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New Acetohydroxy Acid Synthase Activity from Mutational Activation of a Cryptic Gene in *Escherichia coli* K-12

Cheryl L. Robinson and Julius H. Jackson
Department of Microbiology, Meharry Medical College, Nashville, TN 37208, USA

Summary. A mutation in an allele identified as *ilvJ662* causes the expression of acetohydroxy acid synthase activity that is resistant to feedback inhibition by L-valine. The *ilvJ662* allele was transduced as an unselected marker into a strain, CU1126 (*ilvB, ilvHI*), deficient in acetohydroxy acid synthase activity. The *ilvJ662* allele appears to code for a new acetohydroxy acid synthase activity (acetohydroxy acid synthase IV), with physical, kinetic, and physiological properties distinct from the other three isozymes.

The catalytic function of acetohydroxy acid synthase IV is highly stable at 37° C in the presence or absence of ethylene glycol. However, sensitivity to feedback inhibition by valine is rapidly lost at 37° C, but this property is somewhat stabilized by ethylene glycol. The rate of synthesis of acetohydroxy acid synthase IV is uniquely repressed by either leucine or isoleucine. These results suggest that the *ilvJ*$^+$ allele is cryptic for acetohydroxy acid synthase IV, an isozyme distinct from the other acetohydroxy acid synthases.

Offprint requests to: J.H. Jackson

Introduction

Acetohydroxy acid synthase activity in *Escherichia coli* K-12 is attributed to two isozymes, acetohydroxy acid synthase I and acetohydroxy acid synthase III, that are sensitive to feedback inhibition by valine (Leavitt and Umbarger 1962; Umbarger 1978). These enzymes catalyze the first reaction step common to biosynthesis of isoleucine and valine (Fig. 1). The activities of synthase I and synthase III are lost as a consequence of mutation in both *ilvB* and *ilvHI* respectively (Guardiola et al. 1977; DeFelice et al. 1974). A third gene, *ilvG*, codes for acetohydroxy acid synthase II which is not inhibited by valine, but is repressed by the combination of isoleucine, valine and leucine (Blatt et al. 1972; O'Neill and Freundlich 1972; Umbarger 1978).

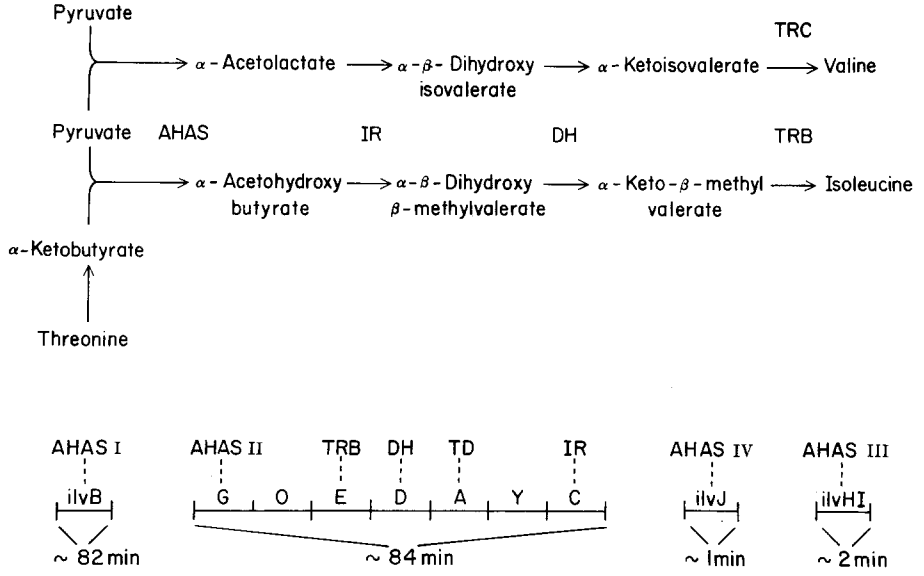

Fig. 1. The biosynthetic pathway for isoleucine and valine and the genes coding for the enzymes involved. The enzymes catalyzing the indicated steps are abbreviated as follows: TD, threonine deaminase (EC 4.2.1.16, L-threonine hydrolase (deaminating); AHAS, acetohydroxy acid synthase (EC 4.1.3.12, 2-aceto-2-hydroxy 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); TRC, transaminase C. The *ilv* genes corresponding to these enzymes and relative map positions are indicated below the scheme

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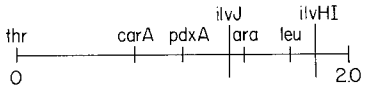

