et al. (4). None of eight revertants tested had levels of pathway-specific enzymes characteristic of the wild type. Thus, *flrB1* is probably not a nonsense mutation.

To search for temperature-sensitive phenotypes, we screened strains resistant to trifluoroleucine for types excreting leucine at 37 C but not at 26 C, and vice versa. Of 116 strains tested, 15 did not excrete leucine at either temperature, 79 excreted leucine at both temperatures, 22 excreted leucine at 37 C but not at 26 C, and none excreted leucine at 26 C but not 37 C. Linkage tests with P22 phage indicated that 7 of the 22 temperature-sensitive strains had *leuS* mutations. Seven of the remaining 15 strains were tested for linkage to *lip* by P1 transduction; all 7 were judged to have *flrB* mutations. Representative data for one strain, CV130, are given in Table 6. As suspected, the level of pathway-specific enzymes in strain CV130 increases with increasing temperature of growth (Fig. 4). We conclude that *flrB* probably specifies a protein.

DISCUSSION

This work clearly establishes *flrB* as a unique locus affecting the regulation of enzymes involved in branched-chain amino acid biosyn-

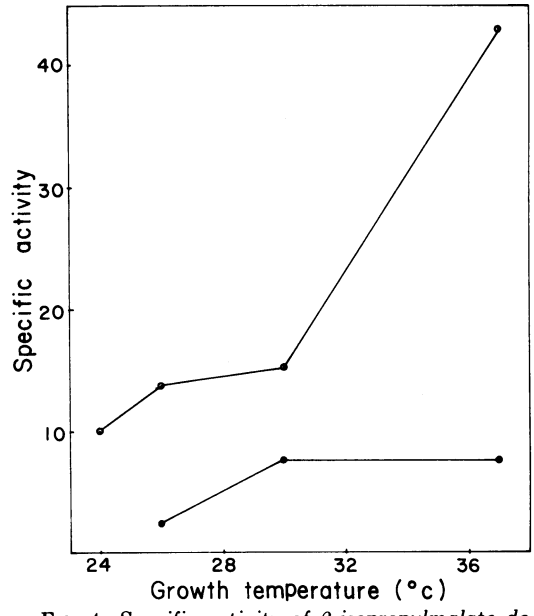

FIG. 4. *Specific activity of β-isopropylmalate dehydrogenase (○) and α-acetohydroxyacid synthetase (●) in crude extracts of strain CV130 as a function of growth temperature. The levels of these enzymes in the parent strain (about 5 and 2, respectively) are invariant over this temperature range (19). Specific activity equals micromoles per hour per milligram of protein.*

thesis. The designation *flrA* has been assigned by Kline to a locus near *thr* that also affects branched-chain amino acid biosynthesis (16).

Strain CV123, the well-studied *flrB* mutant, grows slowly and excretes sizable quantities of branched-chain amino acids, especially valine. The slow growth rate may be related to the overproduction of valine. Presumably, the vast overproduction of an amino acid requires both derepressed pathway-specific enzymes and an initial enzyme at least partially insensitive to end-product inhibition. Such was the case for a strain overproducing leucine (12). *S. typhimurium* has an α-acetohydroxy acid synthetase isozyme that is refractory to end-product inhibition (5) and, presumably, this enzyme is derepressed in strain CV123.

This work again draws attention to the interrelationship between the control of leucine biosynthesis and isoleucine-valine biosynthesis. Past studies indicated that both leucine (13) and leucyl-tRNA synthetase (2) are elements functioning in common in the control of all three pathways. To this list must be added the product of *flrB*.

The function of *flrB* is not known. Besides operator regions, there are three broad classes of loci implicated in the regulation of enzymes functioning in amino acid biosynthesis: loci coding for amino acyl-tRNA synthetases, loci affecting the amount or structure of tRNA, and loci coding for putative repressor proteins (9). *flrB* does not appear to affect leucyl-tRNA synthetase nor have we detected any alteration in tRNA. Certainly, there is no sizable reduction in leucine acceptor activity in strain CV123 as there is in histidine acceptor activity in *hisR* mutants (6). Furthermore, we observed no pleiotropic effects on amino acid excretion and amino acid acceptor activity as was seen for certain *hisT* mutants (6). However, we cannot rule out the possibility that the properties of strain CV123 are due to some subtle alteration in the structure of $tRNA^{Leu}$. The possibility that *flrB* codes for a repressor protein remains open and difficult to test.

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