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Location of the Multivalent Control Site for the *ilvEDA* Operon of *Escherichia coli*

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A strain of *Escherichia coli* K-12 containing a deletion extending from early in the *ilvE* gene toward the *ilvG* gene was shown to exhibit a higher expression of the downstream genes, *ilvD* and *ilvA*, than did an *ilv*$^+$ strain. The elevated expression was under apparently normal *ilv*-specific control, however. The deletion was transferred to the *ilv* region of λ*h80dilv* and shown by restriction endonuclease and heteroduplex analysis to extend through the deoxyribonucleic acid (DNA) shown, in the preceding paper (C. S. Subrahmanyam, G. M. McCorkle, and H. E. Umbarger, J. Bacteriol **142**:547-555, 1980), to contain the *ilvO* determinant. The deletion was also transferred to an *ilv-lac* fusion strain and shown to cause an increase in β-galactosidase formation while allowing retention of *ilv*-specific control. Transducing phages excised from these fusion strains with and without the *ilvO* determinant were compared. The phage carrying the *ilvO*$^+$ determinant contained *ilv* DNA extending only into but not through the *ilvG* gene. It did not exhibit an *ilv*-specific control of β-galactosidase formation. The phage carrying the deletion of *ilvO* but containing *ilv* DNA extending beyond the *ilvG* gene exhibited *ilv*-specific control of β-galactosidase formation. It was concluded that the multivalently controlled *ilv*-specific promoter affecting *ilv* operon expression lies upstream from *ilvG* and that the *ilvO* region in the wild-type K-12 strain is a region of polarity preventing *ilvG* expression and reducing *ilvEDA* expression.

Three of the enzymes in the isoleucine and valine biosynthetic pathway are specified by three genes (*ilvE*, *D*, and *A*) in a single operon (Fig. 1). In the presence of excess isoleucine, valine, and leucine there is a nearly coordinate repression of this operon (5). When one of the branched-chain amino acids is limiting, there is a derepression of the operon, but, particularly with limiting isoleucine, the derepression is not a coordinate one. Rather, *ilvA* exhibits more derepression than *ilvD*, and *ilvD* exhibits more than *ilvE* (5). Derepression of the operon appears to be related to the extent to which the branched-chain aminoacyl tRNA synthetases can charge their cognate tRNA's (reviewed in reference 28).

For several amino acid biosynthetic operons that exhibit derepression upon restricted charging of the cognate tRNA, an important feature of the derepression mechanism appears to be the rate of translation of a leader transcript (1, 7, 9, 12, 17, 31). When translation of the leader is retarded at a critical site (a region rich in codons for the amino acid in question), transcription will usually be terminated at the attenuator, a site preceding the first structural gene in the operon (9, 17). Deletion of the attenuator site in both the *his* and *trp* operons has resulted in an enhanced transcription of the operon owing presumably to loss of the region at which transcription is terminated (11, 12).

Because the technique used for the isolation of the *trp* attenuator deletion strains by Jackson and Yanofsky (11) could be adapted to the *ilv* cluster, experiments were performed to examine strains with deletions that removed the early part of the *ilvE* gene and that part of the promoter-proximal DNA that was suspected of being an attenuator site (25). This paper describes the consequences of one such mutation that was shown to have removed part of the *ilvG* gene and that portion of *ilv* DNA shown in the previous paper to contain the *ilvO* determinant (27). A phage carrying the deletion was isolated so that it was possible to characterize the extent of the deletion physically. The consequences of the deletion on *ilv*-specific control in an *ilv-lac* fusion strain were also examined.

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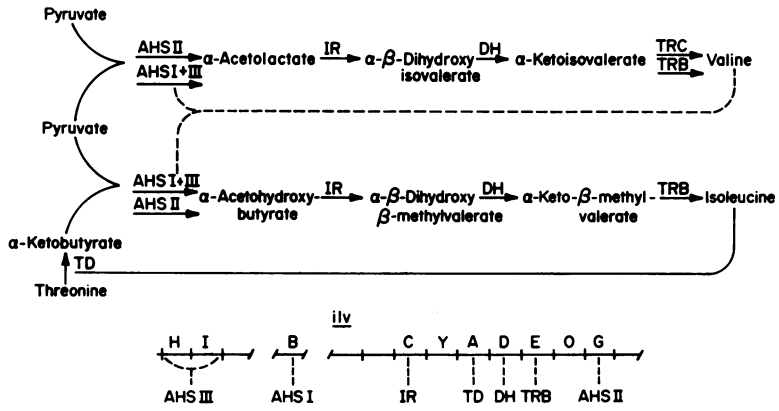

FIG. 1. *Biosynthesis of isoleucine and valine. The enzymes catalyzing the indicated steps are abbreviated and the corresponding structural genes (where known) are indicated in parentheses as follows: TD (ilvA) threonine deaminase; AHS I (ilvB) and AHS III (ilvHI) end product-inhibited acetohydroxy acid synthases; AHS II (ilvG), end product-noninhibited acetohydroxy acid synthase; IR (ilvC), acetohydroxy acid isomeroreductase; DH (ilvD), dihydroxy acid dehydrase; TRB (ilvE), transaminase B; TRC, transaminase C. ilvG exhibits no activity in E. coli K-12 unless a mutation in ilvO has occurred. ilvY specifies a control element for isomeroreductase induction by substrate.*

MATERIALS AND METHODS

Bacterial and bacteriophage strains. All bacterial strains used in this study were *Escherichia coli* K-12 derivatives and are listed in Table 1. The bacteriophages used are listed in Table 2.

Media and growth of cells. The medium of Davis and Mingioli (5) modified by the omission of citrate and by the increase of the glucose concentration to 0.5% was used as the minimal medium and was supplemented as indicated. Unless stated otherwise, all amino acids or their precursors when added were at a final concentration of 0.5 mM except valine, which was 1 mM. Vitamin supplements when added were at a final concentration of 0.1 mM. Thymine and uridine were added to yield a final concentration of 50 μg/ml. 5-Fluorouracil was added to yield a final concentration of 20 μg/ml. Agar (Difco) was added to yield a final concentration of 1.5%. Ionagar (Difco) or Noble agar (Difco) at a concentration of 1.5% was used to prepare X-gal plates which contained 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml. Isoleucine at a concentration of 15 μM and proline at 26 μM were added to limit growth of auxotrophs on solid media. The other branched-chain amino acids were added in excess. The rich medium was L broth (2). MacConkey ribose plates contained 4% MacConkey agar base and 1% ribose.

Growth of cultures was monitored by measuring the optical density at 660 nm (OD_{660}) in a Spectronic 88 spectrophotometer. The growth temperature was 37°C, except for the temperature-sensitive strains, which were grown at 30°C.

For maximal derepression of the isoleucine and valine biosynthetic enzymes, an auxotroph was grown to an OD_{660} of approximately 0.3 in an excess of isoleucine (0.5 mM), leucine (0.5 mM), and valine (1 mM). The cells were then harvested under sterile conditions at approximately 37°C, washed once with warm minimal medium, and then resuspended in a medium in which the concentration of one of the amino acids was limiting for growth. The other two amino acids were present in excess. The limiting concentrations of amino acids were 15 μM for isoleucine and leucine and 34 μM for valine. The cultures were grown under limitation for 3 h.

Enzyme assays. The preparation of cell extracts and the assay of the isoleucine and valine biosynthetic enzyme activities were as described earlier (24). β-Galactosidase activity was determined by measuring the hydrolysis of *o*-nitrophenol-β-D-galactoside as described by Zubay et al. (31). The reaction mixtures of 0.9 ml total volume contained 90 μmol of sodium phosphate (pH 7.3), 0.5 mg of *o*-nitrophenyl-β-D-galactoside, and 1% (wt/vol) mercaptoethanol. The reaction mixtures were incubated at 30°C until a bright yellow color developed. The reactions were stopped with the addition of 0.05 ml of glacial acetic acid, chilled, and centrifuged at room temperature in a tabletop centrifuge. To the supernatant was added 0.9 ml of 10% sodium carbonate, and the color was read at 420 nm in a Gilford model 240 spectrophotometer.

Specific activities are expressed as nanomoles of product formed per minute per milligram of protein. Protein in the crude extracts was determined by the method of Lowry et al. (19). Crystallized bovine serum albumin was used as the standard.

Transduction. Generalized transductions were performed by the method of Rosner (23). P1*cm* lysates were prepared by heat induction of individual lysogens. The lysogen was grown in L broth at 30°C to an OD_{660} of 0.3. The culture was then shifted to 42°C for 30 min and then to 37°C until lysis occurred. Chloroform was then added to complete the lysis, and the cell debris was removed by centrifugation. P1*cm* lysogens were prepared by cross-streaking the phage from a lysate against a culture of the recipient strain on an L-agar plate supplemented with 15 μg of chloramphenicol and 5 mM $CaCl_2$. The recipient was grown in 5 ml of L broth to an OD_{660} of 0.3 to 0.5. $CaCl_2$ was

TABLE 1. *E. coli strains used*

Strain	Genotype	Source or reference
CSH7	*lacY rpsL thi*	Cold Spring Harbor Laboratory (22)
CSH26	F⁻ Δ(*pro-lac*) *ara thi* λ⁻	Cold Spring Harbor Laboratory (22)
CU1	Wild-type K-12 strain	
CU2	Δ*ilvE12 ilv-2025*	McGilvray and Umbarger (21)
CU339	F⁺ *ilvO468 rbs-215* λ⁺	Kline et al. (14)
CU406	F⁻ *ilvA454 galT12* λ⁻	Smith et al. (25)
CU447	*trp* Δ*lac arg thi rbs-221*	Smith et al. (25)
CU519	F⁻ Δ*ilvDAC115 metE201 leu-455 galT12* λ⁻	Smith et al. (24)
CU520	F⁻ *rbs-221 metE201 leu-455 galT12* λ⁻	Smith et al. (25)
CU532	F⁻ Δ*ilvE2050 leu-455 galT12* λ⁻	Smith et al. (25)
CU544	F⁻ *ilvC44 leu-455 galT12* λ⁻	Smith and Umbarger (26)
CU567	F⁺ *ilvO468 ilvE2054 rbs-215* λ⁺	Spontaneous Ilv⁻ derivative of CU339
CU609	F⁺ *ilvO468 ilvE2061 rbs-215* λ⁺	Smith et al. (24)
CU635	F⁻ Δ*ilvDAC115 metE201 leu-455 rpsL galT12* λ⁻	P1-mediated transduction of CU519 with CSH7 as donor
CU639	F⁺ *ilvG2075*::Mu-1 *ilvO468 rbs-215* λ⁺	Smith et al. (24)
CU664	F⁺ *ilvG2097 ilvO468 rbs-215* λ⁺	Valine-sensitive derivative of CU339 after diethyl sulfate mutagenesis
CU667	F⁺ *ilvG2097 ilvO468* λ⁺	P1-mediated transduction of CU664 with CU532 as donor
CU677	F⁺ *ilvE2080* λ⁺	Spontaneous Ilv⁻ derivative of CU1
CU678	F⁺ *ilvE2081* λ⁺	Spontaneous Ilv⁻ derivative of CU1
CU679	F⁺ *ilvE2082* λ⁺	Spontaneous Ilv⁻ derivative of CU1
CU698	F⁻ *metE201 leu-455 galE12*	Smith et al. (24)
CU703	F⁻ *ilvO468 ilvE2061 leu-455 galT12* λ⁻	P1-mediated transduction of CU698 with CU609 as donor
CU704	F⁻ *ilvO468 ilvE2054 leu-455 galT12* λ⁻	P1-mediated transduction of CU698 with CU567 as donor
CU705	F⁻ *ilvE499 rbs-215 galT12* λ⁻	Smith et al. (24)
CU706	F⁻ *ilvE2080 leu-455 rbs-221 galT12* λ⁻	P1-mediated transduction of CU520 with CU677 as donor
CU707	F⁻ *ilvE2081 leu-455 rbs-221 galT12* λ⁻	P1-mediated transduction of CU520 with CU678 as donor
CU708	F⁻ *ilvE2082 leu-455 rbs-221 galT12* λ⁻	P1-mediated transduction of CU520 with CU679 as donor
CU755	*ilvE2089*::Mucts62*K*am1010Δ(*pro-lac*) *ara thi*	Isoleucine, valine, and leucine auxotroph derived by Mu*cts* mutagenesis of CSH26
CU838	Δ*ilvEDAC2049* Δ(*pro-lac*) *ara thi*	Watson et al. (30)
CU856	F⁻ *ilv-2095 ilvG2111 ilvE2109 leu-455 galT12* λ⁻	Smith et al. (24)
CU902	Δ*ilvE2050 trp arg* Δ*lac thi*	Watson et al. (30)
CU925	Δ*ilvOE2130* Δ(*pro-lac*) *ara thi*	Temperature-resistant survivor of CU755 that no longer required valine or leucine
CU930	pGMM4(*ilvE*⁺) Δ*ilvOE2130* Δ(*pro-lac*) *ara thi*	An Ile⁺ derivative of CU925 obtained by transformation by the pGMM4 plasmid. This plasmid carries an intact *ilvE* gene and is described in references 20 and 27.
CU944	Δ(*pro-lac*) *ara thi ilvD2138*::Mu-1cts62*K*am1010	Leathers et al. (16)
CU945	Δ(*pro-lac*) *ara thi ilvD2139*::Mu-1cts62*K*am1010::λp1(209)	Leathers et al. (16)
CU946	Δ(*pro-lac*) *ara thi ilvD2210*::-ΔMu-1-::λp1(209)	Leathers et al. (16)
CU967	Δ*ilvE12 trp arg* Δ*lac*	P1-mediated transduction of CU447 with CU2 as donor
CU1083	Δ(*pro-lac*) *ara rbs-232 thi ilvD2210*::-ΔMu-1-::λp1(209)	Spontaneous Rbs⁻ derivative of CU946
CU1088	Δ*ilvOE2130* Δ(*pro-lac*) *ara thi ilvD2210*::-ΔMu-1-::λp1(209)	P1-mediated transduction of CU1083 with CU925 as donor
CU1095	F⁻ Δ(*pro-lac*) *ara thi thyA* λ⁻	Spontaneous mutant of CSH26 selected in presence of trimethoprim and thymine
CU1099	F⁻ Δ(*pro-lac*) *ara thi thyA* λ⁺	λ lysogen of CU1095
CU1108	Δ*ilvOE2130* Δ(*pro-lac*) *ara thi* λ*h80* λ*h80dilvA2094*	Ilv⁺ lysogen of CU925 obtained by transduction with λ*h80dilvA2094* with helper