Fig. 2. Map of the chromosome region containing *ilvJ*. Region of *E. coli* linkage map to show the relative position of *ilvJ* (Jackson et al. 1981)

Since the *ilvG* gene in K-12 is cryptic, the strain does not synthesize isoleucine in the presence of millimolar concentrations of valine (Blatt et al. 1972; O'Neill and Freundlich 1972; Smith et al. 1979). Therefore, growth of the K-12 strain in a glucose minimal salts medium is inhibited by exogenous valine, and the inhibition is attributed both to the repression of acetohydroxy acid synthase I and acetohydroxy acid synthase III synthesis, and to the inhibition of their activities by valine (Blatt et al. 1972; O'Neill and Freundlich 1972; Umbarger and Freundlich 1965; DeFelice et al. 1977).

The cryptic *ilvG* gene is apparently activated by mutations in the *ilvO* region of *ilvG* and active acetohydroxy acid synthase is then made (Cohen and Jones 1975; DeFelice et al. 1977; Smith et al. 1979; Favre et al. 1976). Strains bearing the $ilvO^-$ mutation are resistant to growth inhibition by valine. We have previously reported the selection of six classes of spontaneous valine-resistant mutants that express acetohydroxy acid synthase activity with decreased sensitivity to valine (Davis et al. 1977). These mutations lie in loci distinct from *ilvB*, *ilvHI* and *ilvG*. This study provides evidence that one mutational allele (*ilvJ662*) defines an inactive or cryptic structural gene that encodes an acetohydroxy acid synthase distinct from the three known isozymes. Because of the unique map position of *ilvJ662* (Fig. 2) and the distinct properties of its gene product, we refer to the product of this allele as acetohydroxy acid synthase IV.

Materials and Methods

Strains, Media, and Cultivation Conditions. The strains of *E. coli* used in this study are listed in Table 1. Routine strain cultivation was done using the minimal salts medium of Davis and Mingioli (1950) modified by omission of citrate and adjustment of glucose concentration to 0.5%. Media were supplemented, where necessary, according to strain growth requirements. Rich media for propagation of bacteriophage Plkc anc P1CMclr100*ts* were made according to Lennox (1955) and Rosner (1972) respectively. L-broth contains 1% tryptone, 0.5% yeast extract and 0.25% sodium chloride. LB broth contains the same components except that the pH is adjusted to 7.4 with 1 N sodium hydroxide. Cultures were routinely grown at 37° C except where otherwise indicated. Where amino acid supplementation was used for growth the concentration was 0.4 mM except for L-valine which was used at a concentration of 1.2 mM. Media were supplemented with thiamine-HCl (2.5 μg/ml) and uracil (0.2 μM) where required. Liquid batch cultures were aerated by rapid shaking in baffled culture flasks.

Strain Construction and Transduction Analysis of Gene Function. The methods of Rosner (1972) were used to construct non-lysogenic strains by transduction with *E. coli* bacteriophage P1CMclr100*ts* (P1CM). The same constructions were also made using phage Plkc by the method of Lennox (1955) and the appropriate transductants were screened for P1 sensitivity to obtain non-lysogens. Strain MJ130 (*ilvJ662, leu*) was made by transduction of *ilvJ662* as an unselected marker linked to *thr* in a cross where strain MJ43 was the donor and strain MJ10, (*thr, leu*) was the recipient. Constructions are described under Results. The *ilvJ662* allele confers the phenotype of resistance to growth inhibition by L-valine (Val^r). Bacteriophage P1kc and P1CM were both used in genetic crosses to determine *ilvJ662* function.

Molecular Weight Approximation. The apparent molecular weights of acetohydroxy acid synthases III and IV were estimated by the comparative sedimentation velocity method of Martin and Ames (1961) on a sucrose density gradient. A crude cell extract was prepared in a buffer consisting of 50 mM potassium phosphate pH 8.0 with 1.0 mM $MgCl_2$, 0.5 mM dithiothreitol, 10 μg/ml flavin adenine dinucleotide (FAD), 0.1 mM thiamine diphosphate, and 30% ethylene glycol. This buffer is subsequently referred to as stabilizing buffer. Cells from a glucose minimal medium culture (1 l) were harvested by centrifugation, washed twice in 100 ml of stabilizing buffer, and resuspended in 2 ml of the same buffer. Cells were disrupted by sonic oscillation using 50 watts of power from a Heat Systems sonifier. Extracts were cleared of cellular debris by centrifugation at 27,000 × g for 15 min at 4° C. Extracts were then dialyzed twice for 2 h against 100 ml of stabilizing buffer per ml of extract. Rabbit muscle lactate dehydrogenase (Sigma Type II suspension) was mixed with extract such that 50 μl of lactate dehydrogenase and 0.2 ml of protein extract were placed on a linear 5% to 20% (w/v) gradient of sucrose (4.5 ml) in stabilizing buffer. Lactate dehydrogenase was used as a reference protein because it approximates the mol wt of the acetohydroxy acid synthetases previously studied. Gradients were centrifuged for 15 h at 45,000 rpm using a Beckman SW 50.1 rotor in a Beckman L5-50 ultracentrifuge. Fractions (0.2 ml) were collected from the top of the gradient tubes using an Isco Density Gradient Fractionator Model 183 and a Gilson Microfractionator to collect samples. Each fraction was assayed for acetohydroxy

Table 1. Strains used

Strain	Genotype	Use and derivation	Source
MJ6	*rbs-215*	Strain construction	H.E. Umbarger
MJ10	*thr, leu*	Strain construction and mapping	H.E. Umbarger
MJ43	*rbs-214, ilvJ662*	Strain construction	Davis et al. (1977)
MJ130	*leu, ilvJ662*	Strain construction and mapping	Jackson et al. (1981)
MJ133	*rbs-221, ilvB, ilvHI, ilvJ662, Δ(pro-lac)*	Strain construction	Jackson et al. (1981)
MJ164	*rbs-221, ilvB, thi, Δ(pro-lac)*	Strain construction	This study
NK5336	su^+ *uga*	λNK370 propagation	W. Nunn
CU1126	*rbs-221, ilvB, ilvHI, ara, thi, Δ(pro-lac)*	Strain construction	H.E. Umbarger

acid synthase and lactate dehydrogenase activity as subsequently described.

Enzyme Assays. Acetohydroxy acid synthase activity was measured in crude extracts (Størmer and Umbarger 1964; Szentirmai et al. 1968) and in detergent permeabilized cells (Davis et al. 1977). Routinely, the stabilizing buffer contained 30% ethylene glycol to increase enzyme stability, except where indicated. Kinetic analyses were done on crude extracts that were dialyzed at 4° C against 2 changes of the stabilizing buffer at 2 h intervals. Procedures for the kinetic analysis were previously described (Davis et al. 1977; Blatt and Jackson 1978). Sucrose density gradient fractions were assayed for acetohydroxy acid synthase activity by using a 50 µl sample in a standard reaction mixture previously described (Davis et al. 1977) for 20 min at 37° C. Fractions were diluted 1:100 and assayed for lactate dehydrogenase activity by using 50 µl of sample in a total reaction volume of 3.0 ml. A complete reaction mixture contained 0.56 m moles of Tris (hydroxymethyl) aminomethane-HCl (Tris-HCl) pH 7.3, 3.0 µ moles sodium pyruvate, and 0.3 µ moles of reduced nicotinamide adenine dinucleotide (NADH). A control for endogenous oxidation of NADH was used as the reference. The decrease in NADH absorbance at 340 nm was followed as a function of time at 30° C to measure the lactate dehydrogenase activity. Protein was measured by the biuret method (Layne 1957).

Tn10 Element Insertions. Bacteriophage lambda NK370 (*b*221, *c*I587 *c*I171::Tn10 *O*uga261) was used as a vector to introduce the Tn10 element into strains for "tet hops" according to procedures described by Kleckner et al. (1978). NK5336 was the host strain for λNK370.

An overnight culture of MJ133 was harvested by centrifugation at 4° C and cells were resuspended in 1/20 volume of fresh medium. The λ NK370 lysate was added to cells such that the multiplicity of infection approximated 0.2 phage/bacterial cell. Adsorption followed for 45 min at 37° C. Dilutions were made and 0.1 ml was spread onto LB agar plates (Rosner 1972) containing a final concentration of 20 µg tetracycline hydrochloride per ml and 2.5 mM sodium pyrophosphate. Plates were incubated for three days at 37° C and replica plated onto selective media and scored. Colonies were picked that grew on minimal medium plates containing 0.4 mM isoleucine and 1.2 mM valine, but did not grow on unsupplemented plates. Permeabilized cell assays were used to measure acetohydroxy acid synthase activity (Blatt and Jackson 1978).

Chemicals. All chemicals were reagent grade or the highest purity commercially available.

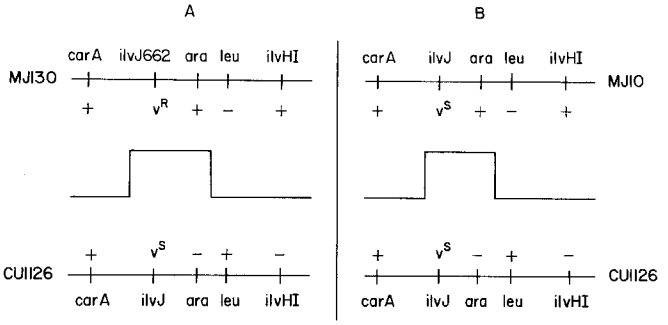

Fig. 3. Test for expression of a cryptic gene for acetohydroxy acid synthases. Cross A: If *ilvJ662* expresses acetohydroxy acid synthase activity then a primary joint selection for Ara^+ Leu^+ should yield Ilv^+ with a frequency >95%. <5% Ilv^+ would be expected to result from a double recombination, and >95% Ilv^+ should be Val^r. Cross B: If *ilvJ* is cryptic for acetohydroxy acid synthase activity then *ilvHI* will be the exclusive source of Ilv^+ when Ara^+ Leu^+ are jointly selected phenotypes. The frequency of such a double unlinked transduction event would be ≪5%

Results

Test for Acetohydroxy Acid Synthase Activity from ilvJ662

The possibility that the mutation in *ilvJ* activates a cryptic gene was examined by introduction of *ilvJ662* into a strain that lacks acetohydroxy acid synthase activity. The construction of such a strain was accomplished as shown in Fig. 3. Strain MJ130, the donor, was constructed by introduction of *ilvJ662* into strain MJ10 by cotransduction with thr^+. P1kc and P1Cm lysates were used to transduce strain CU1126 (*ilvB, ilvHI*). The selection for Ara^+ Leu^+ transductants shown in Fig. 3A permitted *ilvJ662* to transduce into CU1126 (*ilvB, ilvHI*) without the transduction of $ilvHI^+$. The $ilvHI^+$ locus would be expected to transduce into CU1126 only as a consequence of a rare double recombinational event. If acetohydroxy acid synthase activity were associated with *ilvJ662* then the resultant Ara^+ Leu^+ recombinants would be expected to be ilv^+ with a frequency approaching 100%. Experimental results revealed that all Ilv^+ transductants of the MJ130 × CU1126 cross were resistant to growth inhibition by valine (Val^r), a phenotype associated with the *ilvJ662* allele (Table 2, Cross A). The identical selection, from the Cross B (Fig. 3 and Table 2) using the isogenic donor that is $ilvJ^+$ (strain MJ10) yielded no Ilv^+ transductants. Clearly, the design of the cross was proper to exclude transduction of $ilvHI^+$, therefore the Ilv^+ phenotype is most easily explained if acetohydroxy acid synthase activity is encoded by the *ilvJ662* allele.

Table 2. Test for the expression of a cryptic gene for acetohydroxy acid synthase

Cross	Donor (genotype) × Recipient (genotype)	Selected markers	Transductants selected	Unselected markers	Unselected markers appearance	Frequency of appearance
A	MJ130 (*ilvJ662, leu*) × CU1126 (*rbs-221, ilvB, ilvHI, ara, thi, Δ(pro-lac)*)	ara^+ leu^+	393	ilv^+ ilv^+ Val^r	393 393	1.0 1.0
B	MJ10 (*thr, leu*) × CU1126 (*rbs-221, ilvB, ilvHI, ara, thi, Δ(pro-lac)*)	ara^+ leu^+	252	ilv^+ ilv^+ Val^r	0 0	0.0 0.0

Table 3. Acetohydroxy acid synthase specific activity of *ilvJ662* product

Strain (relevant genotype)	+FAD			−FAD		
	−valine[a]	+valine[b]	%I[c]	−valine[a]	+valine[b]	%I[c]
MJ133 4A (*ilvJ662, ilvB, ilvHI*)	1.073	0.217	79.8	1.041	0.198	81.0
5A (*ilvJ662, ilvB, ilvHI*)	1.003	0.203	79.8	1.003	0.193	80.7
20B (*ilvJ662, ilvB, ilvHI*)	1.078	0.199	81.5	1.049	0.216	79.4

[a] One unit of specific activity equals one nmole of acetolactate formed per minute per milliliter culture per Klett unit of culture density with a # 42 filter

[b] One unit of specific activity equals one nmole of acetolactate formed per minute per milliliter culture per Klett unit of culture density with a # 42 filter using 1.0 mM valine in the assay mixture

[c] $\%I = \left[1 - \frac{+\text{valine}}{-\text{valine}}\right] \times 100$

Table 4. Acetohydroxy acid synthase specific activity after Tn10 element insertion (*ilvJ662*::Tn10)

