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Structural Genes for a Newly Recognized Acetolactate Synthase in *Escherichia coli* K-12

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Evidence is reported that shows the presence in *Escherichia coli* K-12 of a newly found acetolactate synthase. This enzyme is the product of two genes, *ilvH* and *ilvI*, both located very close to *leu*. Amber mutations have been found in both genes and therefore their products are polypeptides. Mutations in the *ilvH* gene cause the appearance of an acetolactate synthase activity which is relatively resistant to valine inhibition and can be separated by adsorption on hydroxylapatite from another activity present in the extract and more sensitive to valine inhibition than the former. A mutant altered in the *ilvI* gene was isolated among the revertants sensitive to valine inhibition of an *ilvH* mutant. Such a mutant lacks the resistant acetolactate synthase. A temperature-sensitive revertant of the *ilvI* mutant contained a temperature-sensitive acetolactate synthase. Thus *ilvI* is the structural gene for a specific acetolactate synthase. The activity of the *ilvH* gene product has been measured by adding an extract containing it to a purified *ilvI* acetolactate synthase, which, upon incubation, became more sensitive to valine inhibition. Conversely, a valine-sensitive acetolactate synthase (the product of the *ilvH* and the *ilvI* genes) became more resistant to valine inhibition upon incubation with an extract of a strain containing a missense *ilvH* gene product.

Isoleucine, leucine, and valine are synthesized in *Escherichia coli* K-12 as well as in other *Enterobacteriaceae* through a branched pathway in which several common enzymes are necessary for their biosynthesis (see Fig. 1). Evidence has been reported that the pool size of isoleucine cannot be expanded (11), probably because of the feedback inhibition of its first biosynthetic enzyme (21). It has also been reported that the pool size of valine is regulated (6), and moreover that it is regulated independently from that of isoleucine and leucine. Since the enzyme inhibited by valine, acetolactate synthase (acetolactate pyruvate-lyase [carboxylating], EC 4.1.3.18), is the second enzyme for isoleucine biosynthesis, inhibition of this activity should cause an isoleucine starvation. This is not so in *Salmonella typhimurium* and *E. coli* B because two forms of acetolactate synthase are present in these species (2, 14), one of which is resistant to valine inhibition (Val^r). Mutants of *S. typhimurium* that are missing the Val^r acetolactate synthase do not grow when valine is added to the minimal medium (15). In the K-12 strain of *E. coli* the Val^r acetolactate synthase is not expressed (M. Iaccarino, R. Favre, and M. Freundlich, manuscript in preparation) and therefore the growth of this strain in minimal medium is inhibited by valine.

Isoleucine- and valine-requiring mutants of *S. typhimurium* lacking acetolactate synthase have never been described and this had been one of the indications for the presence of more than one acetolactate synthase. On the other hand, neither have such mutants ever been found in *E. coli* K-12. Evidence for the presence of more than one acetolactate synthase in *E. coli* K-12 has also been reported (16).

In this paper we show the presence of a gene of *E. coli* K-12, *ilvI*, the product of which interacts with the product of the *ilvH* gene (6) to give an acetolactate synthase sensitive to valine inhibition (Val^s). Mutations in these two genes cause an alteration in, or an absence of, a specific acetolactate synthase. The mutated strain still contains acetolactate synthase activity and does not require isoleucine and valine for growth. We report elsewhere data indicating that the remaining acetolactate synthase activity is the product of the *ilvG* gene (9; Iaccarino, Favre, and Freundlich, manuscript in preparation) and of the *ilvB* gene (9; Guardiola, De Felice and Iaccarino, manuscript in preparation).

A preliminary account on these results has been reported (De Felice, Guardiola, and Iaccarino, Lunteren Lectures on Molecular Genetice, Atti Ass. Genet. Ital. vol. XIX, p. 144-145, 1974).

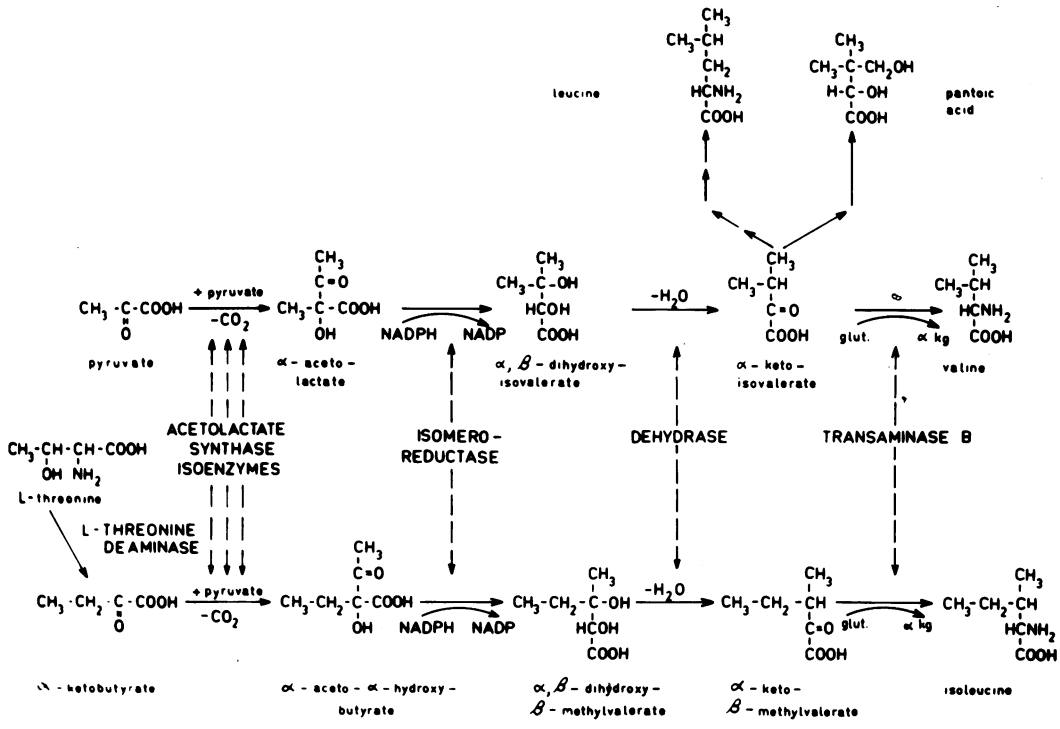

FIG. 1. *Biosynthetic pathway for isoleucine, leucine and valine (adapted from Ramakrishnan and Adelberg) (16).*

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the strains of *E. coli* K-12 used. Strain MI166f was prepared from strain MI166e by conjugation with strain JC5088 and selection of Thy$^+$ recombinants. Some of these were ultraviolet (UV) sensitive (i.e., *recA56*) and one of them was purified twice by single colony isolation and named strain MI166f.

Reagents. Reagents whose source is not mentioned here were of the highest purity commercially available. Amino acids used were all L-form. Hydroxylapatite (Bio Gel HTP) was purchased from Bio Rad Laboratories, Richmond, Calif.

Media. The minimal medium used was "minimal citrate" (22). Usual supplements when required were glucose (0.4%), tryptophan (25 μg/ml), arginine (100 μg/ml), other amino acids (50 μg/ml), nucleosides (50 μg/ml), and thiamine (10 μg/ml).

Transductions. Transductions were performed either with *P1kc* or with *P1CMclr100* (17) as described previously (10).

Isolation of Val^s back mutants. A suspension containing about 10^9 bacteria per ml was irradiated with a dose of UV light that left about 1% survivors. The suspension was diluted and grown in supplemented minimal medium. After growth, the culture was diluted to a concentration of 10^8 bacteria per ml, L-valine was added to a final concentration of 5 mg/ml and, after 1 h of incubation at 37 C with shaking, 2,000 U of penicillin per ml was added for 3 h without shaking. Cells were collected by centrifugation, grown again in minimal medium, and subjected to a new penicillin counterselection at the end of which they were plated on minimal agar. After incubation single colonies were tested for resistance to valine.

Test for amber mutations. To determine whether the phenotype of a mutant was due to an amber mutation, a given strain was lysogenized either with *φ80psu3* (prepared from strain 594) or with *φ80*, as a control. The lysogens were immune to both *φ80s* but sensitive to *φ80vir;* the *φ80* lysogens supported the growth of phage T4 but not of a phage T4 containing the mutation *amB17* in gene 23 (obtained from A. Coppo and J. F. Pulitzer). The *φ80psu3* lysogens supported the growth of both phages. The relevant phenotype of the strain to be tested as well as that of the corresponding *φ80psu3* lysogen was determined. When the phenotype of a mutation changed after lysogenization with *φ80psu3* the mutation was defined amber.

Enzyme assays and preparation of cell extracts. Acetolactate synthase was assayed by determining the rate of acetolactate formation (19). Specific activities are expressed as nanomoles of product formed per min per mg of protein.

Bacteria grown under the desired conditions were harvested by centrifugation at 4 C and then frozen as a pellet at −20 C. To 100 to 150 mg of frozen cell pellet, 1 ml of extraction solution was added and the suspension was mixed in a Vortex mixer. The suspension was treated twice for 45 s with 1-min interval for cooling with an MSE sonic oscillator and then it was centrifuged for 20 min at 15,000 rpm in a Sorvall

TABLE 1. *Bacterial strains*[a]

Strain	*ilvH*	*ilvI*	Genotype	Origin
594	+	+	*phoA, trp, φ80psu3*	From E. Gallucci (1)
AT739	+	+	*thr-10, pyrA53, thi-1,* λ⁻, HfrH	From A. L. Taylor
AT2365	+	+	*thr-4, ara-14, pdxA1Mu*⁺, *leu-8, proA2, lacY1, galK2,* λ⁺, *strA20, xyl-5, mtl, thi-1, F*⁻	From A. L. Taylor
Ca85	+	+	*thi, his, lac*^{am}, HfrH	From J. Beckwith
JC5088	+	+	*ilv-318, thi, spec*^{r}, *recA56*, Hfr PO45	From A. J. Clark
MI166c	+	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9*, F	Ilv⁻ transductant of a *leu* derivative of AB3590 (12)
MI166d	+	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9, azi*, F	Spontaneous *azi* from MI166c
MI166e	+	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9, thyA, F*⁻	Spontaneous *thyA* by trimethoprim selection (18)
MI166f	+	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9, azi, recA56*, F	From MI166e by conjugation with JC5088
MI167	*ilvH612*	+	*ara, thi-1,* λ⁻, HfrH	See Results
MI167f	*ilvH612*	+	*ara, thr-10, thi-1,* λ⁻, HfrH	Pyr⁺, Val^{r} transductant of AT739 with P1 grown on MI167
MI167g	*ilvH*⁺/*ilvH612*	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9, azi, recA56, F'leu*⁺, *ilvH612*	See Results
MI168	*ilvH612*	+	*thi-1,*λ⁻,HfrH	Ara⁻ transductant of MI167
MI183f	*ilvH611*	+	*thr-10, thi-1,* λ⁻, HfrH	Pyr⁻, Val^{r} transductant of AT739
MI183g	*ilvH*⁺/*ilvH611*	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8* or *-9, azi, recA56, F'leu*⁺, *ilvH611*	See Results
MI219a	*ilvH613*	+	*thr-4, proA2, lacY1, galK2,* λ⁻, *strA20, xyl-5, mtl-1, thi-1*, F	Leu⁺, Pdx⁺, Ara⁺, Val^{r} transductant of AT2365 with P1 grown on MI219 (6)
MI219b	*ilvH613*	+	*thr-4, pdxA1Mu*⁺, *leu-8, proA2, lacY1, galK2,* λ⁻, *strA20, xyl-5, mtl-1, thi-1*, F	Ara⁺ transductant of AT2365 with P1 grown on MI219a
MI219c	*ilvH613*	+	*thr-4, leu-8, proA2, lacY1, galK2,* λ⁻, *strA20, xyl-5, mtl-1, thi-1*, F	Pdx⁺, Leu⁻, Val^{r} transductant of MI219b with P1 grown on a wild type
MI219f	*ilvH613*	+	*thr-10, thi-1,* λ⁻, HfrH	Pyr⁺ Val^{r} transductant of AT739
MI219g	*ilvH*⁺/*ilvH613*	+	*thi-1, his-4, trpC, leu, tsx-3, lacZ13, mtl, malA1, str-8*, or *-9, azi, recA56*, F'*leu*⁺, *ilvH613*	See Results
MI244	*ilvH613*	+	*thr-10, thi-1, ilvO603, ilvG605,* λ⁻, HfrH	Pyr⁻ Val^{r} transductant of MI226 with P1 grown on MI219
MI253	*ilvH612*	*ilvI614*	*thr-10, thi-1, ara,* λ⁻, HfrH	This paper
MI253a	*ilvH612*	*ilvI615*(ts)	*thr-10, thi-1, ara,* λ⁻, HfrH	This paper
MI253c	*ilvH612*	*ilvI614*	*thi-1, ara*, HfrH	Thr⁻ transductant of MI253 with P1 grown on a wild type
MI257	*ilvH613*	*ilvI616*(ts)	*thr-4*(?), *pdxA1Mu*⁺, *leu-8, proA2 lacY1, galK2,* λ⁺, *strA20, xyl-5, mtl-1, thi-1, φ80psu3*, F	This paper
MI262	*ilvH612*	*ilvI614*	*thi-1, ilvO603, ilvG605, ilvB619, leu,* λ⁻, HfrH	(9)
MI263	+	+	*thi-1, ilvO603, ilvG605, ilvB619,* λ⁻, HfrH	(9)
MI263a	*ilvH612*	*ilvI615*(ts)	*thi-1, ilvO603, ilvG605, ilvB619,* λ⁻, HfrH	Leu⁺, Ilv⁺ ts transductant of MI262 with P, grown on MI253a
MI263d	*ilvH612*	+	*thi-1, ilvO603, ilvG605, ilvB619,* λ⁻, HfrH	Leu⁺, Ilv⁺ transductant of MI262 with P1 grown on MI167

[a] Symbols for genetic markers are those used by Taylor and Trotter (20).

SS-34 rotor. The extracts contained between 5 and 15 mg of protein per ml. Proteins were determined by the method of Groves et al. (8) with crystalline bovine plasma albumin as a standard.

When extracts were prepared for hydroxylapatite columns, 2 ml of extraction solution was added to 400 to 500 mg of cells. The suspension was treated three times for 1 min with the sonic oscillator and centrifuged, and the pellet was sonicated again with more extraction solution. After centrifugation the two supernatants were combined.

Different extraction solutions were used. For the assay of acetolactate synthase the extraction solution was 0.1 M potassium phosphate, pH 8.0. For hydroxylapatite columns the extraction solution was 42 mM potassium phosphate (pH 7.1) containing 5 mM $MgCl_2$, 20 μg of flavin adenine dinucleotide per ml, 200 μg of thiamine pyrophosphate per ml, and 20% glycerol (14).

RESULTS

Isolation of mutants altered in the ilvI gene. We have previously shown that mutations in the *ilvH* gene cause a Val^r phenotype through the appearance of a Val^r form of acetolactate synthase (6). We have looked for mutants altered in the structural gene for this acetolactate synthase among the Val^s back mutants. To eliminate the class containing the *ilvH*$^+$ revertants, we chose to start with a strain containing the *ilvH612* mutation which is not suppressed by the *su3* amber suppressor. Starting with this strain we looked for a mutation that causes a Val^s phenotype and which is also suppressible by the *su3* suppressor. Since a phenotype similar to that of the *ilvH612* mutation is caused by the *ilvH613* (amber) mutation we assume that the phenotype of the *ilvH612* mutation is caused by the absence of activity of a gene product. Therefore it is unlikely that an amber mutation causing a Val^s phenotype in a strain bearing an *ilvH612* mutation is a revertant of the *ilvH* gene.

Val^s back mutants were isolated from strain MI167f (*ilvH612*) by UV mutagenesis and penicillin counterselection (7) as described. Among eight Val^s mutants found, one became resistant upon lysogenization with ϕ80*psu3* and was retained for further study. This strain was named strain MI253, the gene altered *ilvI*, and the amber mutation causing the Val^s phenotype *ilvI614*. Preliminary conjugation experiments indicated that the *ilvI614* mutation is located on the chromosomal map near the HfrH origin. Therefore, P1 phage grown on strain MI168 (*ara*$^+$, *ilvH612*, *ilvI*$^+$, Val^r) was used to transduce strain MI253 (*ara*$^-$, *ilvH612*, *ilvI614*, Val^s) with selection of Ara$^+$ transductants. Seventy percent (67/96) of these transductants were found to be Val^r, which shows that the *ilvI* gene is 70% contransducible with *ara*. To locate more precisely the *ilvI614* mutation, the transductions described in Table 2 were performed. Since the frequency of cotransduction of *thr* with *leu* is only about 2 to 5%, when both markers are selected the frequency of cotransduction of outside located markers should decrease. The tranductions of Table 2 show that, when Leu$^+$ transductants are selected, the frequency of cotransduction of either *ilvH*$^+$ (P1 grown on strain Ca85) or of *ilvI614* (P1 grown on strain MI253c) is about the same (85% and 83%, respectively). When the selected transductants are both Thr$^+$ and Leu$^+$ the frequency of cotransduction decreases to 44% in the case of *ilvH*$^+$, and to 24% in the case of *ilvI614*.

In conclusion, the *ilvH612* and the *ilvI614* mutations are both located clockwise to *leu* on the standard map of *E. coli* K-12 (20). They are very close to each other and the *ilvI614* mutation is probably located to the right of *ilvH612*. The evidence that *ilvH* and *ilvI* are different genes is reported below (Tables 5 and 6).

The *ilvH612* (Val^r) mutation was rescued from strain MI253 (*ilvH612*, *ilvI614*, Val^s) by the following transduction analogous to those of Table 2. Strain AT2365 (*thr*, *leu-8*, *ilvH*$^+$, *ilvI*$^+$, Val^s) was transduced with P1 phage grown on strain MI253c (*thr*$^+$, *leu*$^+$, *ilvH612*, *ilvI614*, Val^s) and either Leu$^+$ transductants or Thr$^+$ and Leu$^+$ transductants were selected. Since *ilvH612* and *ilvI614* are very close, a crossover between them should be rare. However, its frequency should increase when the selected transductants are Thr$^+$ and Leu$^+$ if *ilvI614* is

TABLE 2. *Transduction of strain MI219c (thr, leu, ilvH613) with P1 grown on strain Ca85 (thr*$^+$*, leu*$^+$*, ilvH*$^+$*) or with P1 grown on strain MI253c (thr*$^+$*, leu*$^+$*, ilvH612, ilvI614)*

P1 phage	Selection	Val^s phenotype (%)
Ca85	Leu$^+$	85 (136/160)
Ca85	Leu$^+$ and Thr$^+$	44 (59/135)
MI253c	Leu$^+$	83 (133/160)
MI253c	Leu$^+$ and Thr$^+$	24 (30/150)

P1(Ca85)	+	+	+	+
MI219c	*thr*	*leu*	*ilvH*	*ilvI*
	−	−	*613*	+
P1(MI253c)	+	+	*612*	*614*
MI219c	*thr*	*leu*	*ilvH*	*ilvI*
	−	−	*613*	+

located clockwise to *ilvH612* on the *E. coli* map (20) as indicated by the results shown in Table 2. Among the selected Leu$^+$ transductants 1% (2/192) were Val^r, whereas among the selected Thr$^+$ and Leu$^-$ transductants 6% (6/96) were Val^r. We conclude that *ilvH612* is present in strain MI253 and that it is located between *leu-8* and *ilvI614*.

A temperature-sensitive (ts) mutation in the *ilvI* gene was isolated by selecting, at 30 C, Val^r mutants from strain MI253. Among 70 Val^r mutants selected, 17 did not grow on plates supplemented with valine if incubated at 37 C. These mutants grew at 30 C not only on plates supplemented with valine but also on plates supplemented with glycylvaline. Consequently they are not altered in the active transport as valine and glycylvaline enter the cell by different transport systems (5, 10). One of these strains, strain MI253a, was purified and retained for further study; the mutation causing resistance to valine was designated *ilvI615*. Transduction of strain MI253a (*ara*, *ilvH612*, *ilvI615*, Val^r) with P1 grown on strain MI166c (*ara*$^+$, *ilvH*$^+$, *ilvI*$^+$, Val^s) with selection of Ara$^+$ transductants gave 84% (81/96) Val^s transductants when analyzed at the permissive temperature. This result suggests that the ts mutation affecting the Val^r phenotype is in the *ilvI* gene. Therefore it appears that, in strains containing an *ilvH* mutation, the expression of the *ilvI* gene makes isoleucine biosynthesis resistant to valine inhibition. This has been confirmed by an experiment that will be reported elsewhere. When the genes coding for other acetolactate synthases are missing and the *ilvH* gene is mutated, a strain containing the *ilvI*$^-$ gene is Ilv$^-$, a strain containing the *ilvI614* allele is Ilv$^-$, a strain containing the ts mutation *ilvI615* is Ilvts and Val^r at the permissive temperature.

Acetolactate synthase activity in ilvI strains. When acetolactate synthase activity of extracts of different strains is assayed in the presence of 1.5 mM valine (see Table 3), the fraction of activity resistant to valine inhibition is 50% in strain MI167f (*ilvH612*, *ilvI*$^+$), 9% in strain MI253 (*ilvH612*, *ilvI614*), and 58% in strain MI253a (*ilvH612*, *ilvI615*). The experiment described in Fig. 2 shows that the acetolactate synthase form eluted at higher ionic strength, which is characteristic of the *ilvH612* mutant (6), is present in strain MI167f, absent in strain MI253, and present again in strain MI253a grown at the permissive temperature. The second activity eluted, as described (6), is 80% when assayed in the presence of 1.5 mM valine (final concentration).

The experiment of Fig. 3 shows the rate of

TABLE 3. *Acetolactate synthase activity in extracts of strains containing different ilvI alleles*

Strain	Sp act	
	No valine	1.5 mM valine
MI167f (*ilvH612*, *ilvI*$^+$)	84	42 (50%)
MI253 (*ilvH612*, *ilvI614*)	65	6 (9%)
MI253a (*ilvH612*, *ilvI615*)[a]	72	42 (58%)

[a] This strain was grown at 30 C.

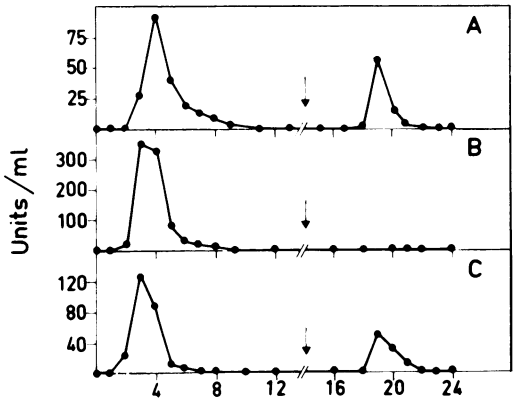

FIG. 2. *Hydroxylapatite separation of acetolactate synthase activities from strains MI167f (A) MI253 (B) and MI253a (C). The extracts prepared as described were layered on a column (0.8 by 12 cm) of hydroxylapatite previously washed with the extraction solution. The column was then eluted with 30 ml of this solution. A second elution with 30 ml of the same solution containing 100 mM potassium phosphate (pH 7.1) was started as indicated by arrows. Fractions of 2 ml were collected and 0.1-ml samples were assayed for acetolactate synthase activity. Units are expressed in nanomoles of product formed per minute.*

inactivation at 40 C of the acetolactate synthase activity of strains MI263a and MI263d. Strain MI263a contains the *ilvH612* (missense) and *ilvI615* (ts) mutations and other mutations in the genes coding for the remaining acetolactate synthases (9). Strain MI263d, otherwise isogenic to MI263a, contains the wild type *ilvI* gene. Although not first order in either case, the rate of inactivation is very different in the two extracts showing that the acetolactate synthase activity is inactivated at a faster rate in an extract of strain MI263a as compared to an extract of strain MI263d. We conclude that *ilvI* is the structural gene for an acetolactate synthase. The lack of linearity cannot be due to the existence of two acetolactate synthases with

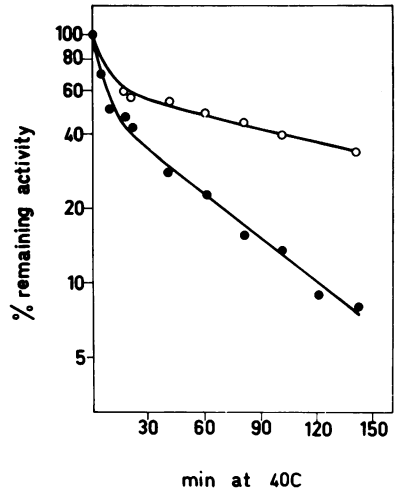

FIG. 3. *Rate of inactivation at 40 C of extracts from strains MI263a (●) and MI263d (○). Protein concentration was 18.1 mg/ml for strain MI263a and 21.5 for strain MI263d. The specific activities were 35 for strain MI263a and 22 for strain MI263d. The extracts were put at 40 C and at different times portions were withdrawn, put at 0 C, and assayed for acetolactate synthase activity.*

different sensitivities to valine in strains MI263a and MI263d, because we obtain the same results as the ones of Fig. 3 in the presence of 1.5 mM valine for both strains. Moreover, if two isoenzymes exist in strains MI263a and MI263d, they should both be ts in strain MI263a because this mutant needs isoleucine and valine for growth at the nonpermissive temperature.

Dominance. The *ilvH* and *ilvI* mutations so far described are so closely located that they might be in the same gene. To understand better the phenotype of the different mutations and the interaction between the *ilvH* product and other gene products possibly involved, we studied the expression of the Val^r phenotype in stable merodiploids containing an *ilvH*$^+$ allele together with either the *ilvH611*, or the *ilvH612*, or the *ilvH613* allele. The stable merodiploids were constructed by mating a *recA*, F$^-$ strain, MI166f, with different HfrH strains containing either the *ilvH611*, *ilvH612*, or *ilvH613* mutation. As shown by Low (13), in an Hfr population there are strains containing F′ episomes of different length and therefore appropriate episomes can be selected in a mating with an F$^-$ strain deficient in recombination.

The three different merodiploids were prepared by mating strain MI166f (F$^-$, *recA*, *leu*) with an HfrH, *thr-10*, *leu*$^+$, which also contained either the *ilvH611* or the *ilvH612* or the *ilvH613* mutation. The mating was interrupted after 6 min and Thr$^+$, Leu$^+$ recombinants were selected on plates containing the other supplements necessary for growth of MI166f (thiamine, histidine, and tryptophan). Randomly chosen Leu$^+$ strains were purified and it was checked that they still needed histidine and tryptophan for growth. That these Leu$^+$ strains were merozygotes was proven by their capacity to donate their episome to strain AT2365 (F$^-$, *leu*$^-$, *pro*$^-$) on plates containing either no leucine or no leucine and proline. In this way the episomes were shown to contain the *leu* genes, but not the *proA* gene (which is located at 7 min). It will be shown below that they contained *ilvH*. Since the HfrH origin is at about 88 min (13) the episomes carry a region of chromosomal genes 3 to 9 min long. The stable merozygotes containing the *ilvH611*, *ilvH612*, or *ilvH613* mutation were called MI183g, MI167g, or MI219g, respectively. The resistance to valine of these strains, of the F$^-$ strain (MI166f), and of the corresponding haploid strains carrying only the mutated *ilvH* allele (MI183f, MI167f, and MI219f) is reported in Table 4. Strain MI166f has a duplication time (93 min) longer than that of its isogenic *rec*$^+$ strain; it has been reported, in fact (3), that *recA* strains grow more slowly than their isogenic *rec*$^+$ parental strains. Table 4 shows that strain MI166f does not grow in a medium containing valine and that the Hfr's containing the *ilvH* mutations do grow, as expected. Strain MI183g (F′, *ilvH*$^+$/*ilvH611*) grows on valine and leucine (129 min duplication time) almost as well as on minimal medium (93 min duplication time). Also strain MI167g (F′, *ilvH*$^+$/*ilvH612*) grows on valine (104 min duplication time) as well as on minimal medium (102 min duplication time). On the other hand, strain MI219g (F′, *ilvH*$^+$/*ilvH613*) does not grow on valine. Therefore the resist-

TABLE 4. *Doubling times of different strains in properly supplemented (see Table 1) minimal medium containing either no valine or valine (25 μg/ml) and leucine (25 μg/ml) as indicated*

Strain	Additions		
	None	Valine	Valine and leucine
MI1664f (F$^-$, *ilvH*$^+$)	93	>600	>600
MI183f (Hfr, *ilvH611*)	51	nd[a]	52
MI183g (F′, *ilvH*$^+$/*ilvH611*)	93	nd	129
MI167f (Hfr; *ilvH612*)	60	56	nd
MI167g (F′, *ilvH*$^+$/*ilvH612*)	102	104	nd
MI219f (Hfr, *ilvH613*)	54	51	nd
M1219g (F′, *ilvH*$^+$/*ilvH613*)	95	>600	nd

[a] Not determined.

ance to valine due to the *ilvH611* and *ilvH612* mutations appears to be dominant, whereas that due to the *ilvH163* mutation appears to be recessive. The presence of the *ilvH611* and *ilvH612* mutations in the corresponding episomes is revealed by their dominance, but it was further checked by donating the episomes to an F^- in a mating with strain AT2365. Leu^+ merozygotes were selected and all of them (48/48) were shown to be Val^r. The presence of the *ilvH613* mutation in strain MI219g was checked in the following way: a mating with strain AT2365 followed by selection of Leu^+ merozygotes showed that all of the clones (48/48) were Val^s, thus confirming the recessive character of the *ilvH613* mutation. One of these merozygotes (which, being now in a rec^+ host should recombine with the chromosome) was purified and grown in rich medium, and then 0.1-ml samples of the suspension were plated on minimal plates supplemented with all the supplements needed by strain AT2365 to grow plus valine to determine the yield of Val^r colonies. The Val^r colonies obtained by plating strain AT2365 were 8 in 10^8 cells, whereas those obtained from the merozygotes were 5×10^3 in 5×10^8 cells. The high frequency of Val^r colonies in the population of the Val^s merozygotes suggests that they are recombinants. When one of the merozygotes was used in a mating with strain MI166c (F^-, *leu*) and Leu^+ colonies were selected, 56% of them (27/48) were Val^r. The Val^r progeny was due to incorporation of the *ilvH613* allele into recombinants haploid for the *ilvH* locus. The Val^s progeny could be either leu^+ $ilvH^+$ recombinants or *ilvH613* $leu^+/ilvH^+$ *leu* merodiploids.

In vitro complementation of the ilvH and ilvI gene products. The experiments reported above suggest that *ilvH* and *ilvI* code for two different polypeptides. The *ilvI* gene product is assumed to be an acetolactate synthase that can interact with the *ilvH* gene product. This would make the "*ilvI*" acetolactate synthase more sensitive to valine inhibition. If this hypothesis is correct it follows that: (i) an *ilvI* gene product extracted from a strain carrying an amber mutation in the *ilvH* gene should be resistant to valine inhibition and it should become more sensitive if incubated with a source of wild-type *ilvH* gene product; (ii) an *ilvI* gene product extracted from a strain carrying a wild-type *ilvH* gene should be sensitive to valine inhibition and it should become more resistant if incubated with an excess of a missense, *ilvH612* gene product.

In the experiment of Table 5 it is shown that the *ilvI* acetolactate synthase becomes more sensitive to valine inhibition after incubation with an extract containing the *ilvH* gene product. The *ilvI* acetolactate synthase was purified from a strain containing the *ilvH613* amber mutation by adsorption and elution from hydroxylapatite (Fig. 2) and in the accompanying paper (6). Since an $ilvH^+$, $ilvI^-$ strain is difficult to prepare the source of *ilvH* gene product was a crude extract of a strain prepared in the following way: Val^s back mutants were isolated from strain MI219b (*leu*, *ilvH613* amber, $ilvI^+$) as described. Four of these back mutants, after purification, were treated with Pl phage grown on strain MI168 (leu^-, *ilvH612*, $ilvI^+$, Val^r) and Leu^+ transductants were selected. Thirty of these transductants were replicated and 80 to 90% of them were found to be Val^r in each case. Therefore these four strains may be *ilvI* mutants (see above isolation of *ilvI614*). From one of them, Val^r mutants were isolated and one of these was found to be Val^r at 30 C but not at 37 C. The Val^r ts mutation was again 90% cotransducible with *leu* and therefore it appears to be an *ilvI* mutation. This strain (*ilvH613 amber*, *ilvI616* ts) was treated with ϕ80*psu3* and a Val^s strain lysogenic for this phage was isolated. This strain was named MI257. It is phenotypically $ilvH^-$ (*ilvH613* suppressed by the *su3* suppressor) and it contains a ts mutation in the *ilvI* gene. Even when grown at 42 C this strain (or the strain containing no ϕ80*psu3*) still contains acetolactate synthase activity which is the product of other gene(s). This activity (Table 5) is sensitive to valine inhibi-

TABLE 5. *In vitro complementation of the ilvH and ilvI gene products from strain MI244 (ilvH amber, $ilvI^+$)[a] and strain MI257 ($ilvH^+$, ilvIts)[b]*

Acetolactate synthase source	Activity (U/ml)	
	Without valine	With valine
MI244	51.4	43.5 (84%)
MI257	56.9	10.3 (18%)
MI244 + MI257[c]	52.7	26.9 (51%) calculated[d]
		15.3 (30%) experimental

[a] *ilvH613*, $ilvI^+$. The enzyme used was the Val^r form purified by adsorption on hydroxylapatite. Specific activity was 40.8.

[b] *ilvH613*, *ilvI616(ts)*, ϕ80*psu3*. This strain was grown at 42 C, and a crude extract was used. Specific activity was 19.1.

[c] Equal volumes of the two enzyme fractions were mixed and incubated for 30 min at 0 C before assay.

[d] 43.5 + 10.3/2.

tion. Therefore, when an extract of strain MI257 is mixed with the *ilvI* acetolactate synthase, the results are affected by the activity still present in strain MI257. When the mixture is preincubated at 0 C for 30 min, if the sensitivity to valine did not change, the result expected can be calculated (Table 5) to be 51%. The experimental result obtained was different (30% instead of 51%) from that calculated, thus suggesting that the *ilvH* gene product present in the extract of strain MI257 has increased the sensitivity to valine of the *ilvI* acetolactate synthase purified from strain MI244. The effect obtained is about two-thirds of the maximum which could have been obtained. Other experiments, not reported, with a lower concentration of the extract of strain MI257 gave a much lower difference between the calculated and experimental values, thus suggesting that the concentration of the *ilvH* gene product is limiting.

The experiment of Table 6 was based on a prediction arising from the dominance experiment (see above): the dominance of the *ilvH612* mutation suggests that the *ilvH612* gene product is able to interact with the *ilvI* acetolactate synthase, but it is not able to make it sensitive to valine inhibition. Therefore, the addition of an excess of *ilvH612* gene product to the *ilvH*$^+$-*ilvI*$^+$ gene products should make acetolactate synthase activity more resistant to valine inhibition. This experiment has been done with strains containing mutations in the genes (*ilvG* and *ilvB*) required for other acetolactate synthase activities (9). One of these strains, MI263, contains the wild type *ilvH* and *ilvI* genes and it shows acetolactate synthase activity sensitive to valine inhibition. An extract of strain MI262, containing the *ilvH612* allele and mutations in the genes coding for other acetolactate synthase, shows only 3% acetolactate synthase activity as compared to that of strain MI263. When these two extracts are mixed as indicated in Table 6 and the mixture is preincubated at 0 C for 30 min, the activity becomes a completely Val^r (108%). This result indicates that the *ilvH612* gene product present in the extract of strain MI262 has replaced the *ilvH*$^+$ gene product of strain MI263 in a complex of the *ilvH*$^+$ and *ilvI*$^+$ gene products, and as a consequence, the acetolactate synthase activity has become resistant to valine inhibition. This result also shows that the *ilvI614* amber mutation has not affected the presence of the *ilvH612* gene product in strain MI262 and confirms the interpretation of the dominance of the *ilvH612* allele. Strain MI262 contains an amber mutation in the *ilvI* gene and therefore the simplest interpretation of this experiment is that *ilvH* and *ilvI* code for two different polypeptides.

TABLE 6. *Interaction of the ilvI acetolactate synthase with an altered ilvH gene product*

Acetolactate synthase source	Activity (U/ml)	
	Without valine	With valine
MI263[a]	108	13 (12%)
MI262[b]	37.2	44 (118%)
MI263 + MI262[c]	54	58.3 (108%)

[a] *ilvH*$^-$, *ilvI*$^-$, *ilvG605*, *ilvB619*. The extract used showed a specific activity of 33.9.

[b] *ilvH612* (missense), *ilvI614* (amber), *ilvG605*, *ilvB619*. The extract used showed a specific activity of 0.96.

[c] One milliliter of extract of strain MI263 (3.18 mg of protein) and 1 ml of extract of strain MI262 (38.8 mg of protein) were mixed and incubated for 30 min at 0 C before assay.

One peculiarity of the experiment of Table 6 is that the activity of the mixture of the two extracts is lower than that expected (54 U/ml instead of 72.6). A possible interpretation of this result is that the turnover number of the *ilvH*$^+$-*ilvI*$^+$ acetolactate synthase is higher than that of the *ilvH612-ilvI*$^+$ acetolactate synthase.

DISCUSSION

We describe in this paper a newly found gene of *E. coli* K-12, namely *ilvI*, which specifies for an as yet undetected acetolactate synthase. This acetolactate synthase can be revealed by separation on hydroxylapatite columns only in strains carrying *ilvH* mutations. This suggests that the *ilvH* gene product might either be a repressor or a subunit of the *ilvI* acetolactate synthase which changes its affinity to hydroxylapatite. Two major pieces of evidence strongly suggest that the *ilvH* gene product is a subunit of this acetolactate synthase. Firstly, a strain missing acetolactate synthase activity (9) requires isoleucine and valine for growth, but becomes capable of growing without addition of isoleucine and valine upon introduction of an *ilvI* wild-type allele. The presence of an *ilvH*$^-$ or an *ilvH*$^-$ allele has no relevance for growth, but changes the valine sensitivity of the acetolactate synthase expressed by the *ilvI* gene (as shown above, the presence of an *ilvH* mutation increases the resistance to valine). Secondly, in vitro complementation experiments (such as those of Tables 5 and 6) show that at least one function of the *ilvH* gene product is to interact with the *ilvI* acetolactate synthase to increase its sensitivity to valine inhibition. In conclusion, the *ilvH* gene product is a subunit of the *ilvI* acetolactate synthase; on the other hand, it

is still possible that the *ilvH* gene product also deserves a repressor-like function.

Since the *ilvH* and the *ilvI* mutations are located very close to each other, they could affect the same gene. The evidence reported is in agreement with the idea that *ilvH* and *ilvI* are two different genes. In fact, amber mutations have been found in both loci, showing a completely different phenotype, *ilvH* amber being Val^r and *ilvI* amber Val^s; furthermore, biochemical experiments (see Tables 5 and 6) strongly suggest that we are dealing with two different gene products. Thus, the hypothesis that the *ilvH* and *ilvI* mutations are in the same gene and that the results of Tables 5 and 6 are due to an in vitro interaction of the same gene product altered at different sites seems unlikely. Genetic complementation analysis of these mutants cannot be done because, as shown by dominance experiments, two of the *ilvH* mutants are dominant. Complementation between the *ilvH613* (recessive) mutant and the *ilvI614* mutant cannot be done because the phenotype of the latter is Val^s as that of an $ilvH^- ilvI^-$ strain.

The *ilvH* and the *ilvI* genes are located close to *leu* and conceivably they might be part of the *leu* operon. If the polarity of transcription is supposed to be in the order *ilvI*, *ilvH*, *leu*, some of the amber mutations in *ilvH* could show a polar effect on the leucine operon and be Val^r, Leu . Although we have not found such a mutant, we do not think we can exclude this possibility.

The experiments reported confirm that the valine sensitivity of *E. coli* K-12 is due to the sensitivity of the acetolactate synthase activity to valine (21). Our results show that the *ilvH* gene product is responsible for the sensitivity to valine of the *ilvI* acetolactate synthase, which is per se relatively valine resistant.

Our results show that the *ilvI* gene is the structural gene for an acetolactate synthase; mutations in this gene do not cause the disappearance of the total amount of acetolactate synthase activity nor an Ilv^- phenotype and therefore *E. coli* K-12, as well as *S. typhimurium* and *E. coli* B, possesses more than one acetolactate synthase. This explains why mutants of *E. coli* K-12 that lack acetolactate synthase activity completely had never been isolated before. Because of the availability of the mutants in the *ilvI* gene described in this paper and in the *ilvG* gene (9; Iaccarino, Favre and Freundlich, manuscript in preparation) we have recently found mutants which are Ilv^- as a consequence of mutations in all the genes coding for acetolactate synthase (9). These mutants show that a multiplicity of acetolactate synthase exists in *E. coli* K-12. An analogous multiplicity exists for the 3-deoxy-D-arabinoheptulosonic acid-7-phosphate synthases in the case of the biosynthesis of the aromatic amino acids (23) and for the aspartokinases in the case of the biosynthesis of threonine, lysine, and methionine (4). This analogy suggests that the acetolactate synthase isoenzymes might have a regulatory role in the biosynthesis of the branched chain amino acids.

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