TABLE 1.—*Continued*

Strain	Genotype	Source or reference
CU1110	F⁻ Δ*ilvDAC115 metE201 leu-455 rpsL galT12* λ*h80* λ*h80dilvOE2130*	Rare Ilv⁺ transductant of CU635 with lysate from CU1108
CU1111	F⁻ *ilvA454 galT12* λ*h80* λ*h80dilvOE2130*	Ilv⁺ transductant of CU406 with lysate from CU1110
CU1112	*ilvC44 leu-455 rbs-221 araC galT12*	Derivative of CU544 able to grow on L-arabinose in presence of D-fucose
CU1151	Δ*ilvOE2130 leu-455* Δ(*pro-lac*) *araC thi*	P1-mediated transduction of CU925 with CU1112 as donor

TABLE 2. *Phage derivatives used*

Phage	Genotype	Source or reference
λp1(209)	Δ(*b-xis*)[(+'Mu)::(*trp'BA' lac'OZY*)]	Casadaban (4)
λp*ilv-lac*3	Δ(*b-xis*)[*ilv'GOED' trp'BA' lac'OZY*]	Leathers et al. (16)
λp*ilv-lac*4	Δ(*b-xis*)[(+'Mu')::(*ilvG'-Δ2130-'ED' trp'BA' lac'OZY*)]	Mitomycin C induction of CU1088
λ*h80dilv*	Δ(8-*att*^{80}*P*)c1857*St68*[*ilvGOEDAYC*]	LoShiavo et al. (18)
λ*h80dilvA2094*	Δ(8-*att*^{80}*P*)c1857*St68*[*ilvGOEDA2094YC*]	Smith et al. (24)
λ*h80dilv-2130*	Δ(8-*att*^{80}*P*)c1857*St68*[*ilvG'-Δ2130-'EDAYC*]	Recombinant *ilvA*⁺ phage arising upon heat induction of CU1108
Mu-1cts	cts62 *K*am1010	Howe (10)
P1*cm*	21 kb::Tn*9 clr*100	Rosner (23), Kondo and Mitsuhashi (15), DeBruijn and Bukhari (6)

added to a final concentration of 5 mM. P1*cmclr*100 transducing phage was added to a multiplicity of infection of 1. Phage absorption was performed at 30°C for 15 min, after which the cells were harvested by centrifugation and resuspended in 0.5 ml of 0.85% NaCl. A 0.1-ml volume of the resuspended cells was spread on the appropriate selection plate. Cell and phage control plates were always included in the experiments.

Isolation of strain CU925. Strain CU755 was prepared by mutagenesis of strain CSH26 with Mucts, followed by penicillin enrichment. Strain CU755 was shown to lack *ilvE* activity and to exhibit negligible levels of *ilvD* and *ilvA* expression. Mu was shown to be inserted very near the promoter-proximal end of the *ilvE* gene. It required all three branched-chain amino acids. To select for derivatives that had spontaneously lost the temperature-sensitive Mu prophage by deletion, strain CU755 was plated on rich medium (L broth) at 42°C. Temperature-resistant survivors were scored for ability to grow on minimal medium plus proline, thiamine, and isoleucine, which required the expression of the *ilvD* gene. A large collection of these derivatives was isolated, none of which was able to grow without the addition of isoleucine. It was concluded that none of the deletions was sufficiently precise to restore *ilvE* function. Many of these strains were characterized with respect to the response of the *ilvD* and *ilvA* genes to limiting levels of isoleucine (D. J. Gayda, unpublished results). Strain CU925 was representative of a class exhibiting elevated enzyme levels under repressing conditions (excess branched-chain amino acids), yet responding to *ilv*-specific control.

Isolation of strain CU946. Another Mu-1cts mutant isolated by J. M. Smith was an *ilvD* mutant, strain CU944, which was used to prepare an *ilv-lac* fusion strain by the method of Casadaban (4). It was lysogenized with λp1 (209) as described earlier (26). One of the Mu-λ double lysogens was strain CU945. Strains containing *ilv-lac* fusions were selected by spreading 10^9 cells of strain CU945 on minimal agar plates containing lactose as carbon source and supplemented with α-ketoisovalerate and α-keto-β-methylvalerate. The plates were then incubated at 42°C for 18 h and then at 37°C until colonies appeared. Those cells in which fusion events deleting the Mu phage (or at least the killing function of the Mu phage) had not occurred were killed as a result of phage induction at 42°C.

Since a functional *ilvE* gene is required for growth on the two keto acids, the deletion event removing the Mu phage could not extend significantly into the *ilvE* gene. Those colonies that appeared were then screened for expression of the *lac* genes associated with the *ilvEDA* regulatory signal. Strain CU946 was such and was saved for further study.

Isolation of strain CU1088. An *rbs* derivative (CU1083) of the fusion strain CU946 was transduced with P1 phage with the deletion strain CU925 as the donor. Transductants were selected on X-gal plates containing excess isoleucine, valine, and leucine with ribose as the carbon source. Blue colonies were scored for an IlvE⁻ phenotype (inability to use α-keto-β-methylvalerate in place of isoleucine). One such strain, CU1088, carried both the *ilvE2130* deletion of strain CU925 and the *ilv-lac* fusion of strain CU946.

Isolation of λp*ilv-lac*4. A 10-ml culture of strain CU1088 in L broth containing 10 mM $MgSO_4$ was grown to early log phase at 30°C. Mitomycin C was added to yield a concentration of 1 μg/ml. Lysis occurred after further incubation. A 2-ml volume of

chloroform was added. The resulting lysate was plated on a lawn of strain CSH26 (*Δpro-lac*) on X-gal plates containing excess isoleucine, valine, and leucine. λ*pilv-lac*4 was picked from a deep blue plaque and purified by single plaque isolation.

Isolation of λ*h80dilv-2130*. Strain CU1108, a λ*h80dilvA2094* lysogen of strain CU925, yielded lysates that exhibited high frequency of transduction of *ilvE* mutants but not *ilvA* mutants. These lysates did exhibit a low frequency of transduction of the *ilvA* marker, either the result of reversion at the *ilvA2094* locus or recombination with the bacterial chromosome. Strain CU635, bearing the *ilvDAC115* deletion, was transduced to *ilv*$^+$ with this lysate. Transductants were scored for temperature sensitivity and the ability to generate phage lysates that were high-frequency transducing for *ilvA* transduction, but not for *ilvE* transduction. λ*h80dilv-2130* was maintained as a double lysogen with its helper, λ*h80*, in strain CU1111, and *ilvE*$^+$ *ilvA454* strain.

Preparation and purification of phages. λ*h80* and its derivatives were prepared as described previously (28).

λ*pilv-lac*3 and λ*pilv-lac*4 were prepared by lytic growth on strain CSH26. Strain CSH26 was infected with phage and spread on an L-agar plate and incubated for 6 to 8 h at 37°C. A 100-ml culture of strain CSH26 grown to an OD_{660} of 1.0 was harvested by centrifugation and resuspended in 30 ml of 0.01 M $MgSO_4$. A 10-ml volume of this cell suspension was added to the confluently lysed lawn of strain CSH26 and allowed to incubate for 20 min at room temperature. Three such plate lysates were then added to 900 ml of L broth. In some experiments mitomycin C was added to a final concentration of 1 μg/ml. This culture was incubated at 37°C until lysis occurred.

The phage was precipitated by adding 133 g of polyethylene glycol 6000 and 110 ml of 5 M NaCl to a liter of lysate. The mixture was stirred gently overnight at 4°C. The mixture was centrifuged for 20 min at 8,000 rpm in a Sorvall GSA rotor. The pellets were resuspended in 11 ml of TMG buffer [10 mM Tris-hydrochloride (pH 7.5) and 10 mM $MgSO_4$, with 10 μg of gelatin per ml]. One-third volume of chloroform was added and mixed thoroughly. The mixture was centrifuged for 10 min at 5,000 rpm. The phage suspension was removed, and the procedure was repeated three to four times. The phage layer was then treated with deoxyribonuclease (0.1 μg/ml) for 10 min at 37°C.

The phage was purified by CsCl block and equilibrium gradient centrifugation as described earlier (28). The phage suspension was layered onto a CsCl block gradient and centrifuged in an SW25.1 rotor for 90 min at 24,000 rpm at 5°C. The block gradient contained 1.5 ml of CsCl n = 1.388, 2.0 ml of n = 1.3788, and 2.0 ml of n = 1.3711 in a cellulose nitrate tube (1 by 3 inches [2.54 by 7.62 cm]). The phage was collected and centrifuged in an equilibrium gradient of CsCl n = 1.388 for 18 to 24 h at 27,000 rpm in a Ti50 rotor. The purified phage was dialyzed in the cold against 100 volumes of TMG buffer with two changes of buffer.

Physical analysis of phage DNA. Restriction endonuclease analysis was performed as described previously (20) with enzymes obtained from New England BioLabs. Digestions were generally done in a total volume of 25 μl containing 0.2 to 2.0 units of enzyme with the appropriately recommended buffers. Heteroduplex analysis was performed as described earlier (20).

In vivo analysis of phages bearing *ilv-lac* fusions. The procedure described by Franklin (8) was used to monitor expression of the *lacZ* gene in vivo from phage DNA. A 100-ml culture of a strain bearing a *thyA* mutation was grown to an OD_{660} of 0.6 in the presence of excess isoleucine, leucine, and valine, and thymine, centrifuged, washed with 0.01 M $MgSO_4$, and resuspended in 10 ml of 0.01 M $MgSO_4$ supplemented with 5-fluorouracil and uridine to yield final concentrations of 20 μg/ml. Phage were allowed to adsorb for 15 min at 37°C (multiplicity of infection = 1). The cells were then chilled and centrifuged to remove unadsorbed phage. The cells were then resuspended in medium supplemented with glucose, 5-fluorouracil, and uridine (to prevent phage and cell replication but not transcription) and either an excess of isoleucine, leucine, and valine, or a limiting amount of one amino acid. The cultures were incubated at 37°C with shaking and enzyme levels were determined as described above.

RESULTS

Enzyme levels in strains bearing the *ilvE2130* deletion. Strain CU925 had been selected as a heat-resistant survivor of a strain bearing Mu-1*cts* inserted at a site early in the *ilvE* gene. The original mutant grew very slowly on isoleucine alone owing to the very limited expression of the *ilvD* gene. Strain CU925 was selected initially as a strain that could grow well on isoleucine alone, thereby indicating a signficant amount of the *ilvD* gene product, dihydroxy acid dehydrase. Although transaminase B activity had not been restored, valine synthesis was possible in strains containing the dehydrase, since transaminase C (21) can convert the α-ketoisovalerate formed by the dehydrase to valine at a significant rate. Strain CU925 had been chosen, since preliminary experiments had shown it to contain a repressed level of threonine deaminase considerably above the level exhibited by strain CSH26, the strain originally used to select the Mucts lysogen.

Table 3 compares the activities of the dehydrase and threonine deaminase in strain CU925 with those in strain CSH26. Of particular interest was the fact that the deletion not only restored the *ilvD* and *ilvA* gene activities but made them two- to threefold higher than those in strain CSH26. Upon isoleucine limitation, the greater response of *ilvA* than that of *ilvD* was similar to the findings with mutants studied earlier (25). The same activities were also examined in a leucine auxotroph that carried the *ilvE2130* deletion and a derivative of strain CU925 carrying an *ilvE*$^+$ plasmid. The *ilv* region in a strain carrying the deletion also undergoes

derepression upon leucine limitation (Table 3). The plasmid-containing strain demonstrated that in a minimal medium the expression of the *ilv* operon was also increased by the *ilvE2130* deletion and that the operon is repressible.

Physical mapping of the *ilvE2130* deletion. For both the genetic and physical characterization of the *ilvE2130* deletion, a derivative of λ*h80dilv* that carried the deletion was isolated as described in Materials and Methods. Phage DNA was prepared from both λ*h80dilv* and λ*h80dilv-2130* and examined by both restriction endonuclease digestion and heteroduplex formation. Figure 2 shows the sites in the non-phage DNA for five restriction endonucleases carried by the two phages. These results indicated that the *Hin*dIII site at coordinate 6.4 kilobase (kb), the *Kpn*I and *Pvu*II sites at 7.4 kb, and the *Sal*I site between were all missing, leading to the fusion of bracketing restriction fragments. In addition, the *Sma*I fragment was shortened internally. As indicated, the newly generated restriction products allowed estimates to be made of between 1.6 and 1.8 kb for the *ilvE2130* deletion, in good agreement with heteroduplex results detailed below.

TABLE 3. *Effect of the ilv-2130 deletion on the expression of the ilvD and ilvA genes*

Strain	Medium	Sp act[a]	
		Threonine deaminase *ilvA*	Dihydroxy acid dehydrase *ilvD*
CSH26 (*ilv*⁺)	Repressing[b]	32	9.2
	Minimal	55	17
	Limiting isoleucine[c]	326	76
CU925 (Δ*ilvE2130*)	Repressing	115	21
	Limiting isoleucine	1560	172
CU930 [Δ*ilvE2130* pGMM4(*ilvE*⁺)]	Repressing	60	15
	Minimal	420	70
CU1151 (Δ*ilvE2130 leu*)	Repressing	70	9.7
	Limiting leucine	371	66

[a] Nanomoles per minute per milligram of protein.
[b] Transaminase B specific activity: 23.
[c] Transaminase B specific activity: 86.

Figure 3 is a diagram of the heteroduplexes that were formed between λ*h80dilv* and λ*h80dilv-2130*. The closed loop structure indicated that the *ilvE2130* deletion is a simple deletion and precluded the possibility that a portion of the inserted Mu had remained in strain CU925. In other experiments the length of the parental λ*h80dilv* was estimated as 51.0 kb. This value exceeded the sum of the double-stranded arms of the heteroduplex (49.3 kb) by 1.7 kb, matching the estimated size of the closed loop in Fig. 2. Since the left arm φ80 portion of λ*h80dilv* was measured as 14.0 ± 0.4 kb, the *ilvE2130* deletion was concluded to begin approximately at coordinate 6.3 kb (in Fig. 1) and to extend rightward 1.75 kb, in complete accord with the restriction endonuclease analysis shown in Fig. 1.

The physical analysis of the *ilvE2130* deletion revealed that it extended into considerable DNA preceding the promoter-proximal end of the *ilvE* gene, which had been concluded earlier to lie between the *Hin*dIII site at 6.4 kb and the *Sal*I site at 6.8 kb. Thus, the deletion had removed the entire region of DNA that was shown in the

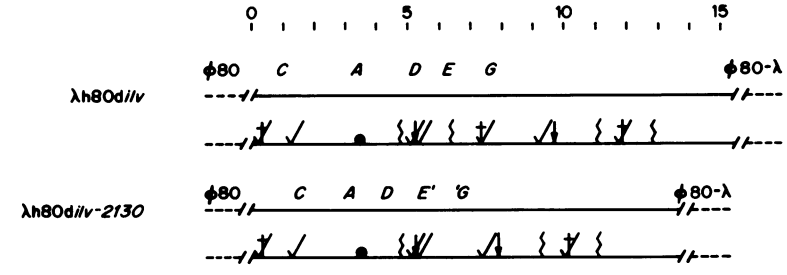

FIG. 2. *Cleavage sites for several restriction endonucleases in the non-phage DNA carried by λh80dilv and λh80dilv-2130. The scale is graduated in kilobases. The zero kilobase coordinate is the φ80-chromosomal DNA junction which is very near the terminus of the ilvC gene. The endonucleases used were: ⌇, HindIII; †, KpnI; √, PuvII; ↓, SmaI; ●, SalI.*

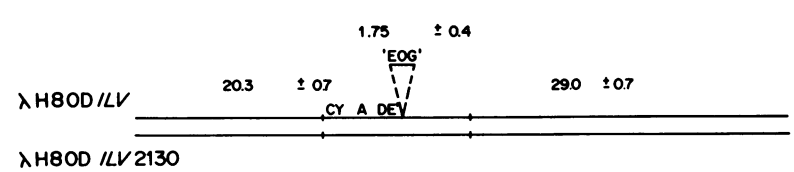

FIG. 3. *Heteroduplex between λh80dilv and λh80dilv-2130. All measurements in kilobases.*

previous paper to contain the *ilvO* determinant as well as part of *ilvE* and is designated hereafter as Δ*ilvOE2130*.

Since the *ilvO* region appeared to be removed by the *ilvOE2130* deletion, it seemed possible that the deletion allowed transcription of the *ilvD* and *ilvA* genes by polymerases originating at a "high-level" promoter upstream of *ilvG* by removing a "low-level" promoter of the wild-type *ilvEDA* operon. Such a low-level promoter might retard polymerases bound to it and block transcription from upstream (13). An alternative was that the *ilvEDA* operon is always transcribed from a promoter outside of the *ilvOE2130* deletion and that the deletion had removed a site that reduced *ilvEDA* expression in *ilvO*$^+$ cells. In other words, except for the fact that the *ilvOE2130* deletion had removed part of the *ilvG* structural gene, it may have mimicked that effect of an *ilvO* mutation that increases *ilvEDA* expression. To decide between these possibilities, strains that carried an *ilv-lac* fusion developed by the general Casadaban (4) technique were examined. From such strains it had been possible to obtain lambda phage that carried *ilv* DNA that extended only a short distance beyond the *ilvO* region. It was also possible to examine phages that carried the *ilvOE1230* deletion but which had excised *ilv* DNA that extended well beyond the *ilvG* gene.

Effect of the *ilvOE2130* deletion on the expression of the *lacZ* gene fused to the *ilvD* gene. Table 4 shows the levels of transaminase B activity (*ilvE* gene product) and β-galactosidase in strain CU946. Both enzyme activities were observed to be increased by growing the cells either with limiting isoleucine or with limiting valine. Clearly the *lac* genes are under *ilv*-specific regulation in this strain. Of some interest was the observation that β-galactosidase in strain CU946 was derepressed almost coordinately with transaminase B. In contrast, the strain from which CU946 had been derived exhibited the "downstream amplification" with limiting isoleucine that is typical of the response to derepression, particularly by limiting isoleucine (25). An obvious difference between the fusion strain and its parent is the absence of threonine deaminase, the product of the *ilvA* gene. Since threonine deaminase has often been postulated to have a regulatory role (3), the effect of the F16 episome was examined. As the table shows, there appeared to be no downstream amplification of β-galactosidase under these conditions, although the levels of the dehydrase and threonine deaminase expressed from the episome revealed such an effect.

Table 4 also shows that the activity of β-galactosidase in strain CU1088, which contained both the fusion and the *ilvOE2130* deletion, was much higher than that in strain CU946, although it was still subject to an *ilv*-specific regulation. Thus, the effect of the *ilvOE2130* deletion on *ilv* gene expression appeared to be the same whether the strains carried intact *ilv* DNA beyond the deletion or the *ilv-lac* fusion. Such an effect of the deletion could have arisen from deletion of a predominant polymerase binding site in the *ilvO* region from which only a small number of transcripts could be made, leaving an

TABLE 4. *ilv-specific regulation of β-galactosidase formation in ilv-lac fusion strains*

Strain	Medium	Sp act[a]			
		Threonine deaminase *ilvA*	Dihydroxy acid dehydrase *ilvD*	Transaminase B	β-Galactosidase
CSH26 (*ilv*$^+$)	L broth	—[b]	—	3.2	—
	Repressing	26	8.7	23	—
	Limiting isoleucine	321	74	86	—
CU946 [*ilvD2139*::λp1(209)]	L broth	—	—	4.3	7.8
	Repressing	—	—	31	17
	Limiting isoleucine	—	—	74	73
	Limiting valine	—	—	93	65
CU1088 [*ilvE2130, ilvD2139*::λp1(209)]	Repressing	—	—		156
	Limiting isoleucine	—	—		553
CU1103 [F'16*ilvE2050/ilvD2139*::λp1(209)]	L broth	—	—	4.1	6.2
	Repressing	39	12	25	21
	Limiting isoleucine	378	61	64	58
CU1150 [F'16/*ilvOE2130*::*ilvD2139*, λp1(209)]	Repressing	23	—		97
	Limiting isoleucine	222	—		340

[a] Nanomoles per minute per milligram of protein.
[b] —, Not done.

upstream binding site from which about three times as many transcripts could be made. Alternatively, the deletion of *ilvO* could have removed a transcriptional or translational barrier which made expression of the *ilvEDA* genes more efficient. To ascertain which alternative seemed more likely, λp*ilv-lac*3, known to carry *ilv* DNA only a short distance beyond the *ilvO* region (16), was compared with λp*ilv-lac*4 which bore the *ilvOE2130* deletion and presumably carried DNA from a region further upstream on the chromosome than did λp*ilv-lac*3.

In vivo analysis of *ilv* gene expression with λp*ilv-lac*3 and λp*ilv-lac*4 as templates. For the in vivo analysis of *ilv* gene expression in λp*ilv-lac*3 and λp*ilv-lac*4, it was necessary to use cells infected with vegetative phage rather than lysogens. Since both phages lacked the phage attachment locus, lysogens would likely result in integration into a region of the chromosome homologous with DNA carried by the phage. Such an integration could lead to control of the *ilv-lac* fusion by a chromosomal structure not carried by the free phage.

The cells containing a *thyA* marker as well as the *pro-lac* deletion were infected as described in Materials and Methods in the presence of 5-fluorouracil and uridine to prevent replication of phage and host DNA but not RNA or protein synthesis. The host was a λ lysogen, so that transcription initiated at a phage promoter, and thus read-through into the *ilv-lac* DNA should have been blocked. In the experiment in which the infecting phage was λp*ilv-lac*3 (Table 5), threonine deaminase was nearly 10 times as high in cells that had been suspended in a limiting isoleucine medium during the 3-h infection period than it was in cells that had been suspended in a repressing medium. Thus, an effective derepression signal had been generated. In contrast, β-galactosidase activity which arose from the *ilv-lac* fusion carried by the infecting phage was actually lower under conditions of an isoleucine limitation. Clearly, the *ilv-lac* genes carried by λp*ilv-lac*3 are not under an *ilv*-specific control.

TABLE 5. *β-Galactosidase and threonine deaminase activities in strains infected with ilv-lac fusion phages*

Infecting phage	Medium	β-Galactosidase[a]	Threonine deaminase[b]
λp*ilv-lac*3	Repressing	65	18
	Limiting isoleucine	45	167
λp*ilv-lac*4	Repressing	169	27
	Limiting isoleucine	581	179

[a] Nanomoles per hour per milligram of protein. This enzyme was specified by the phage.

[b] Nanomoles per minute per milligram of protein. This enzyme was specified by the host.

In the experiment in which λp*ilv-lac*4 was the infecting phage, there was not only a derepression of threonine deaminase but also a derepression of β-galactosidase. Thus, the *ilv-lac* fusion in λp*ilv-lac*4, in contrast to that in λp*ilv-lac*3, is under an *ilv*-specific control. To ascertain which region of DNA carried the *ilv*-specific control site, the DNA of λp*ilv-lac*4 was compared to λp*ilv-lac*3 which had been studied earlier (16).

Restriction analysis of λp*ilv-lac*4 DNA. Figure 4 contains a map of the sites of cleavage by several restriction endonucleases of λp*ilv-lac*4 and, for comparison, that of λp*ilv-lac*3 reported earlier (16). Of particular interest is the absence in λp*ilv-lac*4 of the *SalI* site at the *ilv* 6.8-kb coordinate and the *KpnI* site at the *ilv* 7.4-kb coordinate which are present in λp*ilv-lac*3. These were also shown (Fig. 1) to be missing in λ*h80dilv-2130*. Another difference is the presence of an additional *SmaI* site in λp*ilv-lac*4. This additional site yielded a *SmaI* fragment that is probably identical to the 2.6-kb fragment in λ*h80dilv-2130*. As Fig. 2 shows, this fragment had arisen by the shortening of the 4.3-kb *SmaI* fragment in λ*h80dilv* owing to the deletion event. Thus, λp*ilv-lac*4 carried the *ilvOE2130* deletion but included some *ilv* DNA beyond the *ilvOE2130* deletion terminus not present in λp*ilv-lac*3. This DNA was presumably responsible for the *ilv*-specific control reported in Table 5.

Heteroduplex analysis of λp*ilv-lac*4. Figure 5 summarizes measurements of heteroduplexes between the DNA of λp*ilv-lac*4 and that of λp1(209), the parental Casadaban phage of both λp*ilv-lac*4 and λp*ilv-lac*3. λp*ilv-lac*4 was recognized as a substitution derivative of λp1(209). As anticipated, right-arm homology extended through the *lac* and *trp* material of λp1(209), indicated by the estimated 24.7 ± 0.6 kb of left-arm homology. Right-arm homology of 19.6 ± 0.3 kb indicated that approximately 0.9 kb of original Mu sequence was retained by λp*ilv-lac*4 in its excision from strain CU1088. Consistent with this conclusion, a 2.0 ± 0.6 kb single-stranded bubble arm was observed, representing the remaining portion of Mu from λp1(209) (16). The other single-stranded bubble arm would therefore have included approximately 2.2 kb of *ilvD* sequence (that previously determined as fused to *lac* [16] and extending to the beginning of the *ilvOE2130* deletion), as well as additional DNA from beyond the deletion and extending into *ilvG*. The actual measure-

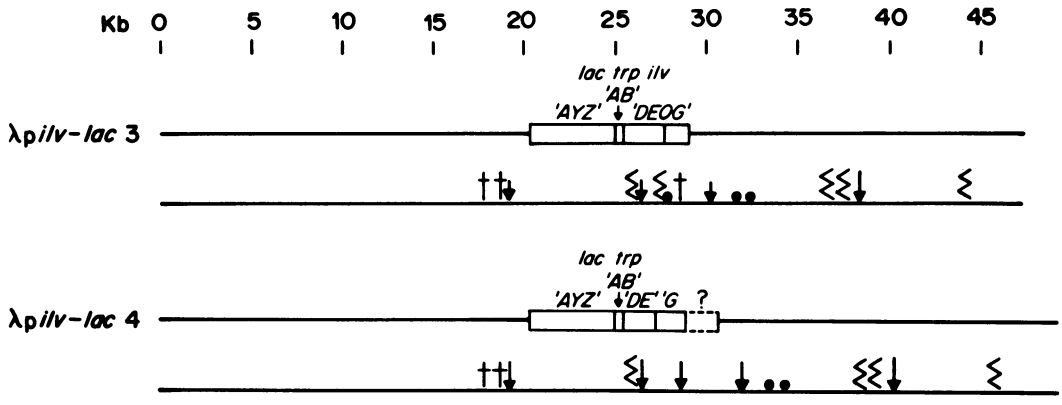

FIG. 4. *Cleavage sites for several restriction endonucleases in λpilv-lac3 and λpilv-lac4. The endonucleases used were: ⦚, HindIII; †, KpnI; √, PuvII; ↓, SmaI; ●, SalI.*

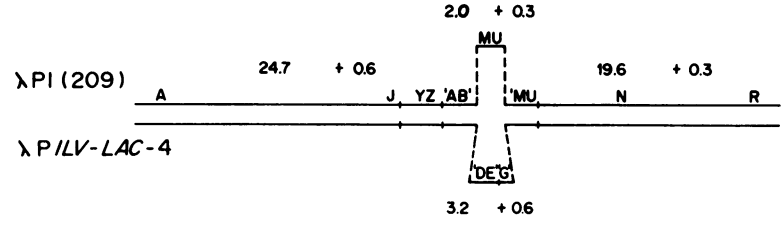

FIG. 5. *Heteroduplex between λp1(209) and λpilv-lac4. All measurements in kilobases.*

ments of this bubble arm yielded a value of 3.2 ± 0.6 kb, which would indicate an additional 1.0 kb of contiguous chromosomal DNA beyond the deletion. This distance would extend only to the 9.05 kb *ilv* coordinate and would not include the *Sma*I site shown in Fig. 4 to be present on λ*pilv-lac*4 DNA. Therefore, in the alignment of the deduced physical studies of non-phage DNA carried by λ*h80dilv* with that of non-phage DNA carried by λ*pilv-lac*4, the value of the bubble arm is increased to 3.8. This value is within the limits of resolution of the heteroduplex measurement and in accord with the restriction analysis. Since this discrepancy is most reasonably explained by error in the heteroduplex measurements, the value for the *ilv* bubble arm was corrected by one standard deviation unit. This value, 3.8, places the end of the chromosomal DNA carried by λ*pilv-lac*4 just beyond the *Sma*I site in Fig. 6, which is an alignment of the *ilv* genes, the *ilvOE2130* deletion, and the *ilv* DNA carried by the fusion of phages with the restriction map of the non-phage DNA carried by λ*h80dilv* previously determined (20).

Genetic analysis of the *ilvOE2130* deletion and of the *ilv* DNA carried by λ*pilv-lac*3. The restriction and heteroduplex analysis of λ*pilv-lac*3 and λ*h80dilv* reported earlier (16, 20) and those of λ*pilv-lac*4 and λ*h80dilv-2130* reported here indicated the extent of the *ilv*-specific DNA carried on the two *ilv-lac* fusion phages. That carried by λ*pilv-lac*3 extended approximately to the 7.7-kb *ilv* coordinate. That deleted by the *ilvOE2130* deletion extended from 6.3 to 8.1 kb. From the alignment of the physical and genetic maps, it would be predicted that the λ*pilv-lac*3 would carry all of *ilvE* and part of *ilvG* as well as the entire *ilvO* determinant. It would be predicted that the deletion covered part of *ilvE*, the *ilvO* determinant, and a significant amount of *ilvG*.

The genetic information actually carried by these phages was examined by applying drops of lysates of either λ*pilv-lac*3 or λ*h80dilv* to lawns of several *ilvE* and *ilvG* mutants on media appropriately supplemented but lacking isoleucine (for recombination at the *ilvG* locus) or isoleucine and valine (for recombination/complementation at the *ilvE* locus). Table 6 shows that indeed λ*pilv-lac*3 exhibited recombination and/or complementation with all the *ilvE* mutants examined and recombination with one of the two *ilvG* mutants tested. λ*h80dilv-2130* exhibited recombination with all but the two most promoter-proximal *ilvE* lesions tested but with neither of the *ilvG* mutants tested. These results are in accord with the physical studies.

DISCUSSION

The experiments reported in this paper provide in vivo evidence that the *ilvO* determinant of the K-12 strain of *E. coli* does not constitute

the *ilv*-specific control region for the *ilvEDA* operon. Rather, it would appear that this control region is upstream from the *ilvG* gene.

The experiments with strains containing the *ilvOE2130* revealed that deletion of the *ilvO* region enhanced the expression of the *ilvEDA* operon. (Actually only expression of *ilvD* and *A* were enhanced, since Δ*ilvOE2130* extends into *ilvE*.) It did not, however, appear to have affected the qualitative character of the multivalent repression signal. While this result was compatible with the idea that a single *ilv*-controlled promoter site lies upstream from *ilvG*, it was also compatible with the idea that the *ilvO* region contained a favored *ilv*-controlled promoter site but from which only a limited number of transcripts could be generated and which might block transcription originating upstream (13).

The second possibility was eliminated when the DNA of a phage containing very little DNA beyond the *ilvO* locus was examined. Such a phage (λp*ilv-lac*3) did not exhibit *ilv*-specific control over the formation of β-galactosidase (which is subject to *ilv*-specific control in the fusion strain from which λp*ilv-lac*3 was derived). A phage of similar size, λp*ilv-lac*4, from which the *ilvO* region had been deleted but which contained *ilv* DNA upstream from *ilvG*, did exhibit *ilv*-specific control over β-galactosidase. It is therefore concluded that the multivalently controlled *ilv*-specific promoter affecting *ilvEDA* expression is in the region of *ilv* DNA lying upstream from *ilvG*.

These results are compatible with those of the previous paper, in which it was shown that transcripts hybridizing to the correct strand of *ilvG* are found in wild-type (*ilvO*$^+$) cells. Thus, even when the *ilvO* determinant does not allow a measurable activity to be formed, there is still transcription of the *ilvG* region. The possibility of an *ilvGEDA* transcript seems very strong. Why such a transcript does not allow expression of an *ilvG* gene product is currently unclear. An obvious possibility is that the K-12 strain contains a site in the *ilvO* region that renders *ilvG* non-translatable and exerts a polar effect on the downstream *ilv* genes, *E*, *D*, and *A*. Whether these effects are due to a nonsense mutation is not known; however, to date no nonsense suppressor mutations have been shown either to enhance *ilvEDA* expression or lead to expression of *ilvG*.

It has yet to be demonstrated that *ilv*-specific

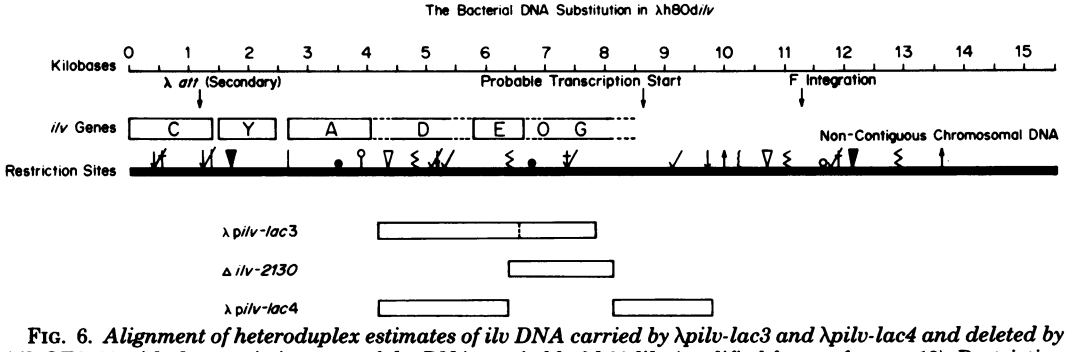

FIG. 6. *Alignment of heteroduplex estimates of ilv DNA carried by λpilv-lac3 and λpilv-lac4 and deleted by ΔilvOE2130 with the restriction map of the DNA carried by λh80dilv (modified from reference 18). Restriction endonuclease code: ↓, SmaI; √, PvuII; †, KpnI; |, EcoRI; ▼, BglII; ●, SalI; ♀, XhoI; ▽, PstI; ≀, HindIII; ↑, HpaI; {, HincII; ○, BamHI. The open bars below indicate ilv DNA carried or deleted as indicated.*

TABLE 6. *Genetic mapping of ilv-2130 deletion on the ilv-lac fusion*

Donor phage	Recipient[a]												
	CU406 *A454*	CU705 *E499*	CU703 *E2061*	CU704 *E2054*	CU706 *E2080*	CU707 *E2081*	CU708 *E2082*	CU902 *ΔE2050*	CU967 *ΔE12*	CU925 *ΔOE21 30*	CU664[b] *G2097 0468*	CU856[b] *G2111 02095*	CU838 *ΔEDA C2049*
λ*h80dilv-2130*	+	+	+	+	+	+	+	−	−	−	−	−	−
λ*h80dilvA2094*	−	+	+	+	+	+	+	+	+	+	+	+	−[c]
λp*ilv-lac3*	−	+	+	+	+	+	+	+	+	+	+[d]	−	−[c]
λp*ilv-lac4*	−	+	+	+	+	ND[e]	+	−	−	−	ND	−	−

[a] The *ilvE* lesions arranged in order of their representation on the chromosomal map: left to right = counterclockwise.
[b] Transductants selected for valine resistance.
[c] Gave transductants that grew on the keto acids.
[d] Actually CU667 used here.
[e] ND, Not done.

control can be exhibited when the region upstream of *ilvG* contained in λp*ilv-lac*4 is combined with the material deleted in the *ilvOE2130* deletion. However, it has been observed that *ilv*-specific control is exerted in a plasmid (pGMM4) that contains the two *Hin*dIII fragments that extend from within the *ilvD* gene at 4.8 kb to 11.1 kb, well beyond the proposed *ilv*-control site. Furthermore, in a subsequent paper it will be shown that *ilv*-specific expression of the *ilv-lac* fusion linked to the *ilvO* region is dependent upon its being contiguous with the DNA upstream from *ilvG* DNA in the proper orientation. In other words, it is, as expected, a *cis*-dependent control site that lies upstream from *ilvG*.

Another finding in this paper that deserves some comment is that of nearly coordinate expression of β-galactosidase and transaminase B. Thus, the downstream amplification of *ilvD* and *ilvA* expression observed particularly during an isoleucine limitation was not observed when β-galactosidase expression was measured instead of dihydroxy dehydrase activity (25). The reason for the different behavior is not clear but may reflect the different demand for isoleucine residues in the various gene products rather than specific control sites as originally suggested. Studies to examine this phenomenon are currently in progress.

ACKNOWLEDGMENTS

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