Molec. gen. Genet. 156, 239–249 (1977)

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Valine-Sensitive Acetohydroxy Acid Synthases in *Escherichia coli* K-12: Unique Regulation Modulated by Multiple Genetic Sites

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Summary. Spontaneous mutants (146) of *Escherichia coli* K-12 were selected that were resistant to inhibition of growth by 1.2 mM L-valine (Val^r). The Val^r isolates, containing acetohydroxy acid synthase resistant to feedback inhibition by L-valine (AHAS^r), were classed according to cotransduction of the mutation with *leu*. Several mutations resulting in an AHAS^r phenotype were found to be cotransducible with *glyA*. However, no mutations causing a Val^r phenotype were linked to *ilv*. AHAS activity was more closely examined in representatives of three classes of mutants with Val^r linked to *leu*, labeled *ilv-660, ilv-661,* and *ilv-662*. The *ilvE503* allele in *E. coli* K-12, known to cause a two- to three-fold derepression of AHAS, was found to affect regulation of synthesis of both valine-sensitive AHAS (AHAS^s) and AHAS^r in the mutants containing *ilv-660* and *ilv-661*, whereas it affected repression of AHAS^s, only, in the mutant containing *ilv-662*. Further, both AHAS^s and AHAS^r in the *ilv-661* mutant were repressed by valine, whereas valine did not repress AHAS^r synthesis in the strain carrying *ilv-660* and only partially repressed AHAS^r in the strain carrying *ilv-662*. Unexpectedly, AHAS^r synthesis in strains carrying *ilv-660* or *ilv-662* was repressible by leucine. The *ilv-660* locus appears to be similar in position to *ilvH* and encodes a product that confers valine-sensitivity upon AHAS activity in the wild-type *E. coli* K-12. The *ilv-660* and *ilv-662* loci may normally encode products that influence both the feedback sensitivity of AHAS and control of AHAS biosynthesis.

Introduction

The peculiar sensitivity to valine that K-12 strains of *Escherichia coli* display has prompted many studies into the fundamental mechanisms of this sensitivity. The second enzyme in the isoleucine-valine biosynthetic pathway, acetohydroxy acid synthase (AHAS, EC 4.1.3.18 acetolactate synthase) is sensitive to feedback inhibition by valine in the K-12 strain (Fig. 1). An additional valine-insensitive activity (AHAS II or AHAS^r) has been reported in non-K-12 strains of *E. coli* and in *Salmonella typhimurium* (Blatt et al., 1972; O'Neill and Freundlich, 1972). The gene for AHAS II is *ilvG*. *E. coli* K-12, although carrying an *ilvG* gene, apparently does not express it sufficiently to meet the need for isoleucine that arises from repression and inhibition of AHAS^s by valine. Six distinct sites have previously been identified as having effects on AHAS activity in *E. coli* K-12: *ilvF* (Pledger and Umbarger, 1973); *ilvG, ilvB* (Guardiola et al., 1974); *ilv-521* (Jackson and Henderson, 1975); and *ilvH, ilvI* (DeFelice, et al., 1974A). We report here evidence for the involvement of two and possibly three more loci.

For some time the *ilvB* gene had been considered to be the singular structural gene for valine-sensitive AHAS (AHAS I or AHAS^s) in *E. coli* K-12 (Ramakrishnan and Adelberg, 1965). Evidence now suggests that more than one structural gene is involved in the synthesis of AHAS^s (DeFelice, et al., 1975). DeFelice, et al. (1974) reported evidence that two genes (*ilvH, ilvI*) involving AHAS synthesis were linked to *leu* instead of *ilv*. Of these two genes, *ilvI* was shown to be a structural gene for an AHAS^s enzymatic activity while *ilvH* was thought to be involved in the valine-sensitivity of the enzyme. It is not yet known how closely-related are the *ilvB* and *ilvI* gene products, though they are isofunctional enzymes.

Mutations resulting in decrease of the inhibitability of AHAS^s by valine had been used on a small scale in the past (Leavitt and Umbarger, 1962; DeFelice et al., 1974, 1974a; Jackson and Henderson, 1975: Kline et al., 1975) to identify genes that encode AHAS^s. By selecting a large number (146) of inde-

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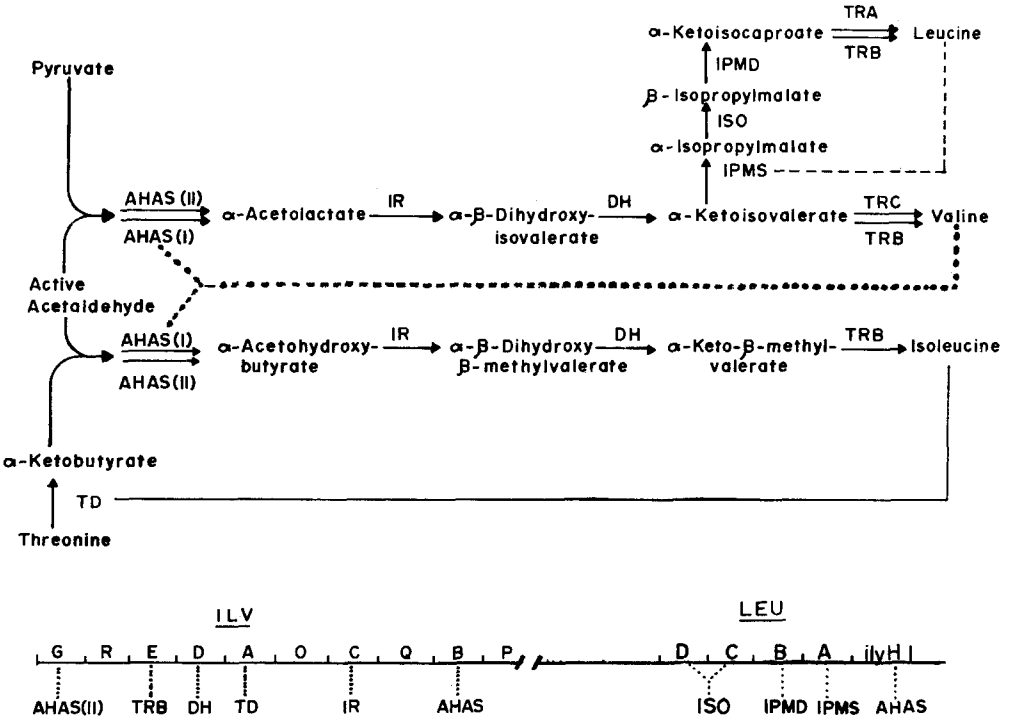


Fig. 1. The biosynthetic pathway for isoleucine, leucine, and valine and genes coding for the enzymes involved. The enzymes catalyzing the indicated steps are abbreviated as follows: TD, threonine deaminase (EC 4.2.1.16, L-threonine hydrolyase (deaminating); AHAS, acetohydroxy acid synthase (EC 4.1.3.12, 2-aceto-2-hydroxy acid 2-oxo acid lyase); IR, acetohydroxy acid isomeroreductase (EC 1.1.78); DH, dihydroxy acid dehydrase (EC 4.2.1.9.1, 3-dihydroxy acid hydro-lyase); TRB, transaminase B (EC 2.6.1.6, branched-chain amino acid amino transferase; TRA, transaminase A; TRC, transminase C; IPMS, isopropylmalate synthase (EC 4.1.3.12); Iso, isopropylmalate isomerase, (EC 4.2.1.33): and IPMD, isopropylmalate dehydrogenase (EC 1.1.1.85). The *ilv* and *leu* genes corresponding to these enzymes are indicated below the scheme. Genes *ilvP* and *ilvO* are repression recognition or modulation genes. The gene order is the reverse of the way it is usually represented on the *E. coli* chromosome map

pendly occurring, spontaneous, valine-resistant mutants, we have attempted to increase the probability of uncovering all gene locations affecting the biosynthesis of AHAS^s. We developed a rapid and accurate permeabilized-cell assay for AHAS activity with reliability comparable to conventional assay of enzyme activity in sonicated extracts. This enabled us to screen a large number of mutants by direct enzyme assay. A preliminary report describing these mutants has been made (Jackson, J.H., J.M. Blatt, E.K. Henderson, and J.J. Whittaker, Ann. Meet. Amer. Soc. Microbiol. p167, 1974). We have examined representatives of three classes of mutants that were linked to *leu* by cotransduction and have determined that these represent genetically and functionally distinct loci. At least one locus (*ilv-662*) represents a previously undefined gene for which we propose the name *ilvJ*.

Materials and Methods

Organisms and Cultivation Conditions. Strains used in this study are listed in Table 1. *E. coli* strain MJ6, a *rbs* derivative of K-12 also known as CU2001, was the parent strain for all mutant selections. A mineral salts glucose medium (minimal medium) previously described (Szentirmai et al., 1968) was used, with supplements as described below, for cultivating cells.

Mutant Selection and Screening. Spontaneous, independent mutants of strain MJ6 were selected that were resistant to inhibition of growth by 1.2 mM L-valine, the normal concentration employed for growth of isoleucine-valine auxotrophs. A total of 146 separate isolates was obtained. Cultures in minimal medium (10 ml) were inoculated from separate colonies and grown to mid-or late-log phase (a turbidity of 150–200 Klett units with a no. 42 filter). A sample (0.1 ml) from each culture was spread onto a minimal agar plate containing 1.2 mM L-valine. Only one isolate was cloned from each plate containing mutants, except when a plate contained two distinct colony sizes, in which case an isolate of each type was cloned. Clonal purity was assured by several successive isolations of single colonies. Mutants were screened for excretion of the branched-chain amino acids by an auxanographic method previously described (Jackson and Umbarger, 1973) and for resistance to inhibition of growth by glycyl-*L*-valine (1.2 mM). Glycyl-*L*-valine resistance was taken to mean that the mutant phenotype was not a consequence of an alteration in transport of valine (Guardiola and Iaccarino, 1971).

Strain Construction. Transduction procedures employing bacteriophage P1 kc were those described by Lennox (1955). The Val^r markers in strains MJ41, MJ42, and MJ43 were transduced into strain

Table 1. Strains used

Strain	Genotype	Use	Source
E. coli			
MJ6	*rbs-215*	Parent of valine-resistant mutants	D. McGilvray (formerly CU2001)
CU8	*ilvA451*	Excretion indicator; mapping	H.E. Umbarger (formerly JHM544)
CU15	*ilvD452*	Excretion indicator	H.E. Umbarger (formerly 20A19)
CU16	*rbs-215, metE*	Mapping	W.J. Pledger
PA200Y	*thr, leu, his, thi, pyrB*	Mapping and strain construction	F.C. Neidhardt
MJ7	*glyA, pro*	Mapping	H.E. Umbarger (formerly SP31)
MJ13	*rbs, leu, thi, his, thr, pyrB*	Recipient in strain constructions derived from PA200Y by DES mutagenesis	J.H. Jackson
MJ15	*rbs, thi, his, thr, pyrB*	Isogenic control strain from MJ6 × MJ13 cross	E.J. Davis
MJ38	*ilvE503*	Strain construction	J.H. Jackson (formerly CU8506)
MJ41	*ilv-661, rbs-215*	Spontaneous Val^r mutants derived from MJ6	This study
MJ42	*ilv-660, rbs-215*	Spontaneous Val^r mutants derived from MJ6	This study
MJ43	*ilv-662, rbs-215*		
MJ46	*ilv-661, rbs, thr, his, thi, pyrB*	Constructed by transduction from MJ41, MJ42,	This study
MJ48	*ilv-660, rbs, thr, his, thi, pyrB*	and MJ43, respectively, to MJ13	
MJ50	*ilv-662, rbs, thr, his, thi, pyrB*		
MJ47	*ilv-661, ilvE503, thr, his, thi, pyrB*	Constructed by transduction from MJ38 to MJ46,	This study
MJ49	*ilv-660, ilvE503, thr, his, thi, pyrB*	MJ48, and MJ50	
MJ51	*ilv-662, ilvE503, thr, his, thi, pyrB*		
MJ56	*ara, leu, his, thi, pyrB, rbs*	Mapping. Derived from MJ13 by transduction from CSH26	This study
CSH26	*ara*, △ (*lac pro*), *thi*	Strain construction	J.H. Miller
S. typhimurium			
leu-447	*leu* ▽ *A, B, C, D*	Excretion indicator	P. Margolin

MJ13, selecting for leucine prototrophy. The recombinants were then screened for the Val^r character. The resulting Val^r, Leu$^+$ transductants were designated MJ46, MJ48 and MJ50. These strains then served as recipients in another series of transductions employing MJ38 as donor. The *ilvE503* mutation of this strain was transferred by cotransduction with *rbs*$^+$. Recombinants were screened auxanographically for the *ilvE503* mutation (Jackson and Umbarger, 1973). Selected transductants containing both Val^r and *ilvE503* were purified and later designated as MJ47, MJ49, and MJ51.

Enzyme assays. AHAS was assayed using crude extract preparations (Størmer and Umbarger, 1964; Szentirmai et al., 1968) and as described below. For screening purposes, the specific activity of AHAS was measured for each mutant using a lysed-cell assay. Sensitivity to feedback inhibition by L-valine was measured using 30 μM and 100 μM concentrations of L-valine to produce approximately half-maximal and nearly maximal inhibition respectively in the wild-type strain MJ6. Protein was measured by the biuret method (Layne, 1957).

For the permeabilized-cell assay, individual cell cultures (10 ml) were grown in a roller drum at 37° C. For larger experiments, samples (10 ml) were taken from batch cultures grown at 37° C with aeration by shaking. In both procedures, the cultures were harvested after reaching a culture density of 150 Klett units. Measurments of cell density were made using a Klett-Summerson colorimeter with a no. 42 filter. Cell cultures were centrifuged at 4° C and supernatant volumes were recorded for each sample. Cells were washed in 1.0 ml and resuspended in 0.8 ml of AHAS stabilizing buffer (Blatt et al., 1972) for assay. Reaction conditions employed were those of Størmer and Umbarger (1964) except for the addition of 0.0071% cetyltrimethylammonium bromide (CETAB) needed to permeabilize cells. The assay mixture contained (in 1.0 ml) 100 mM potassium phosphate, pH 8.0, 10 mM $MgCl_2$, flavin adenine dinucleotide (2 μg/ml) 0.1 mM thiamine pyrophosphate, and 40 mM sodium pyruvate. Usually, four samples (0.1 ml) of cell suspension were used for assay: one blank, one with pyruvate alone, and two with pyruvate and different concentrations of valine. Care was taken to add $MgCl_2$ last in order to avoid precipitation before initiating the reaction by addition of enzyme. Incubation time was 10 min at 37° C and the reactions were stopped by the addition of 0.1 ml of 50% (w/v) H_2SO_4 to each tube. The assay under these conditions was linear for at least 20 min (Blatt and Jackson, manuscript in preparation). Incubation was continued for 30 min at 37° C to decarboxylate acetolactate to acetoin before color development. Samples were clarified by centrifugation before absorbance was measured at 525 nm (1 cm path-length cell). Specific activity, in the case of mutant surveys, was calculated in a manner to reflect proportionality to activity on a per cell basis as follows: Specific Activity = (C) $(A_{525})/(V)$ (K_{42}) where C = 127.0 for a 10 min assay, A_{525} is the absorbance at 525 nm due to assay product formed, K_{42} is the turbidity of the culture in Klett units using a No. 42 filter, at the time of harvest, and V is the culture supernatant volume. Specific activity thus calculated is expressed in units of nanomoles of α-acetolactate formed *per* min *per* ml of culture volume per Klett unit (nmol/min/ml/K_{42}). The sum of contributions from all genes involved in formation of AHAS activity is referred to as AHAS^t. We refer to all measurable AHAS activity in the presence of 1.0 mM valine as AHAS^r. This concentration of valine was chosen since it is at least 10-fold higher than the highest concentration of valine giving half-maximal inhibition of AHAS activity we observed in crude extracts. The difference between AHAS^t and AHAS^r is AHAS^s, the activity inhibitable by valine.

Analysis of Kinetic Data. Kinetic parameters of inhibition experiments were estimated by a FORTRAN computer program adapted from Cleland (1963, 1967) which performs a least squares fit of percent inhibition versus valine concentration data, to a rectangular hyperbola (Michaelis-Menten equation). Program HYPER was obtained from W.W. Cleland, University of Wisconsin, through H.E. Umbarger, Purdue University. We have defined, for comparative use, the valine concentration giving half-maximal inhibition as the $I_{0.5}$ and the maximum percent inhibitability (an asymptotic value) as the I_{max}.

Chemicals. All chemicals were reagent grade or the highest purity commercially available. CETAB was obtained from Sigma Chemical Company.

Results

Mutant Selection, Screening, and Characterization. The specific activity of AHAS was measured in each of 146 independent, spontaneously isolated mutants that were resistant to 1.2 mM valine. The ratio of specific activity of AHAS in each mutant to that of the parent strain MJ6 was used as a screening criterion. The value of AHAS specific activity in strain MJ6 varied over a $\pm 7.5\%$ range, depending on variations in growth. The 17 mutant strains in which this ratio exceeded 2.0 were classified as derepressed for AHAS biosynthesis. Two derepressed mutants also displayed feedback-resistant AHAS (AHAS^r). Approximately 38% of all the valine-resistant (Val^r) isolates, as well as 37% of AHAS^r isolates, exhibited significantly reduced levels of AHAS with a specific activity ratio less than 0.5. AHAS^r isolates were defined either on the basis of their inhibitability of AHAS by valine at the $I_{0.5}$ or at a concentration (1.0 mM) resulting in an inhibition indistinguishable from I_{max}. Since our objective was to identify loci responsible for the feedback inhibitability of AHAS, thus identifying structural genes for AHAS^s or genes that control expression of feedback-sensitivity, this study centered on mutants that did not exhibit abnormally derepressed or repressed levels of AHAS.

All mutants were screened for resistance to glycyl-L-valine. Guardiola and Iaccarino (1971) demonstrated that valine-resistant regulatory mutants were resistant to glycylvaline, whereas mutants that had reduced capacity to transport valine remained sensitive. All mutants classed as having AHAS^r were resistant to glycylvaline.

Bacteriophage P1 lysates were prepared from mutants which displayed AHAS^r and were used to determine cotransduction linkage of Val^r to selected genetic markers. Initially, when *ilvA* was tried, no Val^r recombinants were found. We then tested for linkage of Val^r to other markers close to genes that affect regulation of the branched-chain amino acid biosynthetic pathway. These markers included *pyrB, pyrA, metE, glyA,* and *leu.* We found that Val^r in most of the AHAS^r mutants was cotransducible with both *pyrA* and leu, though with highest frequency to *leu.* Only 12.5% of the AHAS^r mutants had Val^r unlinked to *leu.*

Table 2. Classification of AHAS^r isolates according to cotransduction frequency with leu

Class	Percent cotransduction (Val^{r}Leu$^+$ × 100)	Relative[a] specific activity	Percent[b] inhibition range	No. of isolates
I	83–100	0.33–0.68	5–46	9
II	65–83	0.25–0.75	10–75	23
III	51–54	0.48–0.58	18–44	2
IV	35	0.26	21	1
V	0	0.21–0.33	9–20	4

[a] Relative specific activity = Sp. act. of mutant/Sp. act. of parent
[b] Inhibition of specific activity in the presence of 1.0 mM L-valine

AHAS^r mutants fell into four or five groups based upon linkage of Val^r to *leu* (Table 2). A value of 83% cotransduction with *leu* was used as an arbitrary line of distinction beteen classes I and II. It was not clear, from simple cotransduction frequency between two markers, whether the mutations of classes I and II lay within more than one gene. Cotransduction frequency data usually are consistent enough that mutations falling in the range of 65% to 100% linkage to *leu* represent more than one distinct linkage group. Mutants from class V, unlinked to leu, were heterogeneous. However, none among these was linked to *ilv.* Among these mutants we found one with linkage of Val^r to *metE* and two with linkage to *glyA.* One class V mutant with AHAS^r was derepressed nearly four-fold for AHAS^t whereas other class V mutants displayed one-fifth to one-third of the normal levels of AHAS^t in the wildtype parent strain.

Properties of Mutant AHAS. Representative mutants from each of the classes were examined to compare properties of AHAS in sonicated, clarified extracts and in the lysed-cell assay. Values for maximal percent inhibition of AHAS by valine in crude extracts were generally equivalent to the values obtained in lysed-cell assays (Table 3). AHAS activity in the parental strain MJ6 was generally more sensitive to inhibition in the lysed-cell assay than in the crude, clarified extract. Overall, the lysed-cell assay produced data comparable to that obtained from crude extracts. Therefore, all subsequent measurements of AHAS activity were made using the lysed-cell assay. We measured the sensitivity of AHAS to valine inhibition in mutant isolates and compared

Table 3. Properties of mutant AHAS in different assay systems

Strain	Mutant class	Lysed-cell assay		Sonicated crude extract	
		Sp. Act.[a]	% I[b]	Sp. Act.[c]	% I[b]
MJ6	w.t.	0.065	89	88.5	83
MJ42	I	0.044	46	47.0	37
MJ41	II	0.058	75	62.0	73
MJ43	III	0.069	44	52.3	42

[a] One unit of specific activity equals one nmole of acetolactate formed per min per ml culture per Klett unit of culture density with a no. 42 filter

[b] Percent inhibition was measured using 1.0 mM L-valine to inhibit the reaction

[c] One unit of specific activity equals one nmole of acetolactate formed per min per mg extract protein

Table 4. Sensitivity of AHAS to valine inhibition

Strain	Mutant class	$I_{0.5}$ (μM valine)[a]	I_{max} (% I)[b]
MJ6	w.t.	52.7± 6.2	94.7±2.5
MJ42	I	73.1±13.2	46.5±2.0
MJ41	II	213.5±24.7	86.1±2.7
MJ43	III	81.0±24.6	27.0±1.8

[a] $I_{0.5}$ is the concentration of valine that produces half-maximal inhibition of AHAS activity. This value is derived from a least squares fit of percent inhibition versus the valine concentration to a rectangular hyperbola estimated by a Fortran computer program (Materials and Methods).

The values shown are concentrations of valine ± one standard error of the computer fit of data from representative experiments selected from two to three separate determinations

[b] I_{max} is the maximal percent inhibition of AHAS activity by valine. The values were estimated using the computer program mentioned above. The variations shown indicate one standard error in the computer fit of data from a single, representative experiment

the $I_{0.5}$ and I_{max} to those of AHAS in the parent strain. The AHAS^r mutant phenotype was defined one of two ways. Either it had a maximal inhibitability (I_{max}) by valine of under 60%, or it required a valine concentration greater than 100 μM to achieve half maximal inhibition. These alterations would imply a change in the balance of isofunctional enzymes synthesized or in the structure of a valine binding site responsible for inhibition of the enzyme(s).

Mapping Studies. Representative mutants from each class were assigned allele numbers as follows: MJ42 (class I), *ilv-660*; MJ41 (class II), *ilv-661*; MJ43 (class III), *ilv-662*. Class IV was not included in the present study. Two mutants within class V were found to be 7–16% cotransducible with *glyA* and are presumed to be alleles of *ilvF*. The mutation that was 16% contransducible was assigned the allele

Table 5. Frequencies of cotransduction.

Strain	Selected Marker(s)	Unselected Marker(s)	Transductants scored	Unselected Marker Appearance	Frequency of Cotransduction
MJ42 (Class I)	Leu$^+$	Val^r	765	607/765	0.79
	Thr$^+$	Val^r	1384	81/1384	0.06
	Leu$^+$	Thr$^+$	2120	178/2120	0.08
	Thr$^+$	Leu$^+$	980	86/980	0.09
	Leu$^+$	Ara$^+$	355	223/355	0.63
	Ara$^+$	Leu$^+$	103	52/103	0.50
	Ara$^+$	Val^r	1031	719/1031	0.70
	Ara$^+$	Val^r, Leu$^+$	164	84/164	0.51
	Leu$^+$, Thr$^+$	Val^r	100	62/100	0.62
	Ara$^+$, Leu$^+$	Val^r	1160	780/1160	0.67
MJ41 (Class II)	Leu$^+$	Val^r	682	501/682	0.73
	Thr$^+$	Val^r	1527	72/1527	0.05
	Leu$^+$	Thr$^+$	1943	236/1943	0.10
	Thr$^+$	Leu$^+$	1582	148/1582	0.09
	Ara$^+$	Leu$^+$	281	163/281	0.58
	Ara$^+$	Val^r	1395	1032/1395	0.74
	Ara$^+$	Val^r, Leu$^+$	388	153/388	0.39
	Leu$^+$, Thr$^+$	Val^r	308	52/308	0.17
	Ara$^+$, Leu$^+$	Val^r	1516	928/1516	0.61
MJ43 (Class III)	Leu$^+$	Val^r	631	322/631	0.51
	Thr$^+$	Val^r	701	72/701	0.10
	Thr$^+$	Leu$^+$	1042	110/1042	0.11
	Leu$^+$	Thr$^+$	1091	105/1091	0.10
	Ara$^+$	Leu$^+$	119	87/119	0.73
	Ara$^+$	Val^r	765	570/765	0.75
	Ara$^+$	Val^r, Leu$^+$	644	322/644	0.51
	Leu$^+$, Thr$^+$	Val^r	79	54/79	0.67
	Ara$^+$, Leu$^+$	Val^r	191	170/191	0.89

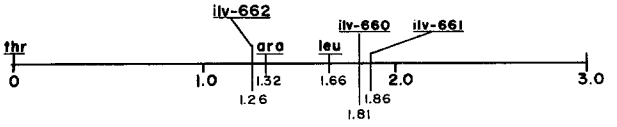


Fig. 2. Transduction linkage map of the *leu* region. Chromosome map positions of three well characterized loci are marked in minutes (*thr*, *ara*, and *leu*). Positions of the three mutant alleles are indicated relative to *leu* and *ara*

number, *ilv-663*. In all cases AHAS^r cotransduced with Val^r. From a series of two-point and three-point, single and joint transductions the relative positions of the Val^r loci were determined (Table 5) subject to confirmation by three-point reciprocal crosses. These studies revealed that *ilv-661* lies to the right (Fig. 2) of *leu*. The class I mutation, *ilv-660*, lies close to and probably to the right (Fig. 2) of *leu*, but these data do not permit unequivocal interpretation. The *ilv-662* locus lies to the left (Fig. 2) of *leu* and probably *ara*. The evidence for this is as follows. Val^r, as an

unselected marker, cotransduced with the selected markers indicated in Table 5. Cotransduction frequencies for *ilv-660* and *ilv-661* were roughly comparable in all crosses involving one selected marker. However *ilv-661* consistently cotransduced less frequently with *leu* than did *ilv-660*. When *leu* and *thr*, or *ara* and *leu* were jointly selected and Val^r was scored as a single unselected marker, the frequencies of cotransduction were lower than those observed for Val^r linked either to *leu* or to *ara*. We interpret these observations to mean that ilv-660 and *ilv-661* are located outside of the *thr-leu* and *ara-leu* boundaries. Considering the higher linkage of these loci to *leu*$^+$ compared to *thr*, a location to the right (Fig. 2) of *leu* seems likely. Note that the frequency of cotransduction with *ara* and *leu*, together, or to a lesser extent with *thr* and *leu* together, would have approached 1.0 if the mutations were located within these respective boundaries. The average reciprocal cotransduction frequency between *ara* and *leu* was 0.57 for *ilv-660* and *ilv-661* strains. Cotransduction of *leu*$^+$ from *ilv-661* and Val^r together linked to *ara* occurred with a frequency of 0.51, slightly lower than the linkage of *leu* alone to *ara*. This reduction in frequency suggested that *ilv-660* might lie to the right (Fig. 2) of *leu*, although the magnitude of the reduction was not large enough to be conclusive. Cotransduction of *leu*$^+$-Val^r from an *ilv-661* strain with *ara* occurred with a frequency of 0.39, a value substantially lower than 0.57 for cotransduction of *leu* with *ara*. This reduced frequency clearly indicated that *ilv-661* is located to the right of *leu* and farther from *leu* than is *ilv-660* (Fig. 2).

The same analyses were used to determine the approximate location of *ilv-662*. The simple cotransduction frequency of *ilv-662* with *leu* was 0.51. The cotransduction frequencies of *ilv-662* with the jointly selected markers *leu-thr* and *ara-leu* were 0.67 and 0.89 respectively. Both values are clearly greater than 0.51. We presume that the reason for the cotransduction frequency with *leu-thr* having been so much less than 1.0 is due to the relatively high probability of crossovers between such distant markers. However, the frequency associated with *ara-leu* was less than 1.0 also. We conclude that *ilv-662* lies to the left of *leu* (Fig. 2) but that its position relative to *ara* is unclear. If *ilv-662* lay to the right of *leu* with 0.51 cotransduction frequency then joint selection of *thr* and *leu* would have reduced linkage dramatically. That clearly did not occur. The average reciprocal cotransduction frequency between *ara* and *leu* in the *ilv-662* strains was 0.69. The cotransduction frequency

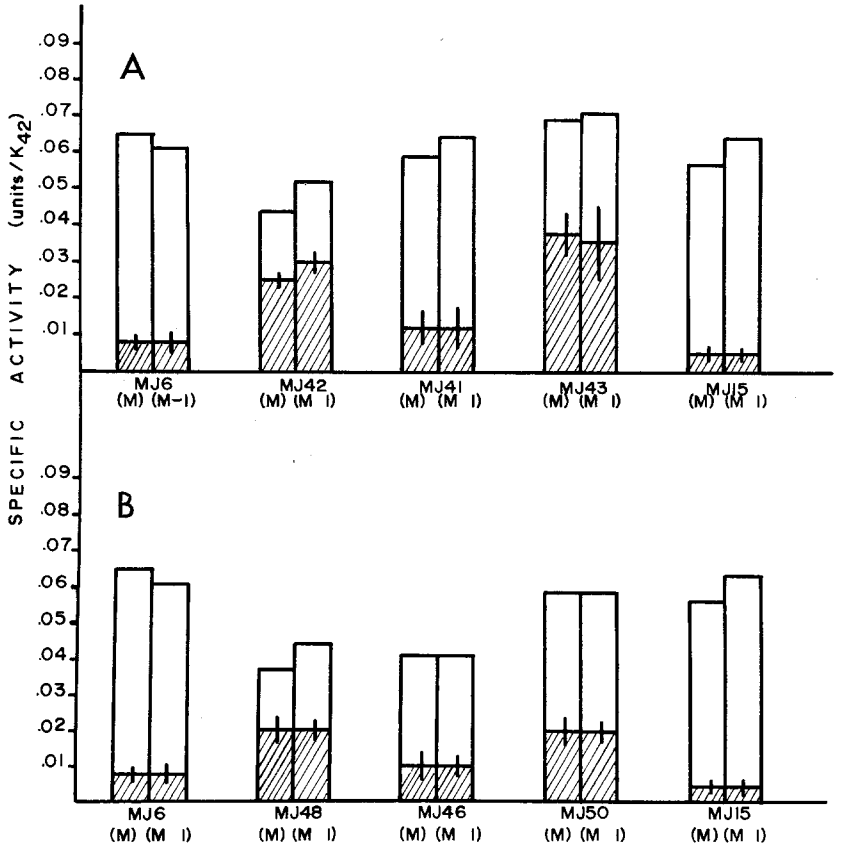


Fig. 3A and B. Modified AHAS activity associated with selection for Val^r in transductants. (**A**) Specific activities of AHAS in original mutant isolates; (**B**) Specific activities of AHAS in Val^r transductants. Total bar height represents the total specific activity of AHAS (AHAS^t). Shaded areas represent AHAS^r, the fraction of AHAS^t which is resistant to inhibition by 1.0 mM L-valine. Clear areas represent AHAS^s, the fraction of AHAS^t which is sensitive to inhibition by 1.0 mM L-valine. The vertical line through the AHAS^r-AHAS^s junction represents the mean deviation in AHAS^r from three to five separate determinations. One unit of specific activity equals 1 nmole of α-acetolactate formed/min/ml culture/Klett unit using a no. 42 blue filter for measurement of culture turbidity. Each strain was cultured in minimal medium (M) and in minimal medium supplemented with L-isoleucine (MI)

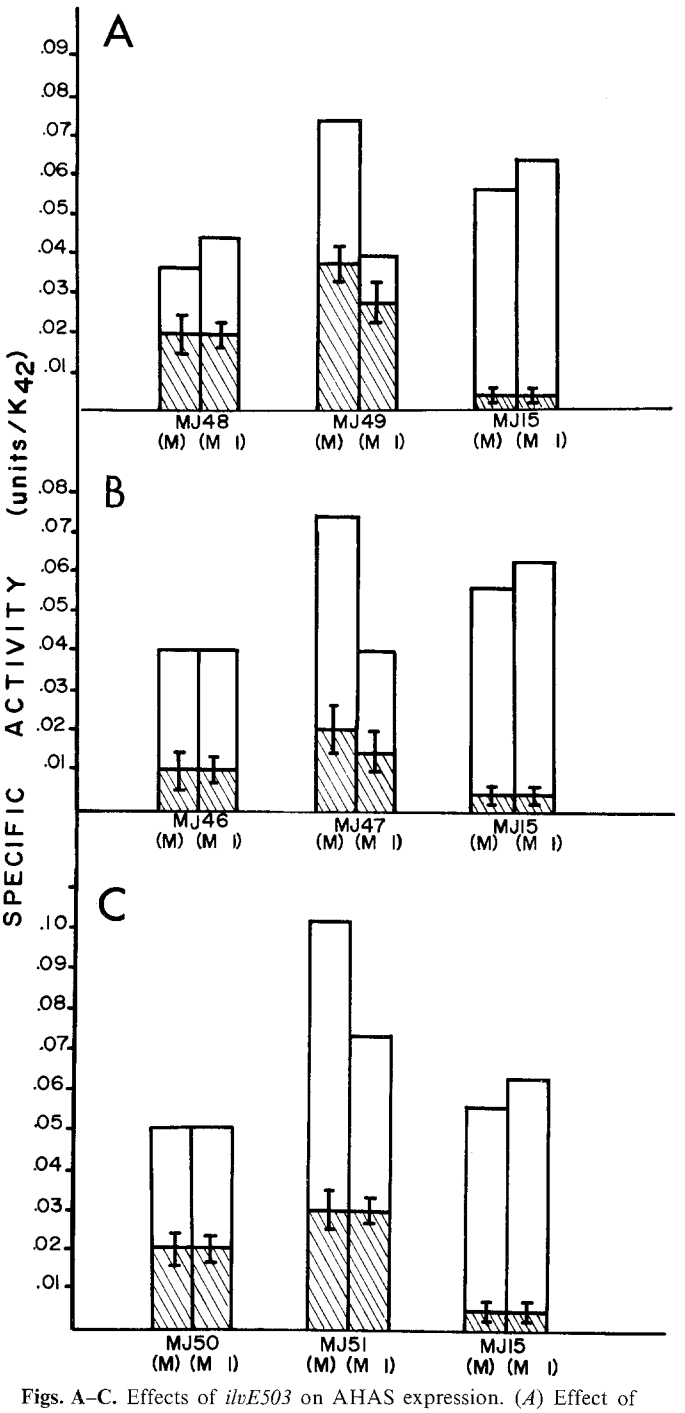
of Val^r and Leu$^+$, jointly cotransduced as unselected markers with *ara*$^+$, was 0.51, a decrease from 0.69. If *ilv-662* lay between *ara* and *leu* an increased frequency would have been expected. Therefore, *ilv-662* apparently lies to the left (Fig. 2) of *ara*.

According to these studies of cotransduction frequency we constructed a map of the *leu* region with the mutations affecting AHAS in position (Fig. 2). The map distances between the mutant loci and previously reported markers were calculated from cotransduction frequencies by use of the Wu equation (Wu, 1966) $F=(1-d/L)^3$ where F=cotransduction frequency, d=distance between markers (min), and L=the length of a transducing fragment. We used 2 min for the value of L (Bachmann et al., 1976). The values of d shown were calculated from F values of Val^r linked to *leu* or *ara*.

Evidence for AHAS^r and Val^r as Pleiotropic Effects of Single Mutational Events. The strains in which the *ilv-660*, *ilv-661*, and *ilv-662* mutations were isolated were tested for correspondence of the AHAS^r phenotype with the Val^r phenotype. The AHAS activity of each Val^r mutant along with the parent strain MJ6 is depicted in Figure 3. Ten random Val^r transductants from each cross were tested. The phenotypic properties of AHAS in all transductants were identical to those in the corresponding original mutant isolates. Properties of AHAS for one representative transductant for each mutant class are shown in Figure 3 along with properties of the parent strain, MJ6, and the recipient strain MJ15, which is isogenic with the transductants.

Regulation of Synthesis of AHAS in AHAS^r Mutants. If a mutation that produces an AHAS^r phenotype simply changes the feedback sensitivitity of a normal AHAS, then whatever increase or decrease occursin the rate of synthesis of AHAS^t should also occur with AHAS^r. In such a system AHAS^r would simply reflect what would normally have been control over synthesis of AHAS^s. This interpretation should be valid even if multiple genes encode AHAS^t, AHAS^r, or both. We sought, then, to determine whether synthesis of AHAS^r by strains carrying *ilv-660, ilv-661,* or *ilv-662* was regulated in the same way as AHAS^t or AHAS^s.

In particular, we sought to determine whether *ilvE503* exerted an effect on AHAS^r in Val^r strains in a manner similar to the derepression of AHAS^s it had been shown to cause in previous studies (Jackson and Umbarger, 1973; Jackson and Henderson, 1975). The two- to three-fold derepression of AHAS^s caused by *ilvE503* occurred concomitantly with



Figs. A–C. Effects of *ilvE503* on AHAS expression. (*A*) Effect of *ilvE503* and *ilv-660* on AHAS in strain MJ49. (**B**) Effect of *ilvE503* and *ilv-661* on AHAS in strain MJ47. (**C**) Effect of *ilveE503* and *ilv-662* on AHAS in strain MJ51. Strains MJ48, MJ46, and MJ50 contain *ilv-660, ilv-661* and *ilv-662*, respectively, in the absence of *ilvE503*. These in turn should be compared to their isogenic Val^r control, strain MJ15. Interpretation of the bar graphs is the same as in Figure 3

an isoleucine restriction and was reversed by supplementation of the growth medium with isoleucine.

Isogenic strains were constructed to contain each Val^r mutation in addition to the *ilvE503* allele (Ta-

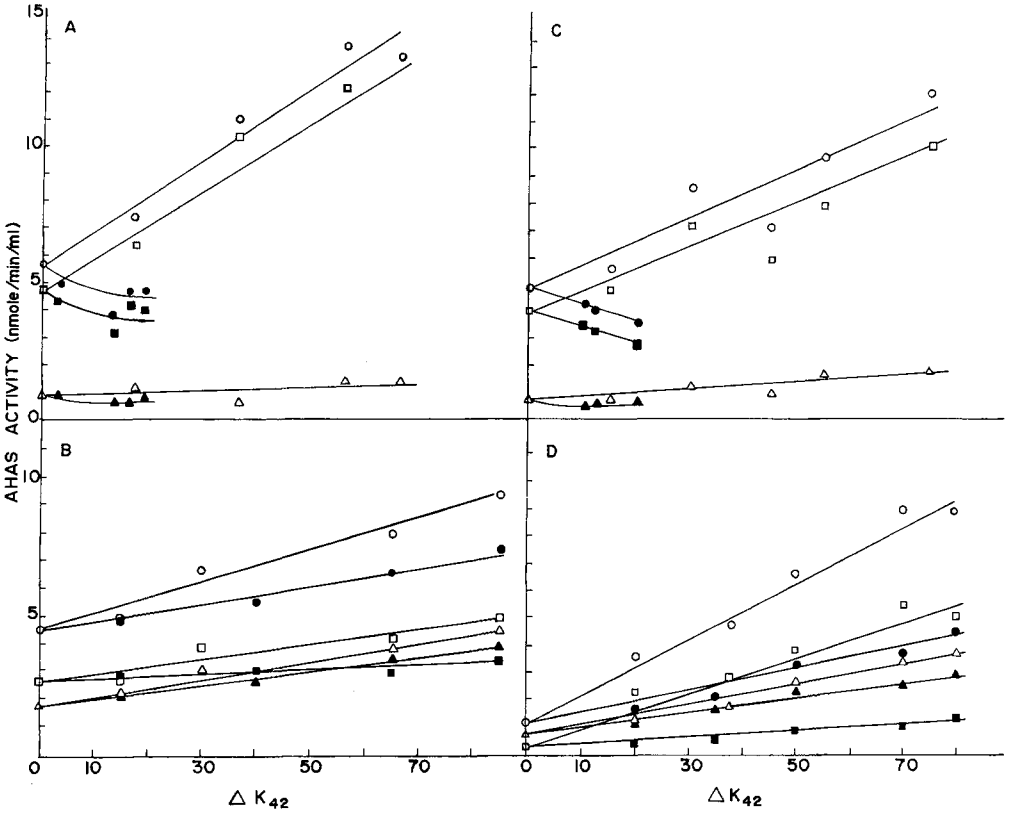


Fig. 5A–D. Effects of valine on AHAS expression. Differential rates of synthesis are plotted as total AHAS activity per ml culture vs. the change in culture turbidity expressed in Klett units using a no. 42 filter. All cultures were grown in minimal medium (minimal) or in minimal medium supplemented with 1.2 mM valine (minimal + valine). Batch cultures were grown to $K_{42} = 100$, the cultures were divided, valine was added to one flask (closed symbols), and both were sampled at intervals. (**A**) Strain MJ6 (parental strain) Symbols: ○, AHAS^t from growth in minimal; ●, AHAS^t from growth in minimal + valine; △, AHAS^r from growth in minimal; ▲, AHAS^r from growth in minimal + valine; □, AHAS^s from growth in minimal; ■, AHAS^s from growth in minimal + valine. (**B**) Strain MJ42 (*ilv-660*). Symbols same as in (**A**). (**C**) Strain MJ41 (*ilv-661*). Symbols as in (**A**). (**D**) Strain MJ43 (*ilv-662*). Symbols as in (**A**)

ble 1). These strains were cultured in minimal medium and in minimal medium supplemented with isoleucine to determine whether the rate of AHAS^r biosynthesis was affected in a manner characteristic of *ilv*E503. Strain MJ49 (*ilv-660, ilvE503*) displayed derepression of AHAS^r concomitant with the expected two-fold derepression of AHAS^t and AHAS^s when compared to an isogenic control strain MJ48 (*ilv-660*) cultured under the same conditions (Fig. 4A). Strain MJ47 (*ilv-661, ilvE503*) behaved similarly but not as distinctly (Fig. 4B). In each of these cases AHAS^r was derepressed approximately two-fold and was slightly repressed in the presence of isoleucine. However, AHAS^s in both mutant classes was repressed by isoleucine to the levels observed in isogenic strains lacking *ilvE503*. In contrast, AHAS^r was derepressed barely 50% in strain MJ51 (*ilv-662, ilvE503*) relative to strain MJ50 while AHAS^s was derepressed approximately 2.5-fold and was partly repressible by isoleucine (Fig. 4C). AHAS^r levels were unaffected by the presence of isoleucine during growth. Thus, the *ilvE503* allele exerted an isoleucine mediated control over AHAS^r and AHAS^s synthesis in strains with *ilv-660* or *ilv-661* mutations. However, in strains containing *ilv-662*, the synthesis of AHAS^s was affected by *ilvE503* while synthesis of AHAS^r was unaltered. These data were indications that the mutation that caused Val^r in MJ43 (*ilv-662*) may have also altered the regulation of synthesis of AHAS^r.

Since one of the three Val^r mutations differed in response to the regulatory influence of *ilvE503*, it was important to determine whether valine exerted normal control over synthesis of AHAS in each Val^r strain. For this purpose the differential rates of synthesis of AHAS^t, AHAS^s, and AHAS^r were measured in cultures of each mutant class growing in minimal medium as well as in minimal medium to which valine was added during the logarithmic growth phase (Figs. 5A through 5D). Differential rate plots for MJ6, the parent strain, revealed that valine caused

Table 6. Effects of valine on strain growth.

Strain	Growth Conditions[a]	Growth Rate Constant (h^{-1})[b]
MJ6 (w.t.)	MIN	0.204
	MIN+V	0.053
MJ42 (ilv-660)	MIN	0.158
	MIN+V	0.161
MJ41 (ilv-661)	MIN	0.180
	MIN+V	0.086
MJ43 (ilv-662)	MIN	0.219
	MIN+V	0.191

[a] A glucose minimal-salts medium (MIN), described in Materials and Methods, was supplemented with 1.2 mM L-valine (V) as indicated

[b] The growth rate constant equals (log 2)/T, where T is the generation time in hours

Table 7. Effect of leucine on expression of AHAS

Strain	Growth[a] Conditions	Specific Activity[b]		
		$AHAS^t$	$AHAS^s$	$AHAS^r$
MJ6 (w.t.)	MIN	0.096	0.085	0.011
	MIN+V	–	–	–
	MIN+L	0.023	0.018	0.005
	MIN+VL	–	–	–
MJ42 (*ilv-660*)	MIN	0.066	0.023	0.043
	MIN+V	0.051	0.015	0.036
	MIN+L	0.022	0.013	0.009
	MIN+VL	0.013	0.003	0.010
MJ43 (*ilv-662*)	MIN	0.074	0.023	0.051
	MIN+V	0.044	0.015	0.029
	MIN+L	0.021	0.016	0.005
	MIN+VL	0.016	0.004	0.012

[a] A glucose minimal salts medium (MIN), described in Materials and Methods, was supplemented with valine (V) or leucine (L) as indicated

[b] Numerical values represent the specific activities of uninhibited AHAS ($AHAS^t$), the fraction of $AHAS^t$ that is sensitive to inhibition by 1.0 mM L-valine ($AHAS^s$), and the fraction of $AHAS^t$ that is resistant to inhibition by 1.0 mM L-valine ($AHAS^r$). Specific activity equals one nmole acetolactate formed per min per ml of culture per Klett unit of culture density with a no. 42 filter

full repression of all forms of AHAS expression (Fig. 5A). Valine effectively repressed synthesis of $AHAS^s$ (and therefore $AHAS^t$) in strains containing *ilv-660*, *ilv-661*, or *ilv-662* (Figs. 5B through 5D). Valine also repressed synthesis of $AHAS^r$, though only in the parent strain and in strain MJ41 (*ilv-661*). Strains that contained *ilv-661* grew very slowly in the presence of 1.2 mM valine (Table 6). In contrast, valine only slightly repressed $AHAS^r$ in strains containing *ilv-660* or *ilv-662* compared to its nearly complete repression of $AHAS^s$ synthesis (Figs. 5B and 5D). Therefore, one of the major consequences of the *ilv-660* and *ilv-662* mutations is an increased rate of $AHAS^r$ synthesis that is modulated poorly or not all by valine.

Since both valine and leucine are involved in the multivalent repression of AHAS, and we found that control by valine was altered in *ilv-660* and *ilv-662* mutant strains, we tested for alteration of control by leucine in these strains (Table 7). We did not include results for the strain containing *ilv-661* in this experiment because regulation of AHAS synthesis by valine was similar to that in the parent strain MJ6. Addition of leucine to a culture of an *ilv-660* strain growing in minimal medium caused a reduction of $AHAS^r$ specific activity from 0.043 to 0.009, whereas the addition of valine had no significant effect (Table 7). The same experiment with an *ilv-662* strain revealed that leucine reduced $AHAS^r$ from a specific activity of 0.051 to 0.005, whereas valine only partially repressed $AHAS^r$ to 0.029 (Table 7). Growth of strains in media containing both leucine and valine did not result in a greater repression than was seen with leucine supplementation alone. In fact, valine may slightly antagonize the leucine effect on *ilv-662*. Thus, *ilv-660* and *ilv-662* each simultaneously decreased the feedback sensitivity of AHAS to valine, and altered the repression of $AHAS^r$ synthesis to respond to a signal for leucine excess.

Discussion

The mutants selected in this study were expected to reveal genes normally involved with $AHAS^s$ synthesis since their abnormal production of $AHAS^r$ would be most likely the result of a change in genes normally expressed in K-12 strains. However, we have not excluded the possibility that these mutations represent the new expression of previously unexpressed $AHAS^r$ genes that may resemble *ilvG* (Fig. 1) in function though not in chromosomal location. If the latter interpretation is correct then a positive gene-dosage effect from the presence of a newly turned-on gene for AHAS might not be observed owing to repression of $AHAS^s$ by an increased intracellular production of valine (and consequently, leucine). To the extent that the total AHAS activity is of the $AHAS^r$ type, the control of end product synthesis resides increasingly in repression as opposed to feedback inhibition of enzyme activity.

Since a complete loss of AHAS activity, resulting in an Ilv^- phenotype, has been reported to occur only when K-12 contains lesions in both *ilvI* and *ilvB* (Smith et al., 1977; Guardiola et al., 1974) the

products of these genes must be indispensable for synthesis of AHAS^s. Further, since no mutations in these genes have been reported to alter the feedback sensitivity of AHAS to valine, it is conceivable that *ilvI* and *ilvB* encode products essential for only the catalytic properties of AHAS^s. It thus becomes reasonable to suppose that the component(s) responsible for regulation of catalysis may be encoded by other genes, including *ilvH* (DeFelice et al., 1974A) and the loci reported in this study. Therefore, we propose as a working hypothesis that catalytic subunits from *ilvB* and *ilvI* may interact with regulatory subunits encoded by the *leu*-linked genes reported here, as well as by *ilvH*, *ilv-521* and *ilvF*. Our mapping data suggest that *ilv-660* may be an allele of *ilvH*.

We conclude that two kinds of regulatory changes occur as a consequence of each Val^r mutation reported here: (1) sensitivity of AHAS^t to feedback inhibition by valine is altered, thus increasing the measurable AHAS^r; and (2) regulation of synthesis of AHAS^r is modified. The first conclusion is based on the clear indication of altered kinetic parameters for allosteric inhibition of AHAS by three classes of Val^r mutants (Table 4). Two lines of evidence support the second conclusion. In an otherwise normal strain the transaminase B allele, *ilvE503*, causes derepression of AHAS^s. If the properties of AHAS were changed by mutation of an AHAS^s structural gene to AHAS^r, then *ilvE503* would be expected to cause derepression of AHAS^r as was observed with *ilv-521* (Jackson and Henderson, 1975). Derepression of AHAS^r occurred in the case of strains carrying *ilv-660* or *ilv-661* but not *ilv-662* (Fig. 4). We interpreted these results to mean that the *ilv-662* mutation alters regulation of AHAS biosynthesis as well as feedback sensitivity. Additionally, supportive of the second conclusion above, valine caused repression of AHAS^r synthesis in strains carrying *ilv-661*, but not *ilv-660* or *ilv-662*, while leucine, alone, fully repressed synthesis of AHAS^r in strains carrying *ilv-660* or *ilv-662*, as well as AHAS^s in strains carrying *ilv-661*.

The data are therefore consistent with our hypothesis in that wild-type alleles of *ilv-660*, *ilv-661* and *ilv-662* encode products (regulatory subunits) which interact with *ilvI* and *ilvB* products (catalytic subunits). The mechanism by which mutations in these genes would bring about changes in repression control remains undefined, though various forms of autoregulation are conceivable.

A possible explanation, suggested previously, is that *ilv-660*, *ilv-661*, and *ilv-662* are genes for AHAS^r not normally expressed in the wild-type K-12 strain, analogous to *ilvG*, in that specific mutations are required to allow expression. However, the incomplete repression of AHAS^s synthesis by valine in *ilv-660* and *ilv-662* strains implies that mutations which allow expression of unexpressed AHAS^r genes must also change the repression control of *ilvB* and/or *ilvI* expression.

Another possible explanation, though not very likely, is that genes specifying other condensing enzymes, which normally utilize different substrates, can be mutated so that their gene products function as AHAS. This could explain increased AHAS^r activity since such enzymes would not be expected to be inhibited by valine. Regulation of their synthesis would certainly be under controls relevant to their normal function, and is difficult to imagine why leucine or valine would be involved.

The map position of *ilv-662*, though not of *ilv-660* and *ilv-661* allows sufficient distinction from *ilvH* or *ilvI* without direct comparison. Since the function and location of *ilv-662* have not previously been reported we propose to name the wild-type allele *ilvJ*. It is interesting to note, however, that *ilvJ* lies in the vicinity of *brnS*, a gene involved in branched-chain amino acid transport (Guardiola and Iaccarino, 1971). The existence of these Val^r loci has been reported previously (Glover, 1962). Therefore, these mutant alleles must represent permanently established genes and not rare anomalies, such as chance duplication of *ilvG*-like genes.

We suggest that normal alleles of at least *ilv-660* and *ilvJ662* encode proteins which serve multiple, noncatalytic functions to regulate synthesis and activity of the proteins that we collectively refer to as AHAS.

Acknowledgments. The technical assistance of Ms. Janice Johnson and Ms. Linda Bell is gratefully acknowledged.

This study was supported by Public Health Service grant GM 20880 from the National Institute of General Medical Sciences, National Science Foundation grant GB 44287, and a Brown-Hazen grant from Research Corporation to J.H.J. It was also supported by National Science Foundation grant GB 44289 to J.M.B., and by a Public Health Service Minority Biomedical Support grant RR08037. E.J.D. was supported by a MARC Faculty Fellowship GM 56020 from the National Institute of General Medical Science.

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Communicated by G. O'Donovan

Received May 2 / July 25, 1977