Strain	Phenotype	Number observed	Specific Activity[a]		% Inhibition
			−valine	+valine	
MJ133	Tet^s	–	126.10	64.82	49.0
MJ133::Tn10	Tet^r	367	–	–	–
	Tet^r, Ilv^-	18	–	–	–
	Tet^r, Ilv^-, AHAS	5	0	0	0

[a] One unit of specific activity equals 1 p mole of acetolactate formed per minute per milliliter per Klett unit of culture density in the absence of the feedback inhibitor valine (−valine) and in the presence of 1.0 mM valine (+valine)

[b] $\%I = \left[1 - \frac{+\text{valine}}{-\text{valine}}\right] \times 100$

Since the previous experiments showed that *ilvJ662* was the source of ilv^+ for CU1126, it was necessary to determine whether acetohydroxy acid synthase activity was associated with the ilv^+ attributed to *ilvJ662*.

Forty Ilv^+ transductants from duplicate crosses described in Fig. 3A were cloned and grown in liquid culture (10 ml) for assay of acetohydroxy acid synthase activity. Results from the permeabilized-cell assay of three transductant clones for acetohydroxy acid synthase activity appear in Table 3. Clearly the transduction of *ilvJ662* into a strain lacking acetohydroxy acid synthase activity ($ilvB^-$ $ilvHI^-$) makes the recipient Ilv^+ by expression of synthase activity from *ilvJ662*. The activity is apparently expressed only as a consequence of the mutation. Therefore the *ilvJ* allele must not code for an active enzyme. The newly expressed activity of *ilvJ662* is independent of FAD and retains approximately 20% activity in the presence of the feedback inhibitor valine (Table 3). Since all of the Ilv^+ Val^r transductants were also resistant to glycyl-valine, the resistance to growth inhibition by valine must be a cytoplasmic property not attributable to membrane exclusion of valine. The transductants exhibit generally the same phenotypic properties that are characteristic of the original *ilvJ662* mutant isolates. However, most transductants grew in clumps when cultured in minimal medium or L-broth. The three strains of Table 3 grew as dispersed suspensions of cells in liquid culture although they did settle more rapidly than the donor, recipient, or other normal K-12 strains.

The mean generation time (T) of 120 min for transductants was 20 min longer than T for the normal recipient strain. The *ilvJ662* transductants generally underwent a long lag phase following transfer from rich medium to minimal medium. The length of the lag phase is apparently shortened by addition of diaminopimelic acid (50 μg/ml).

The strain MJ133-4A was taken as the representative transductant from Table 3 and relabeled simply MJ133. We confirmed that the Val^r phenotype cotransduced with leu^+ from MJ133 into a *leu* $ilvB^+$ $ilvHI^+$ strain (MJ10). Several factors made distinction between the *ilvJ* and *ilvHI* gene products necessary: *ilvJ662* lies close to *ilvHI*; the synthesis of feedback resistant acetohydroxy acid synthase activity in the original mutant is repressed by leucine; *ilvHI* product does not require FAD for activity, whereas *ilvB* and *ilvG* products both require FAD. We especially sought to determine whether properties of the *ilvJ* product differed significantly from properties of the *ilvHI* product, since both genes conceivably arose from a common sequence by gene duplication. Consequently, we constructed strain MJ164 ($ilvB^-$ $ilvHI^+$ *ilvJ*) from the control cross described in Fig. 3B. This strain was thus designed to express only acetohydroxy acid synthase III for comparison with strain MJ133 ($ilvB^-$ $ilvHI^-$ *ilvJ662*), designed to express only acetohydroxy acid synthase IV.

Strain MJ133 was further analyzed for acetohydroxy acid synthase IV expression by Tn10 insertion into *ilvJ662*. If the only biosynthesis of acetohydroxy acid synthase were encoded by *ilvJ662* then Tn10 insertion into that allele would be expected to produce an Ilv^- phenotype attributable to non-functional enzyme. As shown in Table 4, Tn10 element insertion into 367 clones of strain MJ133 produced 18 Ilv^- clones of which 5 lacked acetohydroxy acid synthase activity. Tetracycline resistance (Tet^r) in the 5 clones that lacked acetohydroxy acid synthase activity was linked to *leu* by cotransduction. These results indicate that *ilvJ662* codes for a new acetohydroxy acid synthase activity, acetohydroxy acid synthase IV, that is genetically distinct from acetohydroxy acid synthetases I, II and III.

Properties of Acetohydroxy Acid Synthase IV

We compared the physical and kinetic properties of acetohydroxy acid synthases IV and III. These studies were done on dialyzed crude extracts of strains MJ133 and MJ164 respectively. Strain MJ164 served as the control strain for comparison with published results for properties of acetohydroxy acid synthases I, II and III (Grimminger and Umbarger 1979).

The influence of pH on enzyme activity was measured over pH range of 6.5 to 9.0 in potassium phosphate and

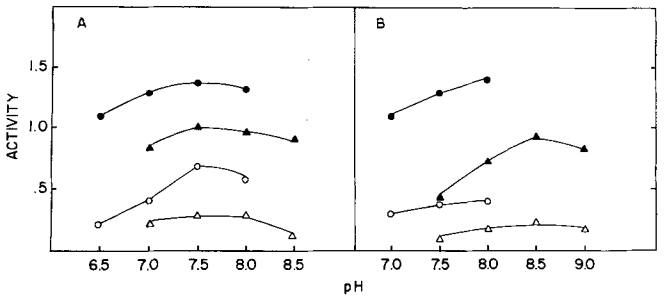

Fig. 4A, B. Effect of pH on the activity of acetohydroxy acid synthase. **A** Acetohydroxy acid synthase IV is dialyzed crude extracts of strain MJ133. Symbols: The reaction mixture contained potassium phosphate buffer used for the pH range shown. ● Uninhibited activity and ○ activity with 1.2 mM valine present in the assay mixture: next the reactions were performed with Tris (hydroxymethyl)-aminomethane buffer used in the pH range shown (▲) uninhibited activity and (△) inhibited activity with 1.2 mM valine present in the assay mixture. **B** Same as **A** except that dialyzed crude extracts of strain MJ164 were used

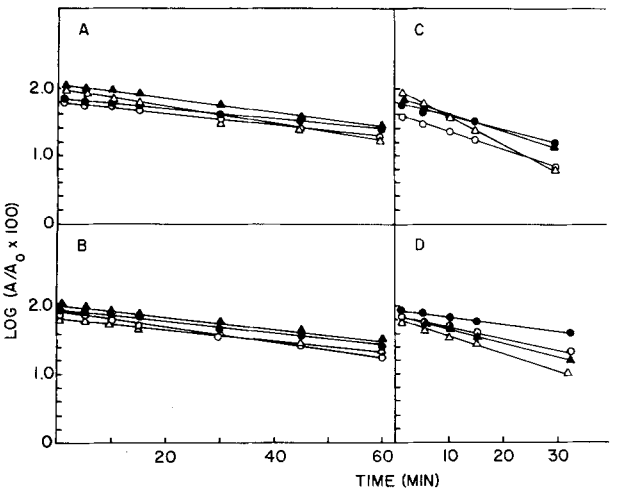

Fig. 5A–D. Heat inactivation curve. **A** Represents the normalized $\log_{10}$ decay of the acetohydroxy acid synthase enzyme in a crude extract suspended in stablizing buffer and assayed after incubation at 37° C for time shown. Symbols: ● Uninhibited activity for MJ133, (○) activity inhibited by 1.2 mM valine present in the assay mixture of MJ133: ▲ Uninhibited activity for MJ164: △ Activity inhibited by 1.2 mM valine present in the assay mixture for MJ164. **B** Incubation at 37° C with the crude extracts suspended in stabilizing buffer containing 30% ethylene glycol. Symbols are the same in **A**. **C** Incubation at 45° C with the crude extracts suspended in stabilizing buffer. Symbols same as in **A**. **D** Incubation at 45° C with the crude extract suspended in stabilizing buffer containing 30% ethylene glycol. Symbols same in **A**

in Tris-HCl buffers (Fig. 4). Maximal enzyme activity occurred at pH 7.5 for acetohydroxy acid synthase IV and at pH 8.5 for acetohydroxy acid synthase III.

The apparent molecular weights of acetohydroxy acid synthases III and IV were estimated by comparison of the sedimentation velocities of these enzymes with rabbit muscle lactate dehydrogenase through a sucrose density gradient. The apparent molecular weight of acetohydroxy acid synthase IV was 78,000 daltons, compared to 162,000 for acetohydroxy acid synthase III.

Neither of these enzymes was activated by FAD at any concentration. Dialysis to remove FAD caused no inactiviation, and no increased activity was detected when the dialyzed extract was assayed in the presence of 2 μg FAD per ml. In contrast, however, both enzymes were inactivated by dialysis in buffer without added Mg^{2+}. We did not determine a K_d for Mg^{2+} but observed that 10 mM Mg^{2+} reactivated acetohydroxy acid synthase III by a factor of 7.5 and reactivated acetohydroxy acid synthase IV by a factor of 5.

Both enzymes were completely resolved of thiamine diphosphate by dialysis and were thus inactivated. The kinetics of dissociation were hyperbolic for each enzyme and a value for K_d was determined. The K_d for synthase IV was 40 times greater than the K_d for synthase III. The K_m for pyruvate and the concentration of valine required for one maximal inhibition were essentially the same for both synthases. These general properties, as measured in crude extracts, are summarized in Table 5.

Effect of Heat on Acetohydroxy Acid Synthase Activity

Activity of acetohydroxy acid synthases III and IV was measured after exposure of the crude extracts to 37° C and 45° C for varying time periods in the presence and absence of ethylene glycol (Fig. 5). Both synthases were comparably stable to 37° C, and ethylene glycol did not significantly enhance stability (Figs. 5A and 5B). At 45° C both synthases were comparably stable in the presence or absence of ethylene glycol (Fig. 5C). Activity of acetohydroxy acid synthase III in the presence of the feedback inhibitor valine was slightly more sensitive to inactivation at 45° C than was the activity of synthase IV. Ethylene glycol stabilized synthase III (Fig. 5D).

Effect of Temperature on Sensitivity to Feedback Inhibition

Feedback sensitivity of acetohydroxy acid synthases III and IV rapidly declined in the absence of ethylene glycol at 37° C. Ethylene glycol completely stabilized the feedback

Table 5. Properties of acetohydroxy acid synthase

Acetohydroxy acid synthase	Gene	Apparent mol wt	pH optimum	$I_{0.5}$ (μM)	K_m for pyruvate (mM)	K_d For		Activation by Mg^{2+}
						Thiamine diphosphate (μM)	FAD	
III (MJ164)	*ilvHI*	162,000	8.5	30	3.0	10	−	+
IV (MJ133)	*ilvJ*	78,000	7.5	47	3.6	400	−	+

Enzyme activities of both strains were completely resolved of pyruvate and thiamine diphosphate upon dialysis

Values for K_m and K_d were determined by a computer fit of the data by a Cleland program (see methods) and by graphical analysis of the data by Eadie-Skatchard and Lineweaver-Burk plots

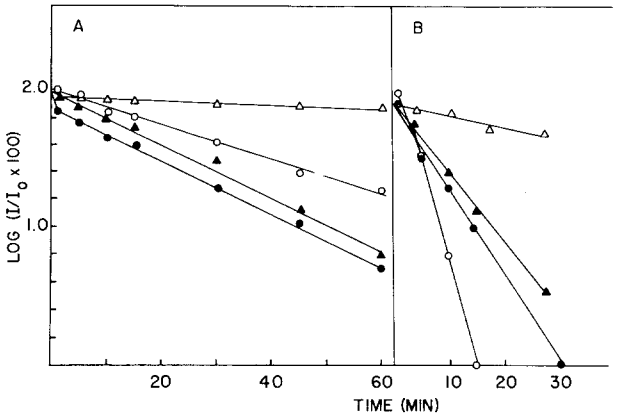

Fig. 6A, B. Valine inhibition decay cuve. **A** Represents the percent inhibition of the acetohydroxy acid synthase isozyme by 1.0 mM valine when the crude extract is heated at 37° C for the time as shown. Symbols: ● Crude extract from strain MJ133 suspended in stabilizing buffer, (○) crude extract from stain MJ133 suspended in stabilizing buffer containing 30% ethylene glycol. ▲ Crude extract from strain MJ164 suspended in stabilizing buffer. △ Crude extract from strain MJ164 suspended in stabilizing buffer containing 30% ethylene glycol. **B** Same as **A** except the crude extracts were heated at 45° C for the time shown

Table 6. Effects of growth conditions on the rate of synthesis of acetohydroxy acid synthase IV

Growth Conditions[a]	Differential rates[b]				
	−valine	Relative −valine	+1 mM valine	Relative +1 mM valine	% inhibition
Min	320	1.0	53	1.00	83.4
Min+I	175	0.55	45	0.85	74.3
Min+V	256	0.80	47	0.89	81.6
Min+L	140	0.44	31	0.59	77.8
Min+VL	141	0.44	39	0.74	72.3
Min+IV	227	0.71	60	1.13	73.2
Min+IL	1,200	3.75	34	0.64	97.1
Min+IVL	149	0.47	0	0	100

[a] Abbreviations are as follows: Minimal salts-glucose growth medium (Min); L-isoleucine (I); L-valine (V); and L-leucine (L)

[b] The differential rate of synthesis is the value of the slope of a linear plot of activity per ml culture vs culture density and has a value of P moles/min/ml/klett unit of culture density. The slope was estimated by the method of least squares from a minimum of four data points and the coefficient of correlation was >0.85 for each value listed. A minimum of two experiments was completed for each growth condition

sensitivity of synthase III but only partially stabilized synthase IV (Fig. 6A). At 45° C the same results were observed with the exception that ethylene glycol apparently increased the instability of acetohydroxy acid synthase IV feedback sensitivity (Fig. 6B).

Effect of Growth Conditions on Rates of Synthesis

Acetohydroxy acid synthase IV activity was measured in constant volume samples taken at varying culture densities from batch cultures of MJ133. A separate batch culture was used for each growth condition, and the rates in Table 6 were determined by calculating slopes of the plot of activity per ml culture against culture density. Each of the branched-chain amino acids was added to batch cultures singly or in combination with another branched-chain amino acid in order to determine the amino acid signals for repression of synthase IV. Repression was measured relative to the unrepressed rate of synthesis of acetohydroxy acid synthase IV in minimal medium. Leucine repressed the rate of synthesis by 56% and isoleucine repressed the rate by 45%. The maximum repressibility observed in minimal medium supplemented with isoleucine (0.4 mM), valine (1.2 mM) and leucine (0.4 mM) was 53%. It is interesting to note that the combination of isoleucine and leucine increased the rate of synthase IV synthesis to 3.75-times the normal rate. This pattern of amino acid mediated repression is unique to acetohydroxy acid synthase IV.

Discussion

All of the evidence we have presented indicates that *ilvJ* is a cryptic gene for acetohydroxy acid synthase IV. Activation of cryptic genes, for acetohydroxy acid synthases that can make acetohydroxy butyrate in the presence of 1.0 mM valine, may account for mechanisms of valine resistance in other mutant classes we previously reported (Davis et al. 1977; Jackson and Henderson 1975). We have preliminary evidence that three other distinct alleles may function similarly (unpublished data). The acetolactate forming activity of acetohydroxy acid synthase IV appears to be as sensitive to valine inhibition as the synthase III. Yet the cells expressing only synthase IV grow in the presence of 1.0 mM valine, whereas cells that express only synthase III do not (DeFelice et al. 1974). The extent of enzyme repression by valine must also be considered in attempts to account for acetohydroxy butyrate synthesis in the presence of valine in the growth medium. Synthase IV synthesis is negligibly decreased by valine and DeFelice and Levinthal (1977) reported that valine does not repress synthesis of acetohydroxy acid synthase III. These data suggest that response of the acetohydroxy butyrate-forming activity to valine inhibition may the critical determinant of the growth sensitivity to valine, and that a comparative measure of the rates of acetolactate and acetohydroxy butyrate formation may differ from one isozyme to another. Studies to examine this possibility are now in progress.

Synthesis of each acetohydroxy acid synthase isozyme is controlled differently than the others. Synthase IV is repressed by either leucine or isoleucine; synthase III is repressed by leucine (DeFelice and Levinthal 1977); synthase II is repressed by the combination isoleucine, valine and leucine (Smith et al. 1979) and synthase I is repressed by valine and leucine (DeFelice et al. 1978). The amino acid involvement in control of *ilvG* expression appears to be as a regulator of aminoacylated tRNA pool size for the branched-chain amino acids. The model for attenuation control of *ilvGEDA* is based upon the aminoacylated tRNA pool size (Lawther and Hatfield 1980; Nargang et al. 1980). Although a similar mechanism may be presumed to regulate *ilvHI, ilvB,* and *ilvJ*, evidence has yet to be demonstrated. It is possible that ancient structural gene duplications of acetohydroxy acid synthase genes evolved to different functions. A question yet unanswered is whether the cryptic genes make a non-functional product, a product functional only in another pathway or make no product at all. We recently suggested that *ilvJ* may be allelic with *brnS* (Jackson et al. 1981), a gene reported to encode a transport pro-

tein for the branched-chain amino acids (Guardiola et al. 1974). Preliminary evidence revealed altered rates of leucine transport as a consequence of *ilvJ662* (James J. Anderson, personal communication). Strains that depend entirely upon *ilvJ662* for acetohydroxy acid synthase activity display tendencies to clump and to settle rapidly from suspension. McEwen and Silverman (1980) reported that mutation in the *cpxA* and *cpxB* genes, for membrane proteins involved in conjugation, cause acetohydroxy acid synthase I to lose function. If cryptic synthase genes do express functional products, some may participate in or influence branched-chain amino acid transport.

This report establishes the presence of a cryptic gene for acetohydroxy acid synthase IV, and establishes the activation of a cryptic gene other than *ilvG* as a mechanism for strain K-12 to become resistant to growth inhibition by valine.

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