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Expression of a Valine-resistant Acetolactate Synthase Activity Mediated by the *ilv O* and *ilv G* Genes of *Escherichia coli* K-12

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Summary. A strain carrying the *ilv0603* mutation has been isolated in *E. coli* K-12 and its characteristics were found to be very similar to those previously reported by Ramakrishnan and Adelberg (1965a) for other *ilv0* mutants.

The strain carrying the *ilv0603* mutation is resistant to valine inhibition (Val^r) and we show that this resistance depends on the expression of a newly recognized gene, *ilvG*, which is located at min 75, between *ilvE* and *ilvD* on the *E. coli* K-12 map. The *ilvG* gene causes the expression of a Val^r acetolactate synthase, which is detectable only when the *ilv0603* mutation is also present in *cis* on the same chromosome. Under these conditions the Val^r acetolactate synthase activity is eluted, on a hydroxylapatite column, at an ionic strength slightly lower than that required for elution of the remaining acetolactate synthase activity (sensitive to valine inhibition). The Val^r peak is missing in a strain carrying an *ilvG* (amber) mutation.

Introduction

In contrast to other *Enterobacteriaceae* the growth of *Escherichia coli* K-12 is inhibited by valine. A detailed explanation for this phenomenon is lacking, in spite of many investigations concerning the mechanism of inhibition (for a review see Umbarger, 1969). Glover (1962) has isolated several classes of mutations conferring valine resistance (Val^r) to *E. coli* K-12. He showed that only some of these exhibited the extreme resistance (10 mg/ml valine) which is characteristic of other *Enterobacteriaceae* and we have shown most valine resistant mutations result from permeability defects (Guardiola and Iaccarino, 1971; De Felice, Guardiola, Lamberti and Iaccarino, 1973; Guardiola, De Felice, Klopotowski and Iaccarino, 1974a and b) or from mutations in the *ilvH* gene, a regulatory subunit of one of the acetolactate synthase isoenzymes (De Felice, Guardiola, Malorni, Klopotowski and Iaccarino, 1974; De Felice, Guardiola, Esposito and Iaccarino, 1974; Guardiola, De Felice and Iaccarino, 1974). In *E. coli* B and *Salmonella typhimurium,* two Val^r *Enterobacteriaceae,* two forms of acetolactate synthase have been demonstrated, one of which is both resistant to inhibition by valine and absent in *E. coli* K-12 (O'Neill and Freundlich, 1972 and 1973; Blatt, Pledger and Umbarger, 1972).

Ramakrishnan and Adelberg (1964, 1965a and 1965b) isolated and characterized *E. coli* K-12 mutant strains (*ilvO*) resistant to 1.17 mg/ml valine which have regulatory defects in the *cis* control region of the *ilvADE* operon. It occurred to us that these regulatory mutations might lead to the expression of a new acetolactate synthase activity, resistant to valine inhibition. Accordingly, we describe in this paper the isolation of a valine sensitive derivative of an *ilvO* strain and we show that the phenotype is due to a mutation in a newly recognized gene in *E. coli* K-12, *ilvG*, located between *ilvD* and *ilvE*. The *ilvG* gene permits the expression of a valine resistant acetolactate synthase activity which causes a Val^r phenotype only in a strain carrying an *ilvO* mutation. Previous evidence (Guardiola, De Felice and Iaccarino, 1974) together with the results of this investigation allow us to conclude that *E. coli* K-12 contains at least three acetolactate synthase isoenzymes. We now report that the Val^r phenotype due to an *ilvO* mutation requires the expression of the *ilvG* gene.

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Materials and Methods

Reagents and Media. Sodium-α, β-dihydroxy-β-methyl valerate was purchased from Reef Laboratories, Santa Paula, California. α-Keto-isovalerate was purchased from Sigma Chemical Co. All other reagents were of the highest purity commercially available. The minimal medium used was that described by Vogel and Bonner (1956); usual supplements, when required, were 0.4% glucose, 25 µg of L-tryptophan per ml; 100 µg of L-arginine per ml, 50 µg of other L-amino acids per ml, 50 µg of nucleosides per ml and 10 µg of thiamine per ml.

Transductions. Transductions were performed either with *P1kc* or with *P1CMclr100* as described previously (Guardiola, De Felice, Klopotowski and Iaccarino, 1974b).

Enzyme Assays. Extracts were prepared and assayed for either threonine deaminase or acetohydroxy acid isomero reductase or acetolactate synthase as described previously (De Felice, Guardiola, Malorni, Klopotowski and Iaccarino, 1974). Specific activities are expressed as nmoles of product formed per min per mg of protein.

Mutagenesis. Ultraviolet and nitrosoguanidine mutagenesis were performed as described previously (De Felice, Guardiola, Malorni, Klopotowski and Iaccarino, 1974).

Search for Amber Mutations. A mutation was classified as an amber mutation if it was suppressed by the *su3* suppressor as previously described (Guardiola, De Felice, Klopotowski and Iaccarino, 1974).

Growth Stimulation or Inhibition Tests. A 0.1 ml sample of a cell suspension grown overnight in minimal citrate medium was diluted in 3 ml of 0.7% minimal citrate agar and layered on supplemented minimal citrate plates and 10 µliters of the solution to be tested for stimulation or inhibition were pipetted onto a small circle (6 mm in diameter) of Whatman 3 MM paper applied on the agar surface. The result was observed after overnight incubation at 37 C.

Bacterial Strains. Table 1 lists the strains used. Symbols for genetic markers are those used by Taylor and Trotter (1972).

MI162a is an isoleucine- and valine-requiring derivative of strain AW206 obtained by nitrosoguanidine mutagenesis and penicillin counterselection. Tramsaminase B, the *ilvE* gene product, and reductoisomerase, the *ilvC* gene product, were assayed in extracts of this strain (experiments not reported) and found to be normal. α-Ketoisovalerate substitutes for valine stimulation on properly supplemented minimal plates, indicating that MI162a contains an active *ilvE* gene product. Sodium-α,β-dihydroxy-β-methylvalerate (which substitutes for isoleucine in the growth stimulation of an *ilvA* strain, MI2) does not stimulate the growth of MI162a and on this basis we conclude that this strain contains a mutation in the *ilvD* gene.

MI226b is an isoleucine- and valine-requiring derivative of strain MI226a obtained by ultraviolet mutagenesis and penicillin counterselection. Growth stimulation experiments similar to the previous ones and enzyme assays indicate that this strain contains an *ilvD* mutation (see Table 3 for assay of the *ilvD* gene product).

Strain MI252 was obtained from strain AW206 by nitrosoguanidine mutagenesis. Auxanographic tests and enzyme assays indicate that it is mutated in *ilvC*.

Strain AB3590 contains a multisite mutation, *ilvDAC115*, isolated by Kiritani, Matsuno and Ikeda (1965) by ultraviolet mutagenesis and then transferred, by P1 transduction, into strain AB3590 by Marsh and Duggan (1972). This mutation was partially characterized as a deletion as it was unable to form recombinants with any *ilvD* or *ilvC* mutant tested. We found no complementation between the *ilvDAC115* mutation and the *ilvA602* mutation carried by a φ80i*λdilv, ilvA602* transducing phage (Favre, Iaccarino and Levinthal, 1974). The *ilvE* gene product, transaminase B, was found to be present in this strain while dehydrase, threonine deaminase or reductoisomerase were absent (Marsh and Duggan, 1972; unpublished data, this laboratory). We found a normal acetolactate synthase specific activity (31 units/mg) and normal derepression

Table 1. Bacterial strains[a]

Strain	Genotype and origin
594	*phoA, trp,* (φ80psu3); From E. Gallucci (Andoh and Ozeki, 1968)
AB1206	F'14, *thi-1, his, proB, str, tsx, gal, lac;* the episome carries the *ilv* genes; from the Coli Gentic Stock Center
AB3516	*thi-1, ilvE316, his-4, trpC, mtl-1, malA1, lacZ13, ton-1?, tsx-3,* λ^R, λ^- ?, *str-8* or *-9;* from the Coli Genetic Stock Center
AB3590	F⁻, *thi-1, mtl-1, malA1, str-8* or *-9, his-4, trpC, lacZ13, tsx-3, ilvDAC115;* derived from strain W2252 of Kiritani, Matsuno and Ikeda, 1965; from the Coli Genetic Stock Center
AT739	*thr-19, pyrA53, thi-1,* (λ^-), HfrH; from A. Taylor
AW206	*thr-10, pyrA53, thi-1, ilv0603,* (λ^-), HfrH; a spontaneous valine-resistant derivative of AT739
MI2	F⁻, *argH, trpA36, ilvA601* (Iaccarino and Berg, 1971)
MI139a	F'14, *thi-1, his, proB, str, tsx, gal, lac, ilv0603;* this paper
MI159	*thr-10, pyrA53, thi-1, ilvC608,* (λ^-), HfrH; from AT739 by nitrosoguanidine mutagenesis
MI162a	*thr-10, pyrA53, thi-1, ilv0603, ilvD606,* (λ^-), HfrH; from AW206 by nitrosoguanidine mutagenesis
MI166a	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, tsx-3, ilvDAC115, leu.* A Leu derivative of AB3590 obtained by UV mutagenesis
MI166b	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, trpC, lacZ13, tsx-3;* Ilv^+ transductant of AB3590 with P1 grown on an Ilv^+ strain
MI199	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, tsx-3, ilvDAC115,* φ80iλCI857t68, φ80iλCI857t68*dilv* (Avitabile *et al.*, 1972)
MI199e	F', *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, ilvDAC115, tsx-3,* φ80iλCI857t68, φ80iλCI857t68*dilv, ilv0603;* described in the text
MI226	*thr-10, pyrA53, thi-1, ilv0603, ilvG605,* (λ^-), HfrH; from AW206 by ultraviolet mutagenesis, as described in the text
MI226a	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, tsx-3, ilv0603, ilvG605;* Ilv^+, Val^s transductant of AB3590 transduced with P1 grown on MI226
MI226b	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, tsx-3, ilv0603, ilvG605, ilvD607;* from MI226a by ultraviolet mutagenesis
MI226e	F⁻, *thi-1, mtl-1, malA1, str-8* or *9, his-4, trpC, lacZ13, tsx-3, ilv0603, ilvG605, ilvD607,* φ80iλCI857t68, φ80iλCI857t68*dilv*
MI252	*thr-10, pyrA53, thi-1, ilv0603, ilvC609,* (λ^-), HfrH; from AW206 by nitrosoguanidine mutagenesis
PS339	*ilvE12, rbs-211, cya;* from Mark Levintahl
PS607	*ilvA454, rbs;* from Mark Levinthal
*E. coli*W	Collection of M.F.

[a] Symbols for genetic markers are those used by Taylor and Trotter (1972).

of this activity when strain MI1166a, a *leu* derivative of strain AB3590, is starved for valine (410 units/mg) or for leucine (650 units/mg). We also found (Guardiola, De Felice and Iaccarino, 1974) that a strain carrying *ilvDAC115* contains both *ilvG* and *ilvB* (besides *ilvHI*).

Strain MI1139a is an *ilv0603* derivative of strain AB1206 which was derived by transduction of the latter with P1 grown on strain AW206 and selection of transductants resistant to valine and glycylvaline. One transductant was purified and the presence of F'14 in it was checked with an appropriate mating.

Strain MI199 contains the phage $\phi 80\lambda$CI857St68*dilv* (Avitabile, Carlomagno-Cerillo, Favre and Blasi, 1972). This phage contains all the *ilv* genes clustered at 75 min (Favre, Iaccarino and Levinthal, 1974; Lo Schiavo, Favre, Kasai, Cascino, Guardiola, Caro and Iaccarino (1975); in this paper we show that it contains also *ilvG*. This phage will be called $\phi 80\lambda dilv$.

Strain MI199e contains an *ilv0603* derivative of $\phi 80\lambda dilv$. It was prepared by treating strain MI199 with P1 grown on strain AW206 and selecting transductants resistant to valine and glycylvaline. One of these transductants was purified, a lysate of $\phi 80\lambda dilv$ was prepared and used to transduce strain AB3590 with selection of Ilv^+ transductants. One of these transductants was purified and named strain MI199e. It was Val^r and derepressed in threonine deaminase. A lysate prepared on this strain gave all Val^r transductants when tested with strain MI2.

Hydroxylapatite Chromatography. The procedure used was the same as described previously (O'Neill and Freundlich, 1972) except that enzyme units are expressed as nmoles of acetolactate formed per min per mg of protein.

Results

A Mutation in the ilv0 Gene

The *ilv0* gene is defined by *cis* dominant constitutive regulatory mutations for the *ilvADE* operon (Fig. 1).

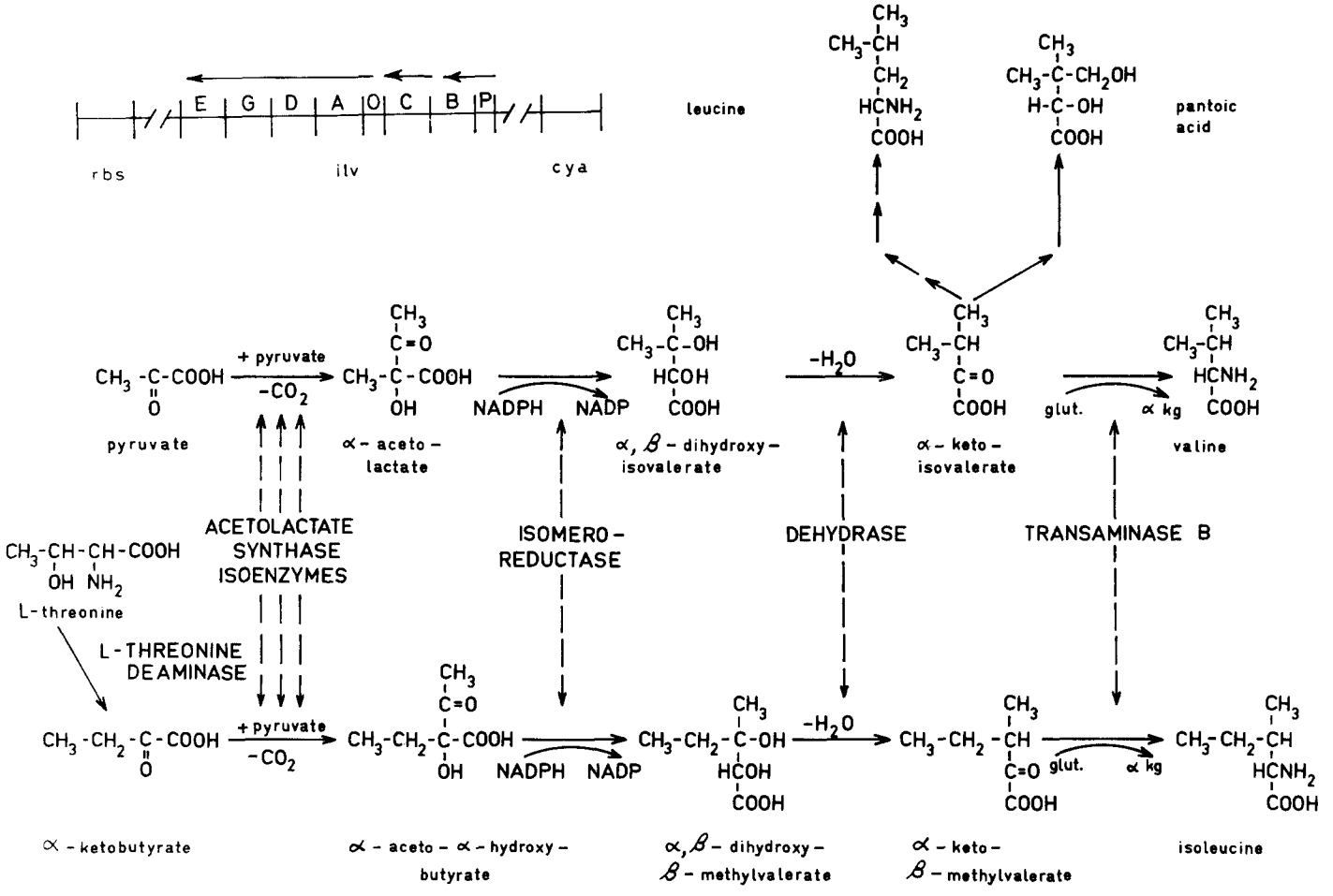


Fig. 1. The biosynthetic pathway for isoleucine, leucine and valine in *Escherichia coli* K-12 (adapted from Ramakrishnan and Adelberg, 1964 and from De Felice, Guardiola, Esposito and Iaccarino, 1974). The *ilv* gene cluster located at min 75 on the *E. coli* linkage map has been adapted from Taylor and Trotter (1972). We added the *ilvG* gene, for which evidence is given in this paper and omitted *ilvY* since it has now been found that it is an *ilvA* mutant (Wasmuth and Umbarger, 1974). The *ilv0* and *ilvP* genes are operator genes for *ilvADGE* and *ilvB* expression, respectively (Ramakrishnan and Adelberg, 1964, 1965a and b; this paper). Arrows indicate direction of transcription (Lo Schiavo *et al.*, 1975). Recent data (Kline *et al.*, 1974) suggest that *ilvE* might not be part of the *ilvADGE* operon, in contrast to what originally shown by Ramakrishnan and Adelberg, 1964, 1965a and b. The *ilvB* gene codes for a Val^s acetolactate synthase isoenzyme (Ramakrishnan and Adelberg, 1965a and b; Guardiola, De Felice and Iaccarino, 1974 and manuscript in preparation). The *ilvA* gene codes for threonine deaminase (Ramakrishnan and Adelberg, 1964, 1965a and b; Calhoun, Kuska and Hatfield, 1975). Mutants altered in the *ilvC*, *ilvD* or *ilvE* genes are devoid of isomeroreductase, dehydrase or transaminase B activity, respectively (Ramakrishnan and Adelberg, 1964, 1965a and b; references quoted in these papers)

Such mutations were isolated and characterized by Ramakrishnan and Adelberg (1964, 1965a and 1965b) as mapping between *ilvA* and *ilvC*. Recently, Kline, Brown, Coleman and Umbarger (1974) reported that this gene is located on the *cya* side of *ilvC*, namely between *ilvC* and *metE* (see Fig. 1) and questioned the regulatory effects caused by mutations in this gene. We have isolated and used (Iaccarino and Berg, 1971; Cervone and Iaccarino, 1972; De Felice, Guardiola, Malorni, Klopotowski and Iaccarino 1974; and below) a strain whose phenotype is very similar to the phenotype originally described by Ramakrishnan and Adelberg for *ilv0* mutants. Our results demonstrate that the *ilv0* mutation we isolated has essentially identical characteristics as those described by Ramakrishnan and Adelberg.

We isolated spontaneous mutations in strain AT739 (see Table 1) resistant to inhibition by valine as described by Glover (1962). The level of valine resistance was tested after two purifications by single colony isolation in the absence of valine. Among 91 strains, 57% were resistant also to glycylvaline and therefore were not valine transport defective mutants (De Felice, Guardiola, Lamberti and Iaccarino, 1973). One of these strains, AW206, had elevated levels of threonine deaminase, and was presumed to contain the *ilv0603* mutation.

Using P1 transduction, we introduced the *ilv0603* mutation both into the F'14 episome (strain MI139a) and into the $\phi 80\lambda dilv$ transducing phage. We crossed strain MI2 (*ilv0*$^+$, *ilvA601*) with strain MI139a (F'14 *ilv0603*, *ilvA*$^+$) or infected it with the $\phi 80\lambda dilv$, *ilv0603*, *ilvA*$^+$ phage and selected Ile$^+$ merodiploids. The Ile$^+$ merodiploids were Val^r and appropriate tests confirmed their diploid character. Thus we conclude that the Val^r phenotype is dominant. The data in Table 2 show that the *ilv0603* mutation causes a 3–4fold derepression of threonine deaminase, dihydroxyacid dehydrase and transaminase B, the products of the *ilvADE* operon (Fig. 1). Acetolactate synthase and isomeroreductase activity are lower in the *ilv0603* mutant as compared to the *ilv0*$^+$ strain. We transferred the *ilv0603* mutation into several different strains and demonstrated that the derepression of threonine deaminase shown in Table 2 always accompanies the valine-resistant phenotype.

The most critical test for an operator mutation is a *cis-trans* test. This is especially true for mutations having a valine resistant phenotype since several different classes of *trans* acting Val^r constitutive mutations have been isolated which are co-transducible with the *ilv* cluster (Mark Levinthal, personal communication). Therefore a *cis-trans* test was performed for the constitutive phenotype. Strain MI226b (*ilv0603*, *ilvD607*) was crossed with $\phi 80\lambda dilv^+$ and Ilv$^+$ merodiploids were selected. The data in Table 3 demonstrate that the *ilv0603* mutation is required in *cis* for the constitutive expression of the *ilvD* gene product (dihydroxy acid dehydrase).

We located the *ilv0603* mutation between *ilvA601* and *ilvC609*. In preliminary crosses between P1 grown on a strain carrying the *ilv0603* mutation and Ilv$^-$ recipients we found the Val^r phenotype to be closely linked to the *ilvC608* mutation of strain MI159 (85/85 Ilv$^+$ transductants were Val^r) and to the *ilvE316* mutation of strain AB3516 (158/159 Ilv$^+$ transductants were Val^r). We performed a three point test

Table 2. Specific activity of the isoleucine-valine biosynthetic enzymes in strains AT739 (*ilv0*$^+$) and AW206 (*ilv0603*)[a]

Strain and growth conditions	Acetolactate synthase	Isomeroreductase	Threonine deaminase	Dihydroxy acid dehydrase	Transaminase B
AT739					
Minimal[b]	79	15	54	69	30
Same, plus corepressors[c]	12	1.6	30	29	20
Rich[d]	0.7	<0.25	12	6	7
AW206					
Minimal[b]	51	6	207	183	71
Same, plus corepressors[c]	20	0.5	155	52	53
Rich[d]	1	1	36	35	12

[a] Preparation of cell extracts and enzyme assays are described under Materials and Methods. The specific activity is expressed as nanomoles of product formed per minute per milligram of protein.
[b] Minimal medium supplemented with glucose, threonine, arginine, uridine and thiamine.
[c] Further additions were isoleucine (50 µg/ml). leucine (50 µg/ml) and valine (100 µg/ml).
[d] L-medium (Lennox, 1955).

Table 3. Cis-trans test of the *ilv0603* mutation

Strains[a]	Relevant genotype	Enzyme activity	
		Threonine deaminase	Dihydroxy acid dehydrase
AT739	*ilv0*$^+$, *ilvD*$^+$	54	69
MI226a	*ilv0603*, *ilvD*$^+$	191	126
MI226b	*ilv0603*, *ilvD607*	266	<1
MI226e	*ilv0603*, *ilvD607* /$\phi 80\lambda dilv$, *ilv0*$^+$, *ilvD*$^+$	317	35

[a] These strains were grown in minimal medium containing the supplements needed for growth.

cross between P1 phage grown on MI252 (*ilvC609, ilv0603*) and the recipient MI2b (*ilvA601, cya*) selecting Ilv^+ transductants and scoring *cya* and Val^r. We found that 100% (126/126) of the Ilv^+ transductants were cya^- indicating the expected order *A601, C609, cya*. We further found 19% Val^s transductants. Since this value is too high for quadruple crossover (given the close linkage to *ilvC* and *ilvE*, as shown above, and 90% linkage between *ilvC* and *cya*, data not shown), these data establish the order *ilvA601, ilv0603, ilvC609, cya*. A reciprocal cross was performed by reversing donor and recipient and equivalent results were obtained.

In summary, the phenotype of the *ilv0603* mutation is identical to that described by Ramakrishnan and Adelberg (1964, 1965a, 1965b) for strains carrying *ilv0* mutations.

Isolation of ilvG Mutants

Following mutagenesis with ultraviolet light, strain AW206 (Val^r, *ilv0603*) was treated with penicillin (Gorini and Kaufman, 1960) in the presence of L-valine (5mg/ml) and regrown. After another round of penicillin counter-selection with valine, we plated the survivors on minimal plates and tested single colonies for valine resistance. Among 236 tested, 5 Val^s colonies appeared, and we assayed these for threonine deaminase. Table 4 shows that isolates 3, 4 and 5 are still derepressed while isolates 1 and 2 are not. We challenged isolates 3, and 4 and 5 with $\phi 80$*psu3* to check if the mutation leading to valine sensitivity was due to an amber mutation, and the Val^s phenotype of no. 5 was suppressed. Therefore, at least three different classes of mutations can suppress the Val^r phenotype caused by the *ilv0603* mutation and suppression can be caused by the loss of a functional protein. We designate the gene involved in the production of this protein *ilvG*, the mutation causing a Val^s phenotype *ilvG605*, and the strain MI226.

The degree of sensitivity to valine of strain MI226 was tested on plates using paper discs impregnated with different concentrations of valine. The diameter of the inhibition zone was equal for strain MI226 and for strain AT739 ($ilv0^+$, $ilvG^+$) over a 100-fold concentration range. Therefore the resistance to valine caused by the *ilv0603* mutation requires the presence of the protein coded by *ilvG*.

Location of the ilvG Gene

We performed a back cross to ascertain if *ilvG* is linked to the *ilv0603* lesion (see Table 5). We grew P1 phage on strain MI226 (*ilv0603, ilvG605*), crossed it with strain MI159 (*ilvC608, ilv0*$^+$, *ilvG*$^+$) and selected Ilv^+ transductants. The Val^r phenotype was scored and 4% of the transductants were found to be Val^r, while in the control cross (P1 phage grown on strain AW206: *ilv0603, ilvG*$^+$) all (85/85) were Val^r. Thus, we conclude that 1) the *ilvG605* mutation is separable from both *ilv0603* and *ilvC608*, 2) it is closely linked to both, 3) it is not located between them. If this interpretation is correct, the genotype of the Val^r transductants should be *ilv0603, ilvG*$^+$. To verify this, we grew P1 on one of the Val^r transductants and repeated the cross with strain MI159 (*ilvC608*). As expected, all (96/96) the Ilv^+ transductants were Val^r.

We proceeded with the precise location of *ilvG* by crossing strain MI226 with strain AB3590 (*ilvDAC115*) (Table 6, cross (a)) and found that 2.8% of the transductants were Val^r. Therefore, the *ilvDAC* deletion is *ilvG*$^+$. The crosses (b) and (c) in Table 6 establish the order *ilvG, ilvD, ilvC*, while cross (d) places *ilvG* between *rbs* and *ilvA*. Finally, cross (e) establishes the order *rbs, ilvE, ilvG*. In summary, the genetic data in Table 6 establish the order *rbs211, ilvE12, ilvG605, ilvD607, ilvA454, ilv0603*. (see Fig. 1 for a summary of the *ilv* gene loci).

Table 4. Specific activity of threonine deaminase in the wild type strain AT739, the *ilv0* mutant AW206, and in different Val^s strains isolated from AW206

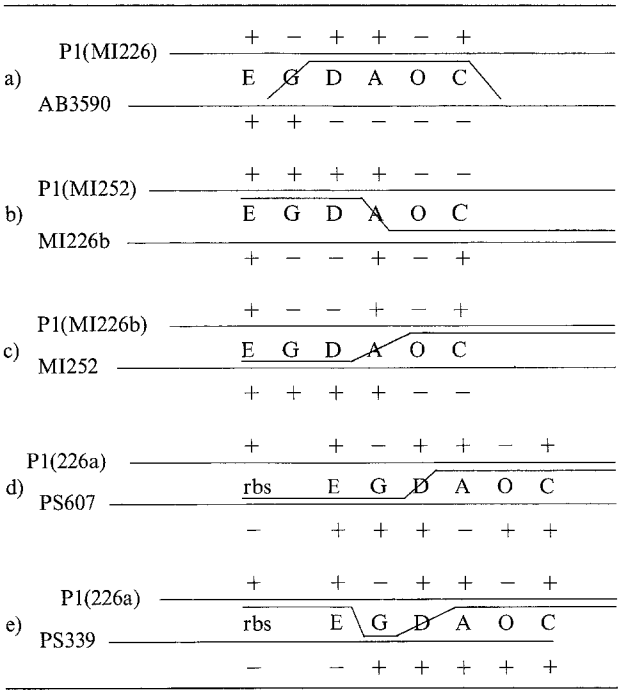
Strains	Threonine deaminase activity (nmoles/min/mg)
AT739	35
AW206	150
Val^s-1	7
Val^s-2	24
Val^s-3	260
Val^s-4	200
Val^s-5 (MI226)	150

Table 5. Transduction of MI159 (*ilvC608*) with P1 phages grown on different strains and selection of Ilv^+ transductants

Donor	Frequency of unselected Val^r transductants					
AW206 (*ilv0603*)	>99% (85/85)					
MI226 (*ilv0603, ilvG605*)	4% (3/80)					
Val^r transductant	>99% (96/96)					
P1 (AW206)	+	+	+	+	−	+
P1 (MI226)	+	−	+	+	−	+
P1 (Val^r transductant)	+	+	+	+	−	+
MI159	+	+	+	+	+	−
	ilvE	*G*	*D*	*A*	*O*	*C*

Table 6. Crosses between different Ilv auxotrophs

	Donor (P1 phage)	Recipient	Selected phenotype	Scored phenotype
a) MI226	(*ilv0603, ilvG605*)	AB3590 (*ilvDAC115*)	Ilv^+	2.8% (6/213) Val^r [a]
b) MI252	(*ilv0603, ilvG$^+$, ilvC609*)	MI226b (*ilv0603, ilvG605, ilvD607*)	Ilv^+	85% (82/96) Val^r
c) MI226b	(*ilv0603, ilvG605, ilvD607*)	MI252 (*ilv0603, ilvG$^+$, ilvC609*)	Ilv^+	70% (88/125) Val^r
d) MI226a	(*ilv0603, ilvG605*)	PS607 (*ilvA454, rbs*)	Ilv^+ and Val^r	29% (28/96) Rbs^+
e) MI226a	(*ilv0603, ilvG605*)	PS339 (*ilvE12, rbs-211*)	Ilv^+ and Val^r	70% (67/96) Rbs^+



[a] One, randomly chosen, of these Val^r transductants was found to have a derepressed level of threonine deaminase.

also repeated an analogous experiment with an *ilv0603* derivative of $\phi 80\lambda dilv$ and found the same results. An assay of threonine deaminase activity confirmed the *ilv0603* genotype of these strains.

Biochemical Characterization

The results in the previous sections show that the *ilvG* gene codes for a protein and that this protein permits the expression of the Val^r phenotype when the *ilv0603* mutation is present in *cis*. Because of the previously reported relationship between a Val^r phenotype and the presence of a Val^r acetolactate synthase activity (O'Neill and Freundlich, 1972, 1973; Blatt, Pledger and Umbarger, 1972) we analyzed this activity. In Fig. 2 we show the acetolactate synthase activity of extracts of different strains, measured as a function of the valine concentration present in the assay mixture. The fraction resistant to valine inhibition is 15% in strain AT739 (*ilv0$^+$, ilvG$^+$*), 41% in strain AW206 (*ilv0603, ilvG$^+$*) and 15% in strain MI226 (*ilv0603, ilvC605*). A similar increase in resistance to valine inhibition of acetolactate synthase activity is found when the *ilv0603* mutation is on the

The Expression of ilvG in Diploids

We constructed a diploid formed by the F'14 *ilvG$^+$, ilvD$^+$, ilv0$^+$* episome of AB1206 and a chromosome containing *ilv0603, ilvG605, ilvD607*. We found the Ilv^+ merozygotes to be Val^s, but they became Val^r when the *ilvG605* mutation was suppressed with $\phi 80psu3$ (4/4 tested). Therefore, the *ilvG* gene can only express the Val^r phenotype when combined with the *ilv0603* mutation in *cis*. Furthermore, when the *ilvG* gene is expressed (in the diploids containing $\phi 80psu3$) its Val^r phenotype is dominant. As a control, we transferred an F'14 episome containing *ilv0603* (from strain MI139a) into strain MI2 (*ilv0$^+$, ilvC$^+$, ilvA601*) and into strain MI226b (*ilv0603, ilvG605, ilvD607*). Such diploids were Val^r, demonstrating that the F'14 contains an *ilvG$^+$* allele. We

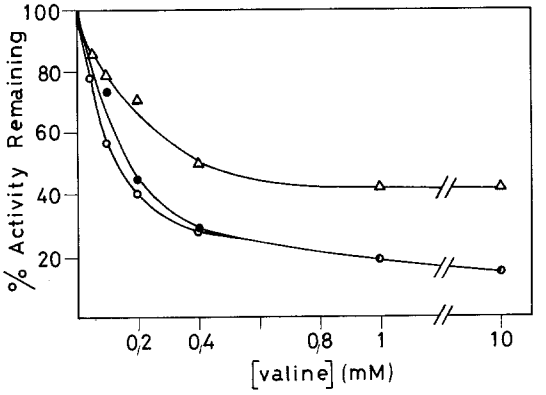


Fig. 2. Inhibition of acetolactate synthase activity by valine in crude extracts of AT739 (○), AW206 (△), and MI226 (●) grown in minimal medium. Specific activities in the absence of valine were 70 (nmol/min per mg) for strain AT739, 44 for strain AW226 and 58 for strain MI226

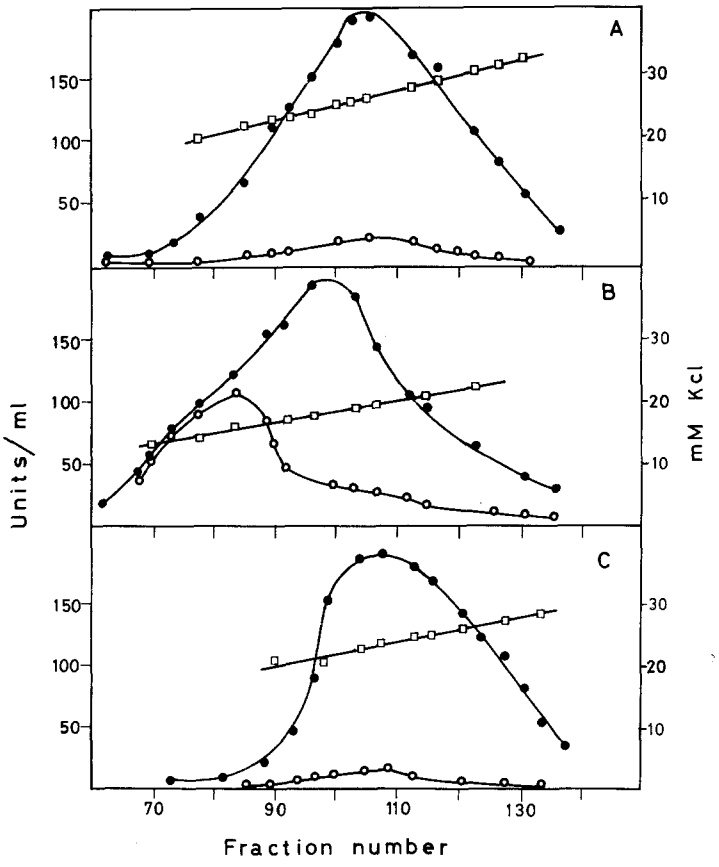


Fig. 3. Hydroxylapatite separation of acetolactate synthase activities from strain AT739 (A), AW206 (B) and MI226 (C). Each fraction was assayed with (○) or without (●) valine (10 mM, final concentration). The molarity of the eluting buffer is also shown (□)

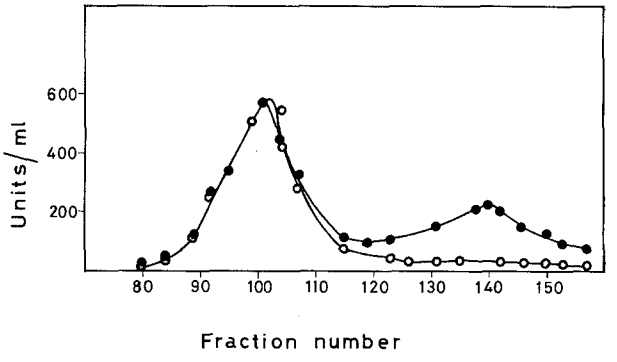


Fig. 4. Hydroxylapatite separation of acetolactate synthase activities from strain *E. coli* W. Each fraction was assayed with (○) or without (●) valine (10 mM, final concentration)

$\phi 80\lambda dilv$ phage. This result suggests that in strain AW206 there is a heterogeneity of acetolactate synthase molecules, namely Val^r molecules and Val^s molecules. The column chromatographic separations reported in Fig. 3 show that this is indeed the case. The profile of acetolactate synthase activity in panel A (*ilv0*$^+$, ilvG$^+$) is identical to that already reported (O'Neill and Freundlich, 1972) for wild type *E. coli* K-12. The *ilv0603*, *ilvG*$^+$ strain (panel B) shows a shoulder of Val^r acetolactate synthase activity which disappears in the *ilv0605*, *ilvG605* strain (panel C). The peak obtained in the latter case is asymmetrical and less wide. The concentration of the eluting buffer in the peak fraction is 25 mM in panel A, 18 mM in panel B and 23 mM in panel C. As a comparison we report, in Fig. 4, a separation of acetolactate synthase activities from *E. coli* W. In this case the Val^r activity is more abundant and better separated than in the case of the *E. coli* K-12 strain AW206 (*ilv0603*, *ilvG*$^+$).

Discussion

The *ilv0603* mutation described in this paper causes a specific derepression of the *ilvA*, *ilvD* and *ilvE* gene products (see below for a discussion of the expression of the *ilvG* gene). In contrast to what was found for the *ilvO* mutants described by Ramakrishnan and Adelberg (1965a) the *ilv0603* mutation causes a slight decrease of total acetolactate synthase activity and a 2.5 fold decrease of isomero reductase activity. Since we did not compare the properties of strain AW206 with those of the strains described by Ramakrishnan and Adelberg, these differences might be due either to the *ilv0* mutation or to other properties of the strain. The decrease in specific activity of total acetolactate synthase activity is probably due to a repression of the *ilvB* and/or *ilvHI* gene products which might be only partially balanced by an increase in *ilvG* expression. However, the result of the *cis-trans* test (Table 3) is in agreement with the results published by Ramakrishnan and Adelberg. In addition, the *ilv0603* mutation is located between *ilvA* and *ilvC*. This location is the same as Ramakrishnan and Adelberg (1965b) reported for their *ilv0* mutants.

The addition of excess corepressors to a culture of strain AW206 causes only very little repression, as expected for an 0^c mutant. However, further derepression can be obtained if an *ileS* mutation is introduced into this strain (Cervone and Iaccarino, 1972), while repression is observed either when strain AW206 (*ilv0603*) or when strain MI164 (*ilv0603*, *ileS1*) are grown in rich medium. The data are consistent with the hypothesis that in rich medium repression is achieved by a different mechanism, although we cannot exclude the possibility that strains carrying the *ilv0603* mutation still have some repressor binding activity. The level of *in vivo* mRNA which specifically hybridizes to the *l* strand of $\phi 80\lambda dilv$ is increased in a strain carrying the *ilv0603* mutation as compared

to the level of a strain carrying an *ilv0*$^+$ allele (Lo Schiavo, Favre, Kasai, Cascino, Guardiola, Caro and Iaccarino, 1975).

Threonine deaminase, the *ilvA* gene product, has been partially purified and characterized from a strain carrying an *ilv0603* mutation (Cervone and Iaccarino, 1972). The experiments on sensitivity to the allosteric effectors, isoleucine and valine, gave results similar to those reported previously in the literature for threonine deaminase purified from other strains. Thus, the *ilv0603* mutation appears to affect the regulation but not the structure of threonine deaminase.

In conclusion, the phenotype and the genetic localization of the *ilv0603* mutation are essentially identical to those described by Ramakrishnan and Adelberg for *ilv0* mutants. However, Kline *et al.* (1974) have reported that the site of numerous *ilv0* mutants lies outside of the region between *ilvA* and *ilvC* and to the *cya* side of *ilvC*. We are unable to explain the reason for the difference found in this study as compared to our data. Recent work by M. Levinthal (personal communication) has shown that three of the *ilv0* mutants used by Kline *et al.* are, in fact, located between *ilvA454* and *ilvC462*. Since the location of *ilv0* by Kline *et al.* (1974) was based on investigations of a strain carrying the *ilvDAC115* deletion we suggest that the data obtained with this strain should be reinterpreted: either the *ilv0* gene is still present in the strain containing *ilvDAC115* (in this case this mutation would not be a deletion but a multisite mutation) or the *ilvE* gene is under the control of another operator, old or new.

The data in Table 6 clearly show that *ilvG* is located between *ilvE* and *ilvD*. The requirement of an *ilv0603* mutation in *cis* for expression of the Val^r phenotype and for detection of a Val^r acetolactate synthase activity, indicates that *ilvG* is part of the previously called *ilvADE* operon. Interestingly, an *ilvG* gene coding for a Val^r acetolactate synthase, has been reported in *Salmonella* and has been located between *ilvE* and *ilvD* (O'Neill and Freundlich, 1973). Therefore, it appears that the valine sensitivity of *E. coli* K-12, as opposed to the resistance shown by *Salmonella,* is not due to the absence of the gene coding for a Val^r acetolactate synthase but to the abnormal expression of the *ilvG* gene. It would be interesting to locate the Val^s phenotype of *E. coli* K-12 with an interspecies cross with *Salmonella*.

The experiments reported in this paper show that the Val^r phenotype caused by the *ilv0603* mutation requires the presence of a Val^r acetolactate synthase activity and that this needs an active *ilvG* gene to be expressed. When the *ilvG* gene is mutated the *ilv0603* strain is as sensitive to valine as the wild type. A strain mutated in the *ilvB* and *ilvI* genes and containing an *ilvG*$^+$ allele in an *ilv0603* background is prototrophic (unpublished experiments). Therefore, the product of the *ilvG* gene appears to be a Val^r acetolactate synthase able to catalize the biosynthesis of both the isoleucine and the valine precursors. We will report elsewhere evidence showing that the product of the *ilvG* gene is indeed an acetolactate synthase (Guardiola, De Felice and Iaccarino, manuscript in preparation). Ramakrishnan and Adelberg (1964) showed that an *ilvO* strain excretes isoleucine but not valine. Because of the results presented here we believe that isoleucine excretion is not a consequence of the derepression but due to the expression of a Val^r acetolactate synthase. While the Val^r acetolactate synthase expressed in an *ilv0603* mutant is required for the expression of the Val^r phenotype, we do not exclude that other gene products are also required. Indeed, Ramakrishnan and Adelberg (1965a) have shown that for the expression of the Val^r phenotype a derepressed level of threonine deaminase is required. These experiments have been confirmed with a merodiploid containing the $\phi 80\lambda dilv^+$ phage (J. Guardiola, unpublished experiments).

The Val^r acetolactate synthase activity reported in this paper is clearly different from the Val^r activity detectable in strains carrying an *ilvH* mutation: the activity is eluted at a concentration of potassium phosphate lower than 18 mM while the Val^r activity of an *ilvH* mutant is eluted at a concentration of potassium phosphate higher than 42 mM (De Felice, Guardiola, Malorni, Klopotowski and Iaccarino, 1974). Moreover, while an *ilv0603, ilvG*$^+$ strain grows on plates containing 3.5 mg/ml of valine an *ilvH* strain does not, suggesting that the Val^r acetolactate synthase activity expressed in an *ilv0603* strain is more resistant to valine inhibition than that expressed in an *ilvH* strain.

The experiments on hydroxylapatite elution of acetolactate synthase activity presented in Fig. 3 show that the Val^r phenotype of strain AW206 (*ilv0603, ilvG*$^+$: panel B) is associated with the presence of a Val^r peak of acetolactate synthase activity. This peak is not present either in strain AT739 (*ilv0*$^+$, *ilvG*$^+$: panel A) or in strain MI226 (*ilv0603; ilvG605:* panel C). Therefore, the acetolactate synthase activity associated with the Val^r peak needs, to be expressed, both an active *ilvG* gene and an *ilv0603* mutation. The profiles of the peaks are complex. Although more evidence is needed, it is possible that the peak obtained in panel A is composed of at least two Val^s forms of acetolactate synthase activity. One of these activities may become Val^r in strain AW206 (panel B) and would be missing in strain MI226 (panel C). Experiments are in progress to clarify this point.

The Val^r phenotype of *E. coli* W also appears

to be correlated with the presence of a Val^r acetolactate synthase activity. The experiment of Fig. 4 shows that in this case the Val^r activity is more abundant than the Val^s one if compared with the two activities of the *E. coli* K-12 strain, AW206 (Fig. 3, panel B). Also, the two activities are well separated in the case of *E. coli* W, very poorly in the case of *E. coli* K-12.

The Val^r acetolactate synthase activity is probably present in any *ilv0* strain of *E. coli* K-12. In fact, strain PS647 (*ilv0264*), carrying the only other *ilv0* mutation for which a *cis-trans* test has been performed, shows a Val^r acetolactate synthase activity in an experiment similar to that shown in Fig. 2 (Mark Levinthal, personal communication).

Presently we are not able to understand why we cannot detect a Val^r acetolactate synthase in an *ilv0*$^+$ strain. On the other hand, we have shown an *ilv0603, ilvG605* strain is as sensitive to valine inhibition as an ilv0$^+$, *ilvG*$^+$ strain. Therefore the Val^r acetolactate synthase is either absent or Val^s in an *ilv0*$^+$, *ilvG*$^+$ strain. When the growth of *E. coli* K-12 is inhibited by valine the products of the *ilvA, ilvD* and *ilvE* genes are derepressed (Dwyer and Umbarger, 1968). If the Val^r acetolactate synthase activity was expressed, *E. coli* K-12 would not be Val^s. Therefore, the expression of the Val^r acetolactate synthase activity appears to be correlated with the derepression caused by the *ilv0603* mutation but not with the derepression caused by isoleucine starvation. We have failed to consistently detect a Val^r activity in crude extracts of *E. coli* K-12 starved for either isoleucine, or leucine, or valine (M. Freundlich, unpublished experiments).

In conclusion, now that the *ilvG* gene has been defined and located and it its gene procuct characterized (Guardiola, De Felice and Iaccorino, manuscript in preparation), further experiments should be done to understand the mechanism and regulation of expression of *ilvG*. Definitive answers to these questions should help to clarify the mechanism of valine sensitivity of *E. coli* K-12.

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