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Regulation of *ilvEDA* Expression Occurs Upstream of *ilvG* in *Escherichia coli*: Additional Evidence for an *ilvGEDA* Operon

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A low-copy-number plasmid was prepared that contained the entire *ilv* gene cluster of *Escherichia coli*. The introduction of an *ilvO* mutation allowed the *ilvG* gene of the plasmid to be expressed and imparted valine resistance to strains carrying it. Insertion of Tn*10* into the *ilvG* gene of the plasmid resulted in a strong polar effect on *ilv* genes *E*, *D*, and *A*. Replacement of a region of *ilv* deoxyribonucleic acid between two *Kpn*I sites on the high-copy-number plasmid carrying the entire *ilv* gene cluster with a *Kpn*I fragment carrying an *ilv-lac* fusion but not extending into the *ilv*-specific control region resulted in a plasmid expressing the *lacZ* gene under *ilv* control when the fusion had been inserted in its normal orientation but not when it had been inserted in the opposite orientation. These experiments indicate that *ilv*-specific control over *ilvE*, *ilvD*, and *ilvA* expression is dependent on these genes being continguous with deoxyribonucleic acid that lies upstream of *ilvG*. The results also add further support to the concept of an *ilvGEDA* operon in *E. coli*.

The *ilv* gene cluster at 83 min on the *Escherichia coli* chromosome (Fig. 1) consists of five genes that specify enzymes responsible for isoleucine and valine biosynthesis. Among these, the *ilvE*, *ilvD*, and *ilvA* genes are expressed as a single transcriptional unit (23). The *ilvC* gene is a separate, inducible transcriptional unit (21). The *ilvG* gene has been located in this cluster more recently. It is expressed only when there is a mutation in the adjacent *ilvO* locus (8). The order of these loci has been established to be *ilvG*, *ilvO*, *ilvEDA*, and *ilvC* (1, 18, 22, 23, 25). All of these genes are transcribed in the same direction (4, 17, 23, 25, 27). Since *ilvG* is transcribed in the same direction as *ilvEDA*, a possibility arises that *ilvG* might be the first structural gene for an *ilvGEDA* operon. This idea was further supported by experiments showing that phages with *ilv* DNA extending through *ilvO* and into but not through *ilvG* did not exhibit *ilv*-specific gene control. In contrast, a phage carrying a deletion of *ilvO* and flanking portions of *ilvE* and *ilvG* but carrying DNA extending beyond *ilvG* exhibited *ilv*-specific gene control (9).

The one observation contradictory to this idea came from several mutants in which valine-sensitive derivatives had been isolated from *ilvO* (valine-resistant) strains after Mu-1 mutagenesis (cited but not documented in reference 22). The lesions were in *ilvG* and were presumed to have been due to Mu-1 insertions. Since selection for growth on minimal medium had necessarily been performed, the polar effect that Mu-1 should induce on *ilvEDA* function was also selected against. However, it is possible that Mu-1 had been excised from *ilvG*, leaving small deletions, or even that the *ilvG* lesions were spontaneous and unrelated to the Mu-1 lysogeny. For this reason, the question has been reexamined using the insertion element Tn*10*. In these experiments, the starting strain was diploid for the *ilvE*, *ilvD*, and *ilvA* genes because of the low-copy-number plasmid it carried. The *ilv* cluster on the plasmid also contained a valine resistance marker in *ilvO*. The Tn*10* insertion event could thus be scored merely for loss of valine resistance, and selection against the predicted polarity was avoided. It was clearly demonstrated that insertion in *ilvG* did exert polarity on the rest of the operon. These experiments, along with other evidence pointing to an *ilvGEDA* operon, are reported in this paper.

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MATERIALS AND METHODS

Bacterial strains. All bacterial strains described in this study are derivatives of the K-12 strain of *Escherichia coli* and are listed in Table 1. The bacteriophage used was λ p*ilv-lac*3, described previously (15). Its genotype is Δ(*b-xis*)[*ilv'GOED' trp 'BA'lac'ZoZY*]. The plasmids used are listed in Table 2.

Genetic manipulations. The techniques used for transduction and transformation were those described earlier (28). Conjugal transfer of RP4 derivatives was

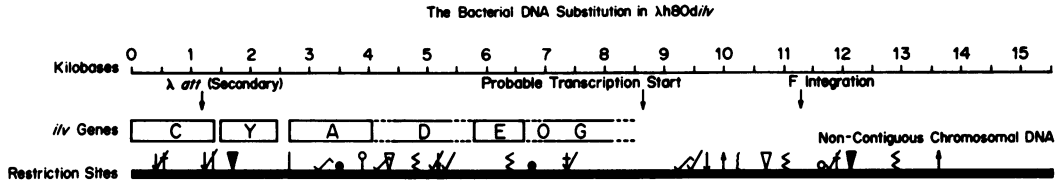

FIG. 1. *Physical map of the non-phage DNA carried by λ h80 dilv. Gene alignments and restriction endonuclease cleavage sites modified from reference 19. Restriction endonuclease code: ↓, SmaI; √, PvuI; √, PvuII; †, KpnI; |, EcoRI; ▼, BglII; ●, SalI; ♀, XholI; ▽, PstI; ≀, HindIII; ↑, HpaI; {, HincII; ○, BamI.*

done by liquid matings as described by Barth (2), except that the rich medium employed was L-broth prepared by the formula of Lennox (16). The minimal medium was that of Davis and Mingioli (6) modified by an omission of citrate and an increase in the concentration of glucose to 0.5%. Antibiotics were used at the following final concentrations: ampicillin, 50 μg/ml; kanamycin, 100 μg/ml; and tetracycline, 10 μg/ml.

Miscellaneous procedures. The growth of cells, preparation of extracts, and assay of enzymes were performed as described previously (22). The methods for restriction analysis and gel electrophoretic separation have been described previously (18, 25). The preparation of plasmid DNA was as described previously (25). Purification of phages and phage DNA was done as described previously (15, 27).

Construction of pJN102 and pJN103. The plasmid pPU183, which carries the *Bam*HI fragment of λ *h80 dilv* that contains the entire *ilv* gene cluster, has been described previously (25) and is represented in Fig. 2. The structure of the DNA of λ p*ilv-lac*3 has also been reported previously (15). It carries a fusion of the *ilv* and *lac* genes that had placed the *lac* genes under *ilv* control. However, the phage carried *ilv* DNA extending only into but not through the *ilvG* gene. It carries the *Kpn*I restriction site in *ilvG* and thus allows construction of a plasmid carrying the *lac* region fused to DNA containing the beginning of the *ilv* gene cluster. Accordingly, *Kpn*I digests of the phage and plasmid DNA were mixed, ligated, and used to transform strain CU838. The transformed cells were selected on L-agar plates containing 50 μg of ampicillin and 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) per ml. Blue colonies were selected, and the plasmid DNA they carried was examined by electrophoresis of a *Sal*I digest. One plasmid, designated pPU35, was shown to contain a 9.1-kilobase (kb) *Sal*I fragment, as did the original pPU18 plasmid, and was inferred to have the *Kpn*I fragment from λ p*ilv-lac*3 inserted in the correct orientation. The 9.1-kb fragment was missing from a second plasmid, designated pPU36, and was inferred to have received the *Kpn*I fragment in the incorrect orientation (Fig. 2).

The construction of pPU34. The same procedure was used to prepare pPU34 from a derivative of pBR313 (pPU5) which also carries the *ilv* gene cluster, except that after cleavage of pPU5 with *Kpn*I, the digest was treated with T4 DNA ligase to yield a shortened plasmid, pPU20, which then lacked the portion of the *ilv* gene cluster flanked by *Kpn*I restriction sites. pPU20 was then opened by *Kpn*I digestion, and the *Kpn*I fragment of λ p*ilv-lac*3 containing the *ilv-lac* fusion was inserted to yield pPU34. An additional difference between pPU34 and pPU35 is that pPU34 contains the *ilv* genes in an orientation such that their transcription occurs in the same direction as the *amp* genes of the plasmid, and pPU35 contains them in the opposite orientation.

RNA polymerase binding studies. The heparin-resistant binding of RNA polymerase to restriction fragments was studied by a modification of the procedures described by Jones and Reznikoff (11). Elution of the fragments from nitrocellulose filters was done by incubating in 0.2 to 0.3 ml of eluting buffer (0.02 M Tris-hydrochloride [pH 8.0] and 0.2% sodium dodecyl sulfate). Sodium dodecyl sulfate was removed by chilling in an ice bath and centrifuging. A portion of the eluate was lyophilized, dissolved in water, and analyzed by electrophoresis on 1% agarose or 5% polyacrylamide gel.

Enzymes and chemicals. All of the restriction enzymes, the T4 DNA ligase, and the RNA polymerase were obtained from New England BioLabs. Heparin was obtained from Sigma Chemical Co. All other chemicals were analytical grade from commercial sources.

RESULTS

Insertion of Tn*10* into a plasmid-borne *ilvG* gene. The procedure chosen to examine the polar effect of an insertion in the *ilvG* gene necessitated use of a cell that was wild type for the *ilv(G)EDA* operon and which carried a plasmid with the entire *ilv* gene cluster. The strain was valine resistant owing to an *ilvO* mutation carried on the plasmid. Such a plasmid was prepared from pPU30, a derivative of RP4 that lacked tetracycline resistance and carried the entire *ilv* gene cluster on a *Bam*HI fragment derived from λ *h80 dilv*. The valine resistance mutation was readily selected in the plasmid *ilvO* gene in a background lacking nearly the entire *ilv* gene cluster on the host chromosome. The plasmid in the valine-resistant strain selected, CU1175, was shown to confer valine resistance upon conjugal transfer to other K-12 hosts and was designated pPU31. The lesion it carried was designated *ilvO2211*.

The *ilvO* plasmid pPU31 was transferred from strain CU1175 by conjugation to strain CU1169, which carried the *ilvC7* marker and Tn*10* in the

TABLE 1. *Bacterial strains used in this study*

Strain	Genotype	Source or reference
CSH52	F⁻ *ara* Δ(*pro-lac*) *thi* λ⁻	Cold Spring Harbor Laboratory
CU344	F⁻ Δ(*ilvDAC*)*115 galT12* λ⁻	Kline et al. (12)
CU505	F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12* λ⁻	Watson et al. (28)
CU519	F⁻ Δ(*ilvDAC*)*115 metE201 leu-455 galT12* λ⁻	Smith et al. (22)
CU558	F⁻ *rbs-221 metE201 leu-455 rpsL galT12* λ⁻	Smith and Umbarger (24)
CU562	F⁻ *ilvA454 arg trp* Δ*lac* λ⁻	Wild et al. (29)
CU713	F⁻ *ilvC2209*::-ΔMu-::λpl (209) *ara* Δ(*pro-lac*) *thi* λ⁻	Watson et al. (28)
CU723	F⁻ *ilvC285 ara* Δ(*pro-lac*) *thi* λ⁻	Watson et al. (28)
CU735	F⁻ *ilvC7 ara* Δ(*pro-lac*) *thi* λ⁻	Smith et al. (22)
CU838	F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Watson et al. (28)
CU925	F⁻ Δ(*ilvGE*)*2130 ara* Δ(*pro-lac*) *thi* λ⁻	Gayda et al. (9)
CU973	pPU29/F⁻ *ilvC285 ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CU723 with pPU29, by M. D. Watson
CU974	pPU29/F⁻ *ilvA454 arg trp* Δ*lac* λ⁻	Conjugal transfer of pPU29 to CU562 with CU973 as donor, by M. D. Watson
CU1112	F⁻ *ilvC44 rbs-221 araC leu-455 galT12* λ⁻	Watson et al. (28)
CU1151	F⁻ Δ(*ilvGE*)*2130 araC leu-455 thi* λ⁻	P1-mediated transduction of CU925 with CU1112 as donor; selection made for L-arabinose utilization in the presence of D-fucose
CU1152	pPU34/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CU838 with pPU34
CU1153	pPU35/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CU838 with pPU35
CU1154	pPU36/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CU838 with pPU36
CU1155	pPU36/F⁻ *ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CSH52 with pPU36
CU1156	pPU34/F⁻ Δ(*ilvGE*)*2130 araC leu-455 thi* λ⁻	Transformation of CU1151 with pPU34
CU1157	pPU35/F⁻ Δ(*ilvGE*)*2130 araC leu-455 thi* λ⁻	Transformation of CU1151 with pPU35
CU1158	pPU36/F⁻ Δ(*ilvGE*)*2130 araC leu-455 thi* λ⁻	Transformation of CU1151 with pPU36
CU1159	pPU5/F⁻ Δ(*ilvGEDAC*)*2049 ara* Δ(*pro-lac*) *thi* λ⁻	Transformation of CU838 with pPU5
CU1160	pPU5/F⁻ Δ(*ilvGE*)*2130 araC leu-455 thi* λ⁻	Transformation of CU1151 with pPU5
CU1167	F⁻ *ilvC7 rbs-221 leu-455 rpsL galT12* λ⁻	P1-mediated transduction of CU558 with CU735 as donor, by Alan Biel
CU1168	F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56 srl-1300*::Tn*10* λ⁻	Conjugal transfer of Tn*10* to CU505 with NK5304 as donor, by Alan Biel
CU1169	F⁻ *ilvC7 rbs-221 leu-455 rpsL galT12 recA56 srl-1300* Tn*10* λ⁻	Conjugal transfer of Tn*10* to CU1167 with NK5304 as donor, by Alan Biel
CU1170	F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56* λ⁻	Spontaneous Srl⁺ Tc^{sa} derivative of CU1168, by Alan Biel
CU1171	F⁻ *ilvC7 rbs-221 leu-455 rpsL galT12 recA56* λ⁻	Spontaneous Srl⁺ Tc^{s} derivative of CU1169, by Alan Biel
CU1172	pPU30/F⁻ *ilvA454 arg trp* Δ*lac thi* λ⁻	D-Cycloserine selection of a derivative of CU974 that had become Tc^{s} but remained Ap^{r} Km^{r} Ilv^{+}, by Alan Biel
CU1173	pPU30/F⁻ *ilvC2209*::-ΔMU-::λpl(209) *ara* Δ(*pro-lac*) *thi* λ⁻	Conjugal transfer of pPU30 to CU713 with CU1172 as donor, by Alan Biel
CU1174	pPU30/F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56* λ⁻	Conjugal transfer of pPU30 to CU1170 with CU1173 as donor
CU1175	pPU31/F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56* λ⁻	Spontaneous valine-resistant derivative of CU1174
CU1176	pPU31/F⁻ *ilvC7 rbs-221 leu-455 galT12 rpsL recA56 srl-1300*::Tn*10* λ⁻	Conjugal transfer of pPU31 to CU1169 with CU1175 as donor
CU1177	pPU32/F⁻ *ilvC2209*::-ΔMu-::λpl(209) *ara* Δ(*pro-lac*) *thi* λ⁻	Valine-sensitive, Ilv^{+} Tc^{r} derivative of CU713 after conjugal transfer with CU1176 as donor
CU1178	pPU33/F⁻ *ilvC2209*::-ΔMU-::λpl(209) *ara* Δ(*pro-lac*) *thi* λ⁻	Independent derivative of CU713 isolated as above

TABLE 1—*Continued*

Strain	Genotype	Source or reference
CU1179	pPU32/F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56* λ⁻	Conjugal transfer of pPU32 to CU1170 with CU1177 as donor
CU1180	pPU33/F⁻ Δ(*ilvGEDAC*)*2049 leu-455 galT12 recA56* λ⁻	Conjugal transfer of pPU33 to CU1170 with CU1178 as donor
NK5304	Hfr PO45 *srl-1300*::Tn*10 recA56 ilv-318 thr-300 thi-1 rel-1 rpsE300*	Nancy Kleckner, Harvard University

[a] Tc[s], Sensitive to tetracycline; Tc[r], resistant to tetracycline; Ap, ampicillin; Km, kanamycin.

srl gene, to yield an Ilv⁺ valine-resistant derivative, strain CU1176. pPU31 was transferred by conjugation from this strain to strain CU713, which carried the *lac* genes inserted in *ilvC*. Selection was for both tetracycline resistance and an Ilv⁺ phenotype. Of 200 such exconjugants, 2 were shown to be valine sensitive. The plasmids they carried were both shown to carry the tetracycline resistance marker and conferred an Ilv⁺ phenotype onto other *ilvC* strains but no longer carried valine resistance. These plasmids, pPU32 and pPU33, were presumed to carry Tn*10* in the *ilvG* gene and were saved for further study.

Physical characterization of pPU32 and pPU33. Plasmid DNA from the new presumed *ilvG*::Tn*10* plasmids was purified and compared with that of the parental plasmid, after digestion with *Hin*dIII and *Sal*I. Figure 3 shows the electrophoretically separated *Hin*dIII fragments of pPU31, pPU32, and pPU18. Of particular interest is the 4.5-kb fragment in pPU31 and pPU18 that appeared to be identical with that carried by pPU15, previously shown to carry the *ilvO* determinant and *ilvG* (25). This fragment was missing in plasmid pPU32. In its place were three additional *Hin*dIII fragments (and a fourth of about 0.5 kb which migrated off the gel), which were presumably derived from cleavage at the three unique *Hin*dIII sites of Tn*10* which had been inserted into the 4.5-kb *Hin*dIII region. The electrophoretic pattern of pPU33 (not shown) appeared to be identical to that of pPU32. Because the three new fragments appeared to be the same size in the two plasmids, it is likely that Tn*10* was inserted in the 4.5-kb

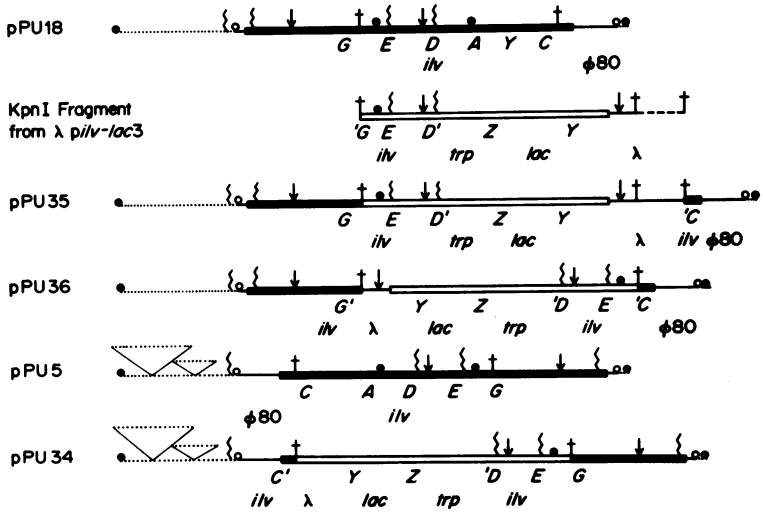

FIG. 2. *Construction of pJN101, pJN102, and pJN103. The ilv DNA between the two KpnI sites of pGMM103 was removed and replaced by a KpnI fragment obtained from λ pilv-lac3. The same was done with pGMM101, except that a shortened plasmid pJN6 (see Table 2) was isolated as an intermediate. pJN6 was then reopened by KpnI, and the insertion was made. Solid bars, Bacterial DNA originally cloned in pGMM103 or pGMM101. Open bars, Bacterial DNA carried by gl pilv-lac3. Solid line, Phage DNA. Dotted line, pBR322 or pBR313 (vector) DNA. Representations of pBR313 and pBR322 DNA are aligned with respect to their homologous regions. The location of nonhomologous regions was suggested by electron micrographs of heteroduplexes prepared by T. D. Leathers. Broken line, Additional λ DNA resulting from incomplete digestion of λ pilv-lac3 by KpnI (inserted in pJN102 but not in pJN103). Symbols: ⦚, HindIII; ●, SalI; ○, BamHI; ↓, SmaI; †, KpnI.*

TABLE 2. *Plasmids used in this study*

Plasmid	Genotype[a]	Description	Source
pPU5	pBR313Ω5[0.375kb:λ *h80* d*ilv* *ilvGOEDAYC* −1.5–11.6kb(−)]	Carries the entire *ilv* cluster from λ *h80* d*ilv* in the *Bam*HI site of pBR313	Formerly pGMM101, McCorkle et al. (18), Subrahmanyam et al. (25)
pPU15	pBR322Ω3[0.029kb:λ *h80* d*ilvO2200* *ilvE2201* *ilvGOE'* 6.4–11.0kb(−)]	Carries the valine resistance determinant and a functional *ilvG* gene in the *Hin*dIII site of pBR313	Formerly pGMM7, Subrahmanyam et al. (25)
pPU18	pBR322Ω7[0.375kb:λ *h80* d*ilvO2200* *ilvE2201* *ilvGOEDAYC* −1.5–11.6kb(−)]	Carries the entire *ilv* cluster from λ *h80* d*ilvO2200* *ilvE2201* in the *Bam*HI site of pBR322	Formerly pGMM103, Subrahmanyam et al. (25)
pPU20	pBR313Ω5[0.375kb:λ *h80* d*ilv* *ilvGOEDAYC* −1.5–11.6kb(−)] Δ1[*ilv'GOEDYAC'* 0.55–7.32kb]	A derivative of pPU5 from which a 6.77-kb *Kpn*I fragment was removed	Formerly pJN6, Subrahmanyam et al. (25)
pPU29	RP4Ω2[6.8kb:λ *h80* d*ilv* *ilvGOEDAYC* −1.5–11.6kb(−)]	The *ilv*-containing *Bam*HI fragment from pPU5 in the *Bam*HI site of RP4	Mixed *Bam*HI digests of pGMM101 and RP4 treated with T4 ligase by M. D. Watson
pPU30	RP4*tet*⁻Ω2[6.8kb:λ *h80* d*ilv* *ilvGOEDAYC* −1.5–11.6kb(−)]	A derivative of pPU29 no longer giving Tc[r] phenotype[b]	See CU1172, Table 1
pPU31	RP4*tet*⁻Ω2[6.8kb:λ *h80* d*ilv* *ilvGO2211EDAYC* −1.5–11.6kb(−)]	A valine-resistant derivative of pPU30	See CU1175, Table 1
pPU32	RP4*tet*⁻Ω2[6.8kb:λ *h80* d*ilv* *ilvG2212*::Tn*10O2211-EDAYC* −1.5–11.6kb(−)]	A Tc[r] Ilv⁻ derivative of pPU31	See CU1177, Table 1
pPU33	RP4*tet*⁻Ω2[6.8kb:λ *h80* d*ilv* *ilvG2213*::Tn*10O2211-EDAYC* −1.5–11.6kb(−)]	A Tc[r] Ilv⁻ derivative of pPU31	See CU1178, Table 1
pPU34	pBR313Ω5[0.375kb:λ *h80* d*ilv* *ilvGOEDAYC* −1.5–11.6kb(−)]Δ1[*ilv'GOEDYAC'* 0.55–7.32kb]Ω7[2.42kb:λ p*ilv-lac*3 *ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–17.28(λ) kb(−)]	*Kpn*I fragment with *ilv-lac* fusion in place of *Kpn*I fragment of pPU5	Digestion of pPU20 with *Kpn*I followed by ligation in presence of a *Kpn*I digest of λ p*ilv-lac*3. The ligated mixture used to transform CU838, CU1152 was a transformant growing on lactose-minimal agar containing ampicillin and the keto acid precursors of isoleucine and valine.
pPU35	pBR322Ω7[0.375kb:λ *h80* d*ilv* *ilvO2200* *ilvE2201* *ilvGOEDAYC* −1.5–11.6kb(+)] Δ9[*ilv'GOEDAYC'* 0.55–7.34kb]Ω15[4.7kb:λ p*ilv-lac*3 *ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–17.28(λ)kb(+)]	*Kpn*I fragment with *ilv-lac* fusion in place of *Kpn*I fragment of pPU18	*Kpn*I fragment from λ p*ilv-lac*3 ligated with a *Kpn*I digest of pPU18. Selection in CU1153 as for pPU34.
pPU36	pBR322Ω7[0.375kb:λ *h80* d*ilvO2200* *ilvE2201* *ilvGOEDAYC* −1.5–11.6 kb(+)]Δ10[*ilv'GOEDAYC'* 0.55–7.34kb]Ω16[4.7kb:λ p*ilv-lac*3 *ilv'GOED' trp'AB' lac'ZoZY* λ' 7.34(K-12)–18.96(λ)kb(−)]	Like pPU35 except *ilv-lac* fragment in opposite orientation	Prepared as pPU35; selection in CU1154

TABLE 2—*Continued*

Plasmid	Genotype[a]	Description	Source
RP4	*bla*$^+$ (−Tem-2) *tet*$^+$ *aph*$^+$	Wild-type, broad host range, conjugative plasmid	Datta et al. (5), DePicker et al. (7), Jacob et al. (10)

[a] All coordinates are given in kb. The chromosomal regions near the *ilv* genes have been given coordinates based upon a zero point at the site of the φ80-*ilv* DNA junction in λ *h80* d*ilv* (almost precisely at the terminus of the *ilvC* gene) (18). Positive coordinates are to the *rbs* side of *ilvC*, and negative coordinates are to the *metE* side of *ilvC* (or in the left arm of φ80). (+) or (−) indicates that the orientation of the plasmid and bacterial genes with respect to each other is the same as or opposite to, respectively, the way it is usually represented on their genetic maps. pBR322 coordinates are from Sutcliffe (26); those for pBR313 are from Jacob et al. (10). The λ coordinate given is based on a zero point at the left arm terminus. The conventions used follow the proposal of Novick et al. (20). Thus, for the complex genotype of pPU34 a 13.1-kb *Bam*HI fragment of λ *h80* d*ilv* extending from 1.5 kb into the left arm of φ80 through the entire *ilv* gene cluster to a point designated 11.6 kb on the physical map shown in Fig. 1 was inserted into the *Bam*HI site of pBR313 (see pPU5). The minus sign (−) indicates that the inserted *ilv* genes are in an orientation opposite to that of the pBR313 genes with respect to the way those genes are represented on their corresponding genetic maps. The portion of the resulting plasmid between the two *Kpn*I sites at *ilv* coordinates 0.55 and 7.32 kb on the physical map of Fig. 1 was deleted. In its place was inserted a fragment derived from λ p*ilv-lac*3 (14) extending from the *Kpn*I site at 7.34 kb on the K-12 *ilv* map of Fig. 1 to a *Kpn*I site at 17.28 kb on the left arm of λ. The orientation of the second insert is also opposite to pBR313 but the same as that of the *ilv* genes in the first insert. Thus, the *ilv* DNA upstream of the *ilv-lac* fusion is like that in the chromosome. In contrast, *ilvE* and *ilvG* are no longer contiguous in pPU36. pPU34, pPU35, and pPU36 are diagrammed in Fig. 2.

[b] Tc^r, Resistant to tetracycline.

*Hin*dIII region and, therefore, in the *ilvG* gene, at the same or very nearly the same site. This conclusion was further strengthened by the finding that TN*10* was not inserted in the *Sal*I fragment spanning the *ilvE*, *ilvD* and *ilvA* genes (data not shown).

Effect of a Tn*10* insertion in *ilvG* on gene expression in the *ilv* gene cluster. The two *ilvG*::Tn*10* plasmids were transferred to a *recA* strain carrying the *ilvGEDAC2049* deletion. The two strains, CU1179 and CU1180, grew only very slowly on isoleucine- and valine-free medium, indicating a very low expression of the *ilvE*, *ilvd*, and *ilvA* genes. Both strains were grown under both repressing and limiting isoleucine conditions, and extracts were prepared. The activities of several isoleucine and valine biosynthetic enzymes were determined and compared with the activities in an extract from the *ilvO* parent (CU1175) and in the strain carrying the plasmid with a wild-type *Bam*HI fragment (CU1174). Since strain CU1175 was resistant to valine, an isoleucine limitation could not be obtained, but the cells were grown in minimal medium plus valine.

Table 3 shows that strain CU1175 with an

TABLE 3. *Effect of an ilvO mutation and an ilvG Tn10 insertion on expression of the ilv operon*

Strain	*ilv* genotype of plasmid	Growth conditions	Sp act[a]				
			Acetohydroxy acid synthase		Trans-aminase B	Dihy-droxy acid de-hydrase	Threo-nine de-aminase
			No va-line	Valine			
CU1174	*ilv*$^+$	Repressing[b]	2.6	0.3	20.2	23.6	102
		Limiting isoleucine[c]	4.8	0.4	31.7	52.1	226
CU1175	*ilvO2211*	Repressing	10.8	6.2	28.1	36	141
		Limiting isoleucine	26.0	16.7	41	80	497
CU1179	*ilvO2211*	Repressing	5.5	0.5	3.2	3.4	22.4
	ilvG2212::Tn*10*	Limiting isoleucine	2.3	0.4	4.8	ND[d]	36.0
CU1180	*ilvO2211*	Repressing	2.0	0.6	4.2	ND	10.8
	ilvG2213::Tn*10*	Limiting isoleucine	2.2	0.4	3.6	ND	8.0

[a] Expressed as nanomoles per minute per milligram of protein.

[b] Medium contained 0.4 mM L-leucine, 0.4 mM L-isoleucine, and 0.8 mM L-valine.

[c] Repressed cells incubated in medium with 0.4 mM L-leucine, 0.8 mM L-valine, and 0.05 mM L-isoleucine.

[d] ND, Not detected.

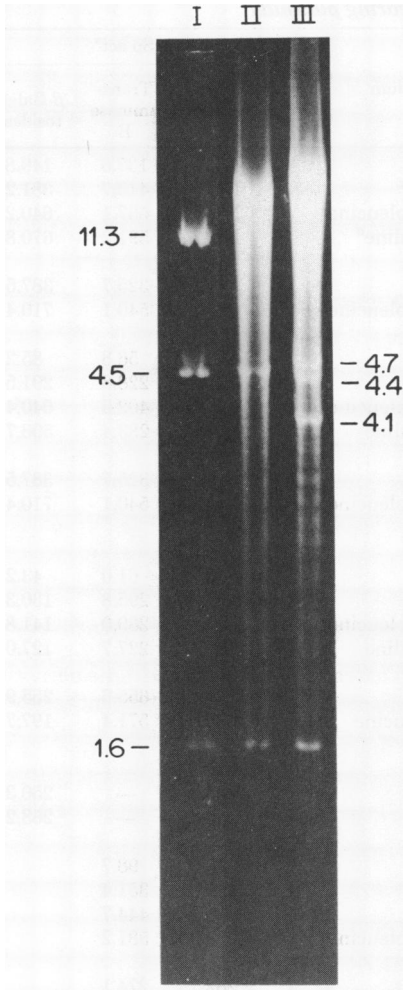

FIG. 3. *Effect of Tn10 insertion on the HindIII cleavage pattern of an ilv-bearing plasmid. pGMM103 (lane I), pCS21 (lane II), pCS22 (lane III). Numbers indicate fragment size in kilobases.*

ilvO mutation carried on the plasmid yielded higher activities of the *ilvE*, *ilvD*, and *ilvA* gene products than did strain CU1174 and, in addition, yielded a valine-resistant acetohydroxy acid synthase. In contrast, the two strains with plasmids carrying Tn*10* in the *ilvG* gene yielded very low activities of the *ilvE*, *ilvD*, and *ilvA* gene products and no evidence of a valine-resistant acetohydroxy synthase.

Restriction enzyme analysis of pPU34, pPU35, and pPU36. Previous studies from this laboratory had shown that *ilv*-specific control was exerted on the *ilv* genes in a plasmid carrying the entire *ilv* cluster (25). Phages that carried the *lac* genes fused to *ilv* DNA that extended through the *ilvO* region but not through the *ilvG* region did not exhibit *ilv*-specific control over β-galactosidase (9). However, if the *ilvO* region was deleted and *ilv* DNA extending beyond *ilvG* was included in the *ilv-lac* fusion phages, *ilv*-specific control over β-galactosidase formation was exhibited. That there was an *ilv*-specific control site before the *ilvG* gene thus seemed clear, but whether that control was independent of the *ilvO* region and independent of all *ilv* DNA downstream of the fusion point was not clear. For this reason, it was important to examine the control of β-galactosidase on a plasmid in which the *lac* genome was fused to *ilv* DNA in which *ilvE*, *ilvO*, *ilvG*, and the control region were contiguous. Plasmids pPU34, pPU35, and pPU36 were thought to be such plasmids and were therefore examined physically.

DNA from all three plasmids was digested separately with *Kpn*I, *Hin*dIII, *Sal*I, *Sma*I, and *Bam*HI endonucleases. Figure 2 shows the cleavage sites of these three plasmids. The detailed analysis verified the fact that the orientation of the *Kpn*I fragment in pPU34 and pPU35 was correct and that in pPU36 was incorrect. It also revealed that the fragment inserted into pPU35 had been cleaved at a different *Kpn*I site downstream from the *lac* gene from that at which the fragments inserted into pPU34 and pPU36 had been cleaved. Both *Kpn* sites had been recognized in an earlier study (15). Figure 2 also shows the structures of the initial plasmid pPU18, pPU5, and of the *ilv-lac* fragment(s) excised from λ p*ilv-lac*3 as previously determined (15, 25). This analysis demonstrated that both pPU34 and pPU35 were the desired plasmids, and that pPU36 would provide a suitable control.

In vivo analysis of gene expression in pPU34, pPU35, and pPU36. The plasmids containing the *ilv-lac* fusion inserted were used to transform strains CU838 [Δ(*ilvGEDAC*)*2049*] and CU1151 [Δ(*ilvGE*)*2130 leu*]. Transformants of the former strain by any of the plasmids were able to grow on the α-keto acid analogs of isoleucine and valine but were still Ilv^-. Transformants of strain CU1151 were Ilv^+ but remained Leu^-. Table 4 shows that growth of strains CU1152 and CU1153 (containing pPU34 and pPU35, respectively) under limiting isoleucine or limiting valine conditions resulted in derepression of both transaminase B and β-galactosidase over the activities of the two enzymes in cells grown with excess branched-chain amino acids (repressing medium). The same plasmids exhibited derepression with limiting leucine (strains CU1156 and CU1157). In contrast, strains containing pPU36 did not exhibit de-

TABLE 4. *Enzyme levels in strains bearing plasmids*

Strain [host genotype]	Plasmid (*ilv* genotype)	Medium	Sp act[a]		
			Threonine deaminase	Transaminase B	β-Galactosidase
CU1152 [Δ(*ilvGEDAC*)*2049*]	pPU34 (*ilvGED' lac'ZoZY ilv'C*)	L-broth	ND[b]	137.6	149.8
		Repressing[c]	ND	413.7	381.2
		Limiting isoleucine[c]	ND	637.1	640.2
		Limiting valine[d]	ND	591.2	670.8
CU1156 [Δ(*ilvGE*)*2130 leu*]	pPU34 (*ilvGED' lac'ZoZY ilv'C*)	Repressing	—[e]	325.7	387.5
		Limiting isoleucine	—	540.1	710.4
CU1153 [Δ(*ilvGEDAC*)*2049*]	pPU35 (*ilvGED' lac'ZoZY ilv'C*)	L-broth	ND	56.8	85.2
		Repressing	ND	228.8	291.5
		Limiting isoleucine	ND	402.5	640.4
		Limiting valine	ND	283.4	598.7
CU1157 [Δ(*ilvGE*)*2130 leu*]	pPU35 (*ilvGED' lac'ZoZY ilv'C*)	Repressing	—	325.7	387.5
		Limiting isoleucine	—	540.1	710.4
CU1154 [Δ(*ilvGEDAC*)*2049*]	pPU36 (*ilvG' lacYZZo' ilv'DEG' 'C*)	L-broth	ND	61.0	43.2
		Repressing	ND	295.8	130.3
		Limiting isoleucine	ND	289.0	141.8
		Limiting valine	ND	227.7	127.0
CU1158 [Δ(*ilvGE*)*2130 leu*]	pPU36 (*ilvG' lacYZZo' ilvDEG' 'C*)	Repressing	101.4	655.6	255.9
		Limiting leucine	333.3	571.4	197.7
CU1155 [*ilv*$^+$]	pPU36 (*ilvG' lacYZZo' ilvDEG' 'C*)	Repressing	35.2	—	250.3
		Minimal	58.3	—	268.2
CU1159 [Δ(*ilvGEDAC*)*2049*]	pPU5 (*ilv*$^+$)	L-broth	75.6	98.7	
		Repressing	410.8	351.4	
		Minimal	516.4	444.7	
		Limiting isoleucine	1,631.3	581.2	
CU1160 [Δ(*ilvGE*)*2130 leu*]	pPU5 (*ilv*$^+$)	Repressing	282.7	274.1	
		Limiting leucine	544.3	419.2	

[a] Expressed as nanomoles per minute per milligram of protein.
[b] ND, Not detected.
[c] See footnote *b* of Table 3.
[d] Repressed cells incubated for 3 h in medium with 0.4 mM L-leucine, 0.4 mM L-isoleucine, 0.1 mM L-valine.
[e] —, Not determined.
[f] Repressed cells incubated for 3 h in medium with 0.4 mM L-isoleucine, 0.8 mM L-valine, and 0.05 mM L-leucine.

repression when any of the three branched-chain amino acids was limiting.

It is clear from Table 4 that the derepression achieved with pPU34 and pPU35 was not very high. That this is probably due to the fact that these are high-copy-number plasmids is indicated by examination of plasmid pPU5, which contains the entire wild-type *ilv* region. The derepression of transaminase B in strains CU1159 and CU1160 is comparable to that observed with the strains carrying pPU34 and pPU35. Plasmid pPU5 with pBR313 as the vector was chosen for this control experiment, since the other starting plasmid, pPU18, contains an *ilvO* mutation which had been replaced with *ilvO*$^+$ in the construction of pPU35 (25).

The experiments show 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 contrast, a comparison of the effects of L-broth on strains bearing the three types of plasmids (CU1153, CU1154, and CU1159) reveals that all three plasmids respond

to the strong metabolic depression of synthesis of the isoleucine- and valine-forming enzymes observed in strains with normal *ilv* regions (9). Thus, this metabolic depression is not a result of *ilv*-specific regulation.

RNA polymerase binding studies. Plasmid pPU15 was shown earlier to carry the 4.5-kb *Hin*dIII fragment of *ilv* DNA, which contains the entire *ilvG* gene as well as the beginning of the *ilvE* gene (25). Since the fragment contained an *ilvO* mutation, it was possible to show that it contained the *ilv*-specific transcription initiation site for the *ilvG* gene. It was furthermore shown that the *ilv* control site and a functional *ilvG* gene were contained in the 2.5-kb length of DNA between the *Sal*I site and the *Pvu*II sites distal to it (Fig. 4). It would thus be expected that a strong RNA polymerase binding site would be carried on the *Pvu*II fragment derived from the *ilv* DNA insert in the pPU15.

Heparin-resistant polymerase binding was examined by incubating a *Hin*dIII-*Pvu*II double digest of pPU15 with various amounts of RNA polymerase for 20 min at 37°C and treating with heparin for 10 min. The incubation mixtures were passed through nitrocellulose filters, and the fragments retained on the filter by polymerase binding were eluted as described above and separated by electrophoresis on agarose gels. Figure 5 shows the fragments that were retained on the filters after incubation with various amounts of RNA polymerase. The strongest binding site was that on the 2.3-kb *Hin*dIII-*Pvu*II fragment (Fig. 5A) containing the *amp* promoter of the vector. As the RNA polymerase/pPU15 molar ratio approached 1.0, binding was observed to the 1.7-kb *Pvu*II fragment (Fig. 5C) thought to contain the *ilvG* promoter. Still weaker was binding to the 2.0-kb *Hin*dIII-*Pvu*II fragment (Fig. 5B) of the vector. This weak binding may have been due to binding at part of the *tet* promoter which is cleaved by *Hin*dIII. Another fragment showing weak binding was the 1.6-kb *Hin*dIII-*Pvu*II fragment (Fig. 5D) of

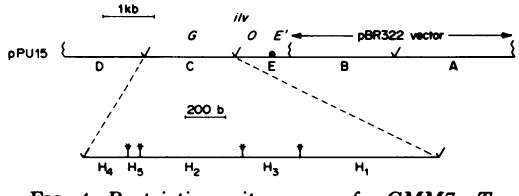

FIG. 4. *Restriction site map of pGMM7. Top, pGMM7 approximate location of genetic loci indicated above. Letters below indicate fragment derived by doubly digesting with HindIII and PvuII. Bottom, detail of HinfI digestion of PvuII fragment C. H_1–H_5, HinfI fragments. Symbols: ⌇, HindIII; √, PvuII; ●, SalI; ↑, HinfI.*

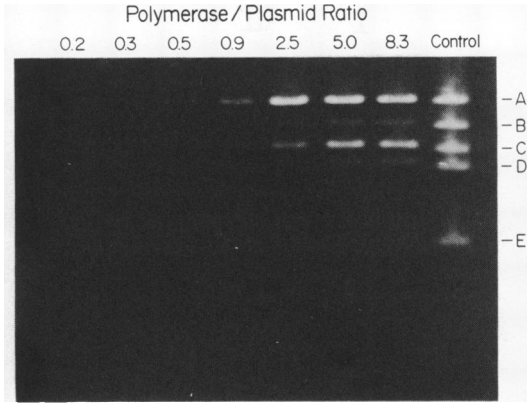

FIG. