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Isoleucine and Valine Metabolism in *Escherichia coli*

XXI. Mutations Affecting Derepression and Valine Resistance

W. J. PLEDGER AND H. E. UMBARGER

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

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The activity of acetohydroxy acid isomeroreductase, an essential enzyme for isoleucine and valine biosynthesis in *Escherichia coli*, was examined in a series of mutants containing derepressed levels of acetohydroxy acid synthetase activity but which differed from each other in the sensitivity of the synthetases to valine inhibition. The finding that isomeroreductase was highest in the strain with the synthetase that was least sensitive to valine inhibition supported the model of internal induction of the isomeroreductase by its acetohydroxy acid substrates. The mutation leading to the acetohydroxy acid synthetase least sensitive to valine was found to be unlinked to the *ilv* gene cluster and appeared to result in a synthetase that differed from the normal enzyme in several properties. The locus of this mutation is designated *ilvF*. The loci leading to derepression were designated *azl*. A pleiotropic, apparently single-step, mutation was found that led to restoration of end-product sensitivity to the synthetase, loss of end-product sensitivity of threonine deaminase [EC 4.2.1.16, L-threonine hydro-lyase (deaminating) and loss of isomeroreductase activity.

The study of the biosynthesis of isoleucine and valine in *Escherichia coli* has been stimulated by the fact that the K-12 strain of that organism and most of its derivatives are inhibited by valine (4). Subsequent biochemical and genetic studies have elucidated the enzymatic steps in these pathways of biosynthesis which are represented in Fig. 1. The figure also shows that region of the *E. coli* chromosome map (min 74) which represents the *ilv* region of the chromosome specifying the enzymes of the two pathways (21).

Valine-resistant mutants of the K-12 strain have been shown by Glover (6) to occur spontaneously with a frequency of about 5×10^{-7}. He identified six genetically distinct groups of valine-resistant mutants in this strain. Three of these groups contained lesions linked by transduction to *thr* and *leu*, two groups had lesions similarly linked to the *ilv* gene cluster, and one group contained a lesion near the *met-ilv* region but unlinked to it by transduction.

Subsequent to this study, Ramakrishnan and Adelberg (16) identified two kinds of valine-resistant mutants in which regulation of gene expression in the *ilv* gene cluster was altered. In one class, the mutation was in *ilvP*, which appears to be the repression recognition site for the acetohydroxy acid synthetase structural gene (*ilvB*). The *ilvP* mutation led to derepression of this enzyme. The other class defined *ilvO*, the repression recognition site for the *ilvADE* operon. The *ilvO* mutants exhibited derepressed levels of the enzymes encoded by *ilvA*, *-D*, and *-E*. A third class of valine-resistant mutants with lesions linked to the *ilv* region would be those affecting the *ilv-B* gene itself in which the synthetase was not inhibited by valine.

Most of the mutants we have found with valine-insensitive acetohydroxy acid synthetases contain lesions in the *ilvB* gene. However, one mutant has been found in which the mutation leading to a valine-insensitive synthetase is unlinked to the *ilv* gene cluster. This locus we have designated *ilvF*. We report in this paper data describing the location of *ilvF* and its effect on acetohydroxy acid synthetase. We also describe a mutation suppressing the effects of *ilvF*; this suppressing mutation is linked to the *ilv* gene cluster.

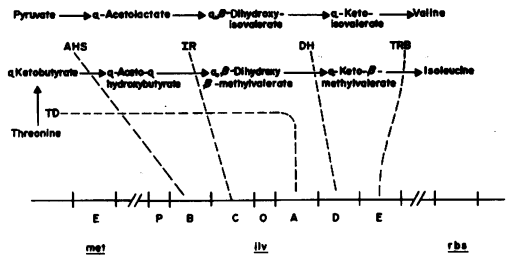

FIG. 1. *The biosynthetic pathways for isoleucine and valine. The enzymes catalyzing the indicated steps are abbreviated as follows: TD, threonine deaminase (EC 4.2.1.16 L-threonine hydro-lyase [deaminating]); AHS, acetohydroxy acid synthetase; IR, acetohydroxy acid isomeroreductase; DH, dihydroxy acid dehydrase; TRB, transaminase B. All the enzymes except threonine deaminase catalyze corresponding steps in the valine and isoleucine pathways. Threonine deaminase is specific for the isoleucine pathway alone. The broken lines indicate the structural genes in the ilv gene cluster that specify the respective enzymes. Gene loci, ilvO and ilvP, are repression recognition sites controlling the expression of genes ADE and gene B, respectively. The gene order shown is the reverse (i.e., left to right is counterclockwise) of the way it is usually represented on the E. coli chromosome map. Adapted from Ramakrishnan and Adelberg (17).*

The sensitivity of acetohydroxy acid synthetase to valine is of further interest in view of the fact that its products, acetolactate and acetohydroxybutyrate, are inducers of the next enzyme in the pathways to valine and isoleucine, the acetohydroxy acid isomeroreductase (2). Since acetohydroxy acid synthetase in *E. coli* K-12 is strongly inhibited by valine, even strains with genetically derepressed levels of acetohydroxy acid synthetase (owing, for example to an *ilvP* mutation) would produce only low levels of acetohydroxy acids if such organisms were grown in the presence of valine. Under these conditions, the *ilvC* gene product would be repressed (or uninduced). This was indeed the case in the experiments of Ramakrishnan and Adelberg (16). However, the isomeroreductase should be induced even in the presence of excess valine in a double mutant that has a feed-back-insensitive, genetically derepressed acetohydroxy acid synthetase. We report evidence to substantiate this idea not only for *ilvB* mutants containing valine-resistant synthetases but for the *ilvF* mutant as well.

MATERIALS AND METHODS

Organisms and media. The organisms used in these studies were all derived from the K-12 strain of *E. coli*. The mutants that were used in these experiments are listed in Table 1. The organisms were maintained in L broth agar stabs (10).

The minimal medium of Davis and Mingioli (5) was used except that the citrate was omitted and the glucose was increased to 0.5%. For growth conditions with excess branched-chain amino acids, 0.6 mM L-leucine and L-isoleucine and 1.2 mM L-valine were added. For isoleucine limitation, that amino acid was reduced to 0.05 mM. For valine limitation, 0.1 mM glycl-L-valine was added instead of valine.

The organisms were grown in 2-liter triple-baffled Erlenmeyer flasks in a New Brunswick gyratory shaker.

Chemicals. Amino acids, pyridoxal 5'-phosphate, vitamins, purines, and pyrimidines were obtained from Calbiochem. Flavine adenine dinucleotide (FAD), diphosphothiamine, and sodium pyruvate (type II) were obtained from Sigma Chemical Co. The other chemicals were all of reagent grade.

Mutagensis. Fresh stationary-phase cultures were diluted into medium containing 10 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) per ml. Cells were treated for 30 min and diluted into fresh minimal medium containing excess isoleucine, leucine, and valine. After the culture was grown to the stationary phase, penicillin enrichment was employed in a method similar to that of Gorini and Kaufman (7), and the survivors were gorwn on minimal agar plates supplemented with suboptimal amounts (0.01 mM) of isoleucine, leucine, and valine. Small colonies were picked for further testing.

Transduction. The techniques for P1-mediated transduction described by Lennox (10) were used.

Preparation of cell extracts and enzymatic assays. The techniques for harvesting the cells, the preparation of extracts, and enzyme assay conditions have been reported previously (16, 17). However, except where otherwise stated, the FAD concentration used in the acetohydroxy acid synthetase assay was 20 μg/ml rather than 2 μg/ml as previously employed. Protein was determined by the method of Lowry et al. (11) or by the biuret (8) method. Specific activities were expressed in micromoles of product per minute per milligram of protein.

Sucrose gradient centrifugation. A Beckman density gradient maker was used to prepare the 5 to 20% sucrose gradients. The samples were layered on top of the gradients with the aid of a Spinco synthetic boundary cap. Centrifugation at the indicated temperatures in a Spinco model L2HV centrifuge was performed for 20 h at 0 C or for 12 h at 20 C by using an SW39 rotor. Fractions were collected from the top by pushing the gradients from the bottom with a dense sucrose solution by using an Isco model D fractionator. Crystallized rabbit muscle lactic dehydrogenase (Sigma) was used as a standard.

RESULTS

Isolation of mutants with valine-resistant acetohydroxy acid synthetases. Spontaneously arising, valine-resistant strains

(Val^r) were isolated by plating cells of strain CU5002 on agar plates containing 1.0 mM valine. Strain CU5002 has genetically derepressed levels of threonine deaminase, dehydrase, transaminase B, and acetohydroxy acid synthetase, but it had a repressed level of

TABLE 1. *List of strains*

Strain	Sex	Genotype	Relevant distinctive characteristics	Source or reference	Use
K-12	F$^+$	Prototrophic			Recipient for *ilv-465* (Table 6)
AT2861	F$^-$	*purC, glyA*		A. L. Taylor	Genetic mapping experiments (Table 4)
CU4	F$^-$	*gal*, prototrophic		J. Blatt	Parent of CU16
CU5	Hfr	*met*	An Hfr derivative of K-12 with an uncertain pedigree	Purdue culture collection	Parents of CU5000 series of mutants
CU8	F$^-$	*ilvA451*		Formerly JHM544 Umbarger and Brown (23)	Genetic mapping experiments (Table 3)
CU16	F$^-$	*metE200, rib-215, gal*	Threonine deaminase negative	Multiple mutagenesis of CU4	Used as parental type for *ilv* mutants
CU35	F$^-$	*gal, ilvC495, rib-215, metE200*	Isomeroreductase negative	N.G. mutagenesis of CU16	Genetic mapping experiments (Table 3)
CU59	F$^-$	*proC, ilvF465*	Valine resistant	By transduction of SP31 from CU5117	Show effect of *ilvF465* in cells with normal repression (Table 7)
CU60	F$^-$	*proC, ilvF465*	Valine resistant	By transduction of SP31 from CU5117	Show effect of *ilvF465* in cells with normal repression (Table 7)
CU61	F$^-$	*azlA1*	Azaleucine resistant	By transduction of UTH4662 from CU5117	Show separability of azaleucine resistance and valine resistance (Table 8)
CU62	F$^-$	*ilvF465*	Valine resistant	By transduction of UTH4662 from CU5117	Show separability of azaleucine resistance and valine resistance (Table 8)
CU5001	Hfr	*met, azlA2, azlB4, azl-6*	Constitutively repressed *ilvADE* Constitutively derepressed *ilvB*	S. Dwyer	
CU5002	Hfr	*met, azlA1, azlB3, azl-5*	Constitutively derepressed *ilvADE* and *B*	S. Dwyer	Source of CU5117
CU5117	Hfr	*met, azlA1, azlB3, azl-5, ilvF465*	Valine resistant	Spontaneous mutant derived from CU5002	Determine effect of valine resistant acetohydroxy acid synthetase on isomeroreductase (Table 2)
CU5118	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilv-466*	Isomeroreductase negative	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation in feedback insensitive strains
CU5119	Hfr	*met, azlA1, azlB3, azl-5, ilvB478*	Valine resistant	Spontaneous mutant derived from CU5002	Determine effect of valine resistant acetohydroxy acid synthetase on isomeroreductase (Table 2) plus genetic mapping experiments (Table 3)
CU5120	Hfr	*met, azlA1, azlB3, azl-5, ilvB479*	Valine resistant	Spontaneous mutant derived from CU5002	Determine effect of valine resistant acetohydroxy acid synthetase on isomeroreductase (Table 2)
CU5121	Hfr	*met, azlA1, azlB3, azl-5, ilvB480*	Valine resistant	Spontaneous mutant derived from CU5002	Determine effect of valine resistant acetohydroxy acid synthetase on isomeroreductase (Table 2)
CU5124	Hfr	*met, azlA1, azlB3, azl-5, ilvB512*	Valine resistant	Spontaneous mutant derived from CU5002	Determine effect of valine resistant acetohydroxy acid synthetase on isomeroreductase (Table 2) plus genetic mapping experiments (Table 3)
CU5125	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilvA511*	Threonine deaminase negative	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation on feedback insensitive strains (Table 10)

TABLE 1—*Continued*

Strain	Sex	Genotype	Relevant distinctive characteristics	Source or reference	Use
CU5127	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilvS514*	Isoleucine auxotroph	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation on feedback insensitive strains (Table 10)
CU5128	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilvD515*	Dehydrase negative	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation on feedback insensitive strains (Table 10)
CU5129	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilvC516*	Isomeroreductase negative	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation on feedback insensitive strains (Table 10)
CU5130	Hfr	*met, azlA1, azlB3, azl-5, ilvF465, ilvD517*	Dehydrase negative	N.G. mutagenesis of CU5117	Determine effect of *ilv* mutation on feedback insensitive strains (Table 10)
CU5131	Hfr	*met, azlA2, azlB4, azl-6, ilvF465*	Valine resistant	By transduction of CU5001 from CU5117	Show effect of *ilvF465* in a second type of Azl^r organism (Table 7)
CU5132	Hfr	*met, azlA1, azlB3, azl-5, ilvF465*	Valine resistant, prototrophic	By spontaneous reversion of CU5118	Obtain presumptive evidence that the *ilvY466* was a single mutation (Table 11)
CU5133	Hfr	*met, azlA1, azlB3, azl-5, ilvF465*	Valine resistant, prototrophic	By transduction of CU5118 from K-12	Shown linkage of *ilvY466* to *ilv* (Table 11)
SP31	F$^-$	*glyA, proC*		R. L. Somerville	Genetic mapping experiments (Tables 7 and 9)
UTH4067	F$^-$	*his, tyrA, trp, purC, guaA*		E. P. Goldschmidt	Genetic mapping experiments (Table 4)
UTH4662	F$^-$	*nadB3*		E. P. Goldschmidt	Genetic mapping experiments (Tables 8 and 9)

isomeroreductase. It had been isolated by Susan Dwyer (Ph.D. thesis, Purdue University, 1969) through resistance to azaleucine (Azl^r). Extracts were prepared from cells grown from several of these Val^r stocks and examined for feedback insensitivity of the acetohydroxy acid synthetases to valine and for levels of isomeroreductase when the cells were grown in the presence of excess branched-chain amino acids. Table 2 shows that the amount of isomeroreductase activity in repressed cells bore roughly an inverse relationship to the valine sensitivity of the acetohydroxy acid synthetase activity. This relationship would be expected if the isomeroreductase were, in fact, regulated by internal induction.

The locations of loci giving rise to resistant acetohydroxy acid synthetases. Table 3 shows the frequency of co-transduction with the *ilv* region of three lesions giving rise to a valine-insensitive acetohydroxy acid synthetase. Two strains (CU5119 and CU5124) have mutations that are linked to the *ilv* region and are presumably in the *ilvB* gene itself. However, as can be seen from these data, the *ilv-465* lesion, either in strain CU5117 or in its *ilvA* derivative (strain CU5125), is not linked to the *ilv* region. All other strains with valine-resistant acetohydroxy acid synthetases that we examined have lesions genetically linked to the *ilv* gene cluster.

TABLE 2. *Relation between the specific activities of isomeroreductase and the sensitivity of acetohydroxy acid synthetase to valine*

	Growth medium[a]	Acetohydroxy acid synthetase		Isomeroreductase specific activity
		Specific activity[b]	Inhibition[c] (%)	
CU5	Minimal	0.046	90	0.048
	Supplemented	0.011	90	0.0042
CU5002	Minimal	0.200	90	0.063
	Supplemented	0.200	90	0.0055
CU5117	Minimal	0.228	5	0.065
	Supplemented	0.209	5	0.065
CU5119	Supplemented	0.199	16	0.040
CU5120	Supplemented	0.184	20	0.032
CU5124	Supplemented	0.236	67	0.014
CU5121	Supplemented	0.211	80	0.0077

[a] The supplement was excess branched-chain amino acids.

[b] Expressed as micromoles of product formed per minute per milligram of protein.

[c] Acetohydroxy acid synthetase assayed in the presence of 10^{-3} M valine.

As shown in Table 4, the lesion giving rise to the Val^r phenotype, *ilv-465*, was co-transducible with *purC* and *glyA* (Fig. 2). We have designated the locus containing *ilv-465* as *ilvF*.

Effects of the ilvF465 lesion on the ace-

TABLE 3. *Attempts to demonstrate linkage of several valine-resistant mutations to the ilv region*

Donor	Pertinent lesions	Recipient	Pertinent lesions	*ilv*+ Recombinants selected	Unselected marker[a]
CU5117	*azlA1, azlB3, azl-5, ilv-465*	CU35	*ilvC495, rbs-215*	351	*rbs*+, 298
K-12	None	CU5125	*ilvF465, ilvA511*	320	*ilvF465*, 0 *ilvF465*, 0
CU5119	*azlA1, azlB3, azl-5, ilvB478*	CU8	*ilvA451*	221	*ilvB478*, 200
CU5119	*azlA1, azlB3, azl-5, ilvB478*	CU35	*ilvC495*	234	*ilvB478*, 230
CU5124	*azlA1, azlB3, azl-5, ilvB512*	CU8	*ilvA451*	234	*ilvB512*, 209
CU5124	*azlA1, azlB3, azl-5, ilvB512*	CU35	*ilvC495*	234	*ilvB512*, 230

[a] With the exception of the *rbs*+ marker, the unselected marker was scored by ability of the *ilv*+ recombinants to grow on minimal agar containing 10^{-3} M valine and 2×10^{-4} M methionine.

TABLE 4. *Genetic linkage of the ilvF465 lesion to markers in the 48- to 49-min region of the E. Coli chromosome*

Recipient	Marker selected[a]	Recombinants selected	Valine-resistant prototrophs
AT2861	*purC*+	210	44 (21%)
AT2861	*glyA*+	117	30 (26%)
UTH4067	*purC*+	398	87 (22%)

[a] The Val^r strain CU5117 (*ilvF465*) served as donor. Val^r phenotype scored by incubating the Pur+ and Gly+ recombinants on minimal agar containing 4×10^{-4} M L-valine and the other supplements required by the recombinants (see Table 1).

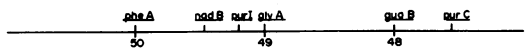

FIG. 2. *The map of the E. coli chromosome showing the location of the purI, glyA, and nadB markers (23).*

tohydroxy acid synthetase. In addition to the long-recognized acetohydroxy acid synthetase with an optimal activity at pH 8, a second optimum at pH 6 has sometimes been observed, particularly with extracts that have not been prepared from cells derepressed for the isoleucine- and valine-forming enzymes (15). We have found that the activity at pH 6 can be detected more readily when the assay mixture includes FAD at a concentration 10 times that recommended originally (18). This activity at pH 6 was enhanced significantly by the *ilvF465* lesion present in strain CU5117 (Table 5). Figure 3 shows the activities of acetohydroxy acid synthetase in strain CU5117 and its parent strain, CU5002, at various pH values, assayed without FAD and with two different concentrations of FAD. For both strains the activity at pH values below 6.5 was more strongly influenced by the concentration of FAD than is the activity near pH 8, which has been considered optimal for the wild-type acetohydroxy acid synthetase (22).

A comparison of the apparent affinity of acetohydroxy acid synthetase for FAD is given in Table 6. It had been observed that passage of the extract through a Sephadex column is more effective for making acetohydroxy acid synthetase at pH 8 dependent upon FAD addition with a CU5002 extract than it is with a CU5117 extract. Whether the activity at pH 8 in the absence of added FAD in Sephadex-treated extracts was because of retention of FAD on the enzyme or because the modified enzyme in strain CU5117 exhibits more activity at pH 8 than does the normal enzyme is not known. However, the pH 6 activity of extracts of strain CU5117 showed the same high apparent K_m for FAD as did the wild-type extract. The activity in strain CU5117 at pH 6 was as high as or higher than the activity at pH 8 when the FAD concentration was sufficiently high. In contrast, the pH 6 activity in the parent strain was always lower than that at pH 8 even when the FAD concentration was high.

The acetohydroxy acid synthetase activities both at pH 6 and at pH 8 showed heat denaturation properties in strain CU5117 much like the pH 8 activity of the acetohydroxy acid synthetase of strain CU5002. The pH 6 activity of strain CU5002 is much more labile so that the pH-activity profile shows an even sharper maximum pH 7.5 and 8 after heat treatment than before. It was observed further that, at either pH, the enzymatic product formed by the extract containing the unusual synthetase activity was dependent upon acid treatment to yield a color in the determination of acetolactate formation, just as has been noted with the "normal" *E. coli* enzyme (23).

The most striking difference between the activities of wild-type extracts and of strain

TABLE 5. *Acetohydroxy acid synthetase activity at pH and pH 8 with varying concentrations of FAD*[a]

FAD (μg per ml)	Acetohydroxy acid synthetase activity[b]			
	CU5002		CU5117	
	pH 6	pH 8	pH 6	pH 8
0.2	0.008	0.063	0.015	0.163
2	0.014	0.180	0.114	0.237
20	0.041	0.187	0.206	0.237

[a] Cells were grown in minimal medium. Extracts were passed over G-25 Sephadex.

[b] Activity expressed as micromoles of product formed per minute per milligram of protein.

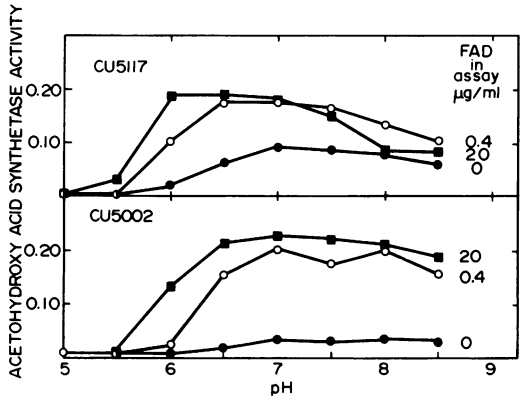

FIG. 3. *The effect of pH on acetohydroxy acid synthetase activities in extracts of strains CU5002 and CU5117. Both extracts were passed over Sephadex G-25 to remove FAD. FAD was added as indicated. The pH values were those of the buffer. The oridinate is specific activity.*

CU5117 extracts was revealed by the difference in sedimentation properties. As will be demonstrated later in this report, the acetohydroxy acid synthetase of strain CU5117 exhibits a faster sedimentation during sucrose density gradient centrifugation than did the wild-type acetohydroxy acid synthetase present in its parent, strain CU5002.

Separation of ilvF465 and the markers causing azaleucine resistance. The *ilvF* locus is readily separated from the lesions in strain CU5117 that cause derepression of the *ilvADE* and *ilv-B* genes. The *ilvF465* lesion was transferred with P1 transducing phage grown on strain CU5117 into cells of strain SP31 (*glyA*, *proC*). Gly$^+$ transductants were selected and scored for the Val^r phenotype on agar plates containing 5×10^{-4} M valine. Val^r derivatives of strain CU5001 were prepared in a similar way except that valine resistance was selected directly. Strain CU5001 is another azaleucine-resistant organism isolated by Susan Dwyer. It has a constitutively derepressed *ilvB* gene product, but the *ilvADE* cluster is constitutively maintained at a repressed level. The Val^r transductants derived from this strain had presumably received a valine-resistance marker unlinked to *ilv* as was indicated by subsequent transduction experiments with one of the Val^r derivatives as donor.

Two valine-resistant transductants derived from strain SP31 and one derived from strain CU5001 were tested to determine the properties of their acetohydroxy acid synthetases. Both the valine resistance and the enhancement of activity at pH 6 (relative to that at pH 8) introduced with the *ilvF465* marker are independent of the state of azaleucine resistance (Table 7). However, azaleucine resistance in strain CU5001, like that in strain CU5002, resulted in derepression of acetohydroxy acid synthetase activity even when that activity was under the influence of *ilvF465*.

The threonine deaminase remained repressed in the Val^r derivative of strain DU5001 (CU5131), indicating it had not received the unknown element from strain CU5117 that accounts for the difference between the two Azl^r stocks.

Strain CU5117 was also used as donor for P1-mediated transduction of the *nadB*$^+$ marker of strain UTH4662. This marker is close to *glyA* (Fig. 2). The nicotinate independent (Nad$^+$) transductants were picked and screened (on agar containing 5×10^{-3} M azaleucine or 10^{-3} M valine) for the Azl^r or Val^r phenotype. Two Val^r Nad$^+$ colonies were picked and shown not to be Azl^r, and two Azl^r

TABLE 6. *Apparent affinity of acetohydroxy acid synthetase for FAD in crude E. coli extracts*

Strain	Apparent K_m for FAD (nM)[a]	
	pH 6	pH 8
K-12	213	47
CU5002	208	50
CU5117	197	47

[a] In each case, the K_m is the concentration of FAD required to yield half maximal stimulation of activity. With the extract from strain CU5117, there was about 50% maximal activity at pH 8 without FAD, whereas at pH 6 and at both pH values for the other extracts there was almost complete dependence on added FAD.

Nad^+ colonies were shown not to be Val^r. Acetohydroxy acid synthetase activities and sensitivity to inhibition by valine were determined for one transductant from each class (Table 8). The Val^r transductant exhibited a valine-resistant synthetase that appeared to be subject to repression control essentially like that of the recipient. The Azl^r transductant exhibited a synthetase that was as sensitive to valine as that in the recipient, but the repression control was clearly different from either recipient or donor. The level of enzyme in the absence of the branched-chain amino acids was similar to that in the recipient, but that level was not repressible. That they were not derepressed indicates the existence of some unknown marker causing derepression.

These experiments demonstrate that the *ilvF465* lesion can be readily separated from the markers giving rise to azaleucine resistance. However, they also showed that an unexpected form of azaleucine resistance could also be transferred by linkage to markers in the *nadB-glyA* region of the chromosome (represented in Fig. 2). In a further study of these markers, strains SP31 and UTH4662 were used as recipients. The donors were the four Azl^r strains, CU5001, CU5002, CU5117, and CU5118. Gly^+ and Nad^+ transductants were screened on agar plates containing 5×10^{-3} M and 9×10^{-3} M azaleucine. Markers giving rise to both high and low levels of resistance were found linked to the *glyA* and *nadB* loci (Table 9). None of the transductants from donor phage prepared from strain CU5001 or CU5002 was resistant to valine, whereas valine-resistant transductants could be found when P1 lysates were prepared from strain CU5117 or strain CU5118.

Additional studies will be required to define

TABLE 7. *The effect of transferring the ilvF465 mutation to strains with different ilv regulation*

Strain	Pertinent genotype	Growth medium	Acetohydroxy acid synthetase			
			pH 8	Inhibition by valine[a] (%)	pH 6	Inhibition by valine[a] (%)
CU5001	*azlA2, azlB4, azl-6*	Minimal	0.32	88	0.0077	96
		Supplemented[b]	0.38	86	0.0081	100
CU5131	*azlA2, azlB4, azl-6, ilvF465*	Minimal	0.30	3	0.32	0
		Supplemented	0.32	0	0.32	0
SP31	*glyA, proC*	Minimal	0.041	95	0.009	—[c]
		Supplemented	0.011	86	—[c]	—[c]
CU59	*proC, ilvF465*	Minimal	0.030	13	0.029	0
		Supplemented	0.010	0	0.0065	0
CU60	*proC, ilvF465*	Minimal	0.042	14	0.036	0
		Supplemented	0.012	0	0.0057	0

[a] Extracts assayed in the presence of 10^{-3} M L-valine.
[b] The supplement was excess branched-chain amino acids.
[c] Too low to measure.

TABLE 8. *Acetohydroxy acid synthetase in Val^r or Azl^r transductants receiving resistance from strain CU5117*[a]

Strain	Growth medium	Acetohydroxy acid synthetase			
		pH 8	Inhibition by valine (%)	pH 6	Inhibition by valine[b] (%)
CU5117 (Donor)	Minimal	0.204	6	0.200	−19
	Supplemented	0.195	5	0.200	−19
UTH4662 (Recipient)	Minimal	0.041	89	0.002	100
	Supplemented	0.006	88	—[c]	—[c]
CU61 (UTH4662-Azl^r)	Minimal	0.046	90	0.014	80
	Supplemented	0.036	88	0.001	—[c]
CU62 (UTH4662-Val^r)	Minimal	0.040	18	0.040	−3
	Supplemented	0.0089	0	0.008	—[c]

[a] Conditions as in Table 6.
[b] Negative values indicate percentage of stimulation by valine.
[c] Too low to measure.

the nature of these markers, but it does appear that they are similar (as expected) in all four donors. The separation of low- and high-resistance phenotypes among the transductants indicates that there are probably two separate loci controlling azaleucine sensitivity. For the present, the two loci will be designated *azlA* (controlling low resistance) and *azlB* (controlling high resistance). Their relative positions in the *nadB-glyA* legion have yet to be established.

That the two classes of *azl* transductants were also biochemically distinct was demonstrated by selecting one high- and one low-resistant transductant from each donor-recipient combination and examining threonine deaminase and acetohydroxy acid synthetase activities and leucine excretion. Very briefly (data not shown), it was clear that neither the low- nor high-resistant types were like the Azl^r donors. Both types overproduced and excreted only small amounts of leucine. The low-resistance class showed essentially the same activities of threonine deaminase and acetohydroxy acid synthetase as did their recipient parental strains in minimal medium. Unlike the parental strains, their levels were virtually not repressible. They resembled the transductant from strain UTH4662 shown in Table 8. The high-resistance class showed about a twofold derepression of acetohydroxy acid synthetase in minimal medium, but these levels were repressed by excess branched-chain amino acids to be repressed levels of the recipient parental strains. Threonine deaminase levels, on the other hand, were only a little higher than those in the parental strains under repressing and nonrepressing conditions.

That neither the high- nor low-resistant transductants exhibit the derepression exhibited by the donors indicates that there is at least one additional locus (presumably unlinked to *nadB* or *glyA*) that contributes to the phenotype of the Azl^r mutants. Additional transduction experiments (data not shown) in which resistance to 5×10^{-3} M azaleucine was selected directly have led to the isolation of heavy leucine-excreting and highly derepressed transductants with both strains CU5001 and CU5002 as donors. The location of these lesions has not been determined nor is it known whether those in the two strains are allelic.

A pleiotropic mutation that suppresses the ilvF465 lesion. In the course of examining a series of Ilv$^-$ auxotrophs derived from strain CU5117 (*azlA1*, *azlB3*, *azl-5*, *ilvF465*), one unusual mutant was encountered. This strain, CU5118 (*azlA1*, *azlB3*, *azl-5*, *ilvF465*, *ilv-466*), was one of the two shown to lack isomeroreductase. Both were initially presumed to be *ilvC* derivatives. However, the *ilv-466* lesion was clearly pleiotropic (Table 10). Thus, the isomeroreductase in strain CU5118, like that in strain CU5129, exhibited only marginal isomeroreductase activity, but in strain CU5118 it was found that the *ilv-466* mutation had restored the feedback sensitivity of acetohydroxy acid synthetase to valine and had made the threonine deaminase insensitive to feedback inhibition by isoleucine. Table 10 shows that the other auxotrophic mutations did not affect the feedback sensitivity pattern shown by the parent strain CU5117. That all three effects observed in strain CU5118 were indeed due to the *ilv-466* lesion was supported by the isolation of spontaneous, prototrophic revertants of strain CU5118 which were found to have the properties of strain CU5117. Similarly, when prototrophic transductants of strain CU5118 (with the wild-type strain of *E. coli* as the

TABLE 9. *Genetic linkage of azaleucine-resistance markers to nadB and glyA*

Donor strain[a]	Strain UTH4662 (*nadB*) as recipient			Strain SP31 (*glyA*, *proC*) as recipient		
	Nad$^+$ colonies examined	Nad$^+$ transductants growing on azaleucine agar		Gly$^+$ colonies examined	Gly$^+$ transductants growing on azaleucine agar	
		5×10^{-3} M azaleucine	9×10^{-3} M azaleucine		5×10^{-3} M azaleucine	9×10^{-3} M azaleucine
CU5001	70	52 (74)[b]	20 (28)[b]	90	72 (80)[b]	70 (77)[b]
CU5002	58	48 (83)	16 (27)	128	122 (95)	96 (75)
CU5117	90	74 (82)	30 (33)	154	152 (98)	123 (86)
CU5118	100	72 (72)	32 (32)	120	102 (85)	92 (76)

[a] Strains CU5001 and CU5002 were independently derived Azl^r strains, strains CU5117 and CU5118 were derived from strain CU5002.

[b] Numbers in parentheses are percentages of co-transduction.

donor) were examined, they too resembled the parental strain CU5117. Results with a typical revertant and a typical transductant are also given in Table 11.

Figure 4 compares the effects of valine on the acetohydroxy acid synthetase from cells of strains CU5118, CU5117, CU5002, and two revertants of strain CU5118. There was nearly a complete loss of valine sensitivity of acetohydroxy acid synthetase activity with the *ilvF465* mutation (in strain CU5117). The sensitivity was nearly restored by the *ilv-466* lesion (in strain CU5118). The enzymes in the prototrophic revertants of strain CU5118 were essentially like those in strain CU5117.

As noted earlier, the acetohydroxy acid synthetase in strain CU5117, in addition to being valine resistant, sedimented in the centrifuge more rapidly than did the valine-sensitive enzyme of strain CU5002. This property, too, was lost as a result of the *ilv-466* mutation in strain CU5118. In every property of the acetohydroxy acid synthetase examined, the enzyme in strain CU5118 appeared to be like that in the original valine-sensitive strain CU5002. The results of sucrose density gradient centrifugation of the acetohydroxy acid synthetases from strains CU5002, CU5117, and CU5118 are shown in Fig. 5. This figure also shows the behavior of the threonine deaminases of the

TABLE 10. *Sensitivity of acetohydroxy acid synthetase and threonine deaminase to end product in several ilv auxotrophs derived from the valine resistant strain CU5117*

Strain	Auxotrophic *ilv* lesion	Phenotype	Threonine deaminase		Acetohydroxy acid synthetase	
			Specific activity[a]	Inhibition[b] (%)	Specific activity	Inhibition[c] (%)
CU5117[d]	None	Val[r] prototroph	0.150	95	0.211	2
CU5118[e]	*ilv-466*	Isomeroreductase negative	0.977	5	0.521	90
CU5127[f]	*ilvS514*	Isoleucine bradytrophic lesion linked to *thr*	0.621	97	0.628	10
CU5128[e]	*ilvD515*	Dehydrase negative	0.571	96	0.628	10
CU5129[e]	*ilvC516*	Isomeroreductase negative	0.400	90	0.421	5
CU5130[e]	*ilvD517*	Dehydrase negative	0.420	96	0.450	0
CU5125[f]	*ilvA511*	Threonine deaminase negative			0.499	2

[a] Expressed as micromoles of product formed per minute per milligram of protein.
[b] Threonine deaminase was assayed in the presence of 10^{-3} M isoleucine.
[c] Acetohydroxy acid synthetase was assayed in the presence of 10^{-3} M valine.
[d] Cells were grown in minimal medium.
[e] Cells were grown in limiting valine medium.
[f] Cells were grown in limiting isoleucine medium.

TABLE 11. *The effect of the ilvF465 and ilv-466 alleles on the end-product inhibition of threonine deaminase and acetohydroxy acid synthetase*

Strain[a]	Pertinent lesions	Threonine deaminase		Acetohydroxy acid synthetase		Isomeroreductase activity
		Specific activity[b]	Inhibition[c] (%)	Specific activity	Inhibition[d] (%)	
CU5002	*azlA1, azl-B3, azl-5*	0.161	95	0.200	89	+
CU5117	*azlA1, azlB3, azl-5, ilvF465*	0.150	95	0.211	2	+
CU5118	*azlA1, azlB3, azl-5, ilvF465, ilv-466*	0.977	5	1.510	90	−
CU5132[e]	*azlA1, azlB3, azl-5, ilvF465*	0.155	90	0.187	5	+
CU5133[f]	*azlA1, azlB3, azl-5, ilvF465*	0.174	95	0.219	11	+

[a] Strain CU5118 was grown in limiting valine, the other strains in minimal medium.
[b] Expressed as micromoles of product formed per minute per milligram of protein.
[c] Extract assayed in the presence of 10^{-3} M isoleucine.
[d] Extract assayed in the presence of 10^{-3} M valine.
[e] Strain CU5132 is a spontaneous Ilv$^+$ revertant of strain CU5118.
[f] Strain CU5133 is an Ilv$^+$ transductant of strain CU5118 obtained in a cross with *E. coli* wild type strain K-12 as the donor.

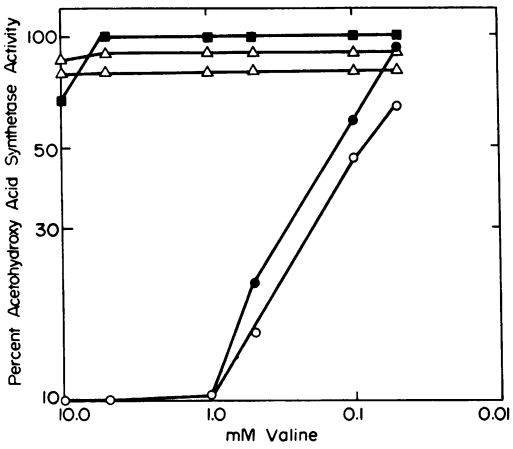

FIG. 4. *Effect of valine on acetohydroxy acid synthetase activity in extracts of several E. coli strains. Symbols: ●, CU5002; ■, CU5117; ○, CU5118; △, two randomly chosen, spontaneously arising prototrophic derivatives of CU5118.*

three strains during sucrose density gradient centrifugation. As may be seen, the isoleucine-insensitive enzyme of strain CU5118 sedimented more slowly than do the isoleucine-sensitive enzymes in strains CU5002 and CU5117. These centrifugation studies reveal physical differences between the two end-product-sensitive enzymes and their end-product-insensitive counterparts in the mutants studied. However, the different rates of sedimentation cannot be immediately correlated with size differences for the rates indicated here are very much dependent upon the type of buffer employed. Thus, the addition of 10^{-4} M mercaptoethanol to the phosphate buffer causes the isoleucine-insensitive threonine deaminase to sediment at the same rate as the normal enzyme (G. W. Hatfield, personal communication). Again, the use of the buffer employed by Blatt *et al.* (3) during sucrose density gradient centrifugation causes the valine-resistant acetohydroxy acid synthetase to sediment more slowly than the valine-sensitive enzyme (the opposite of the results shown in Fig. 5).

DISCUSSION

In this study evidence has been presented for several previously unrecognized loci that affect isoleucine and valine biosynthesis. Unfortunately, not all of these have been sorted and separated from each other. For example, the mutations present in the original azaleucine-resistant strains have been only partially defined. Evidence was found for two loci that affect azaleucine sensitivity in the genetic region represented between 48 and 49 min on the *E. coli* chromosome map (21). Although lesions in both loci lead to azaleucine resistance, neither result in the kind of derepression that was found in the azaleucine-resistant mutants. Clearly, at least a third unidentified locus must be responsible. Further studies will be required to elucidate the mechanism of derepression in these mutants. In some of their properties, they resemble the *flr* mutants of *Salmonella typhimurium* described by Alexander and Calvo (1; Susan Dwyer, Ph.D. thesis, Purdue University, 1969).

These strains were useful, nevertheless, for proving that the isomeroreductase, the product of the *ilvC* gene, was regulated by substrate induction as had been postulated earlier (2). The azaleucine-resistant mutant, strain CU5002, even with a strong derepression signal affecting the other isoleucine and valine forming enzymes, has very low isomeroreductase activity. The K-12 strains of *E. coli* have only a valine-sensitive acetohydroxy acid synthetase (3, 13). Thus, it appeared that there was sufficient control of acetohydroxy acid formation to prevent the internal induction. Internal induction is found in *S. typhimurium* (which has a second, valine-insensitive acetohydroxy acid synthetase) when the other isoleucine- and valine-forming enzymes are derepressed. The additional support for this idea was provided by the finding that a mutation unlinked to the

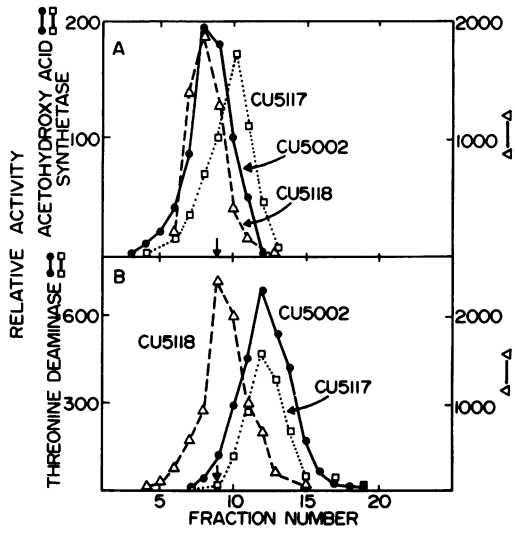

FIG. 5. *Separation of threonine deaminase and acetohydroxy acid synthetase in several derivatives of E. coli K-12. Graph A, centrifugation was at 35,000 rpm at 0 C for 20 h in an SW39 rotor; graph B, centrifugation was at 35,000 rpm at 20 C for 12 h in an SW39 rotor. Fraction no. 9 showing maximal lactate dehydrogenase activity indicated by arrow.*

ilv region (*ilvF465*), which rendered acetohydroxy acid synthetase valine insensitive resulted in induction of the isomeroreductase. That the *ilvF465* lesion was unlinked made it certain that its effect on *ilvC* function was via some cytoplasmic process and not a *cis*-directed effect as expected of "operator" or "promotor" mutations.

Two obvious possibilities can be suggested for the function of the *ilvF* locus. One is that its product modified the acetohydroxy acid synthetase in a way analogous to the way that Nakae (12) suggests the *galF* product modified uridine diphosphate glucose pyrophosphorylase. Whether the apparently heavier enzyme which is feedback insensitive or the "normal" enzyme which is presumed to be the *ilvB* gene product would be the "modified" enzyme cannot be postulated until dominance studies with the *ilvF* locus have been completed.

A second possibility is that the *ilvF465* mutation has permitted the formation of an acetohydroxy acid synthetase specified either by the *ilvF* locus itself or by some gene other than *ilvB*. (The latter possibility [a gene other than *ilvB*] would indicate a suppressor role for *ilvF465* in which case the tentative designation "*ilvF*" would probably not be appropriate.) This model would imply that the gene for a valine-insensitive isozyme is not expressed in the valine-sensitive *E. coli* K-12. The apparent absence of any valine-sensitive acetohydroxy acid synthetase in *ilvF465* strains is difficult to explain, but it may be that an overproduction of valine and leucine, because of the insensitive enzyme, leads to an internal repression of the sensitive enzyme. An analogy might be found in the demonstration in *E. coli* by Lansford et al. (9) that the aspartokinases repressible by threonine and methionine are internally repressed unless exogenous lysine is supplied to repress the normally predominant lysine-repressible aspartokinase. However, it seems unlikely that such a repression could occur in the presence of the *azl* lesions. Another possibility would be that the *ilvF* product and the *ilvB* product share some common subunit.

To distinguish between these possibilities, more information on acetohydroxy acid synthetase of *E. coli* will be necessary. Unfortunately, this enzyme has until now proven refractory to purification. Hopefully, the comparative studies now in progress with the valine-sensitive and the valine-insensitive acetohydroxy acid synthetases of *S. typhimurium* will enable a good comparison to be made with the single *E. coli* enzymes in *ilvF*$^+$ and *ilvF465* strains. It thus might be that the *ilvF* function revealed by the mutation in strain CU5117 can eventually be interpreted in terms of the acetohydroxy acid synthetase that is "missing" in the valine-sensitive strains. Indeed, any of the possibilities for *ilvF* function discussed above can be expressed in these terms.

Since the *ilv-466* mutation had arisen during mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the possibility that the pleiotropic effects were due to multiple mutagenic events should be considered. However, as shown in Table 4, the mutation did appear to undergo reversion to a state very similar to wild type although at a rather low frequency. Thus, the mutation is tentatively assumed to be a single one. Its role in suppressing the *ilvF465* mutation has not been studied further. Its role in controlling feedback sensitivity has been found to be extremely complex, but its role in preventing the formation of the isomeroreductase has been more clearly defined by the genetic and biochemical studies that have been performed. These studies are the subject of the following paper (14).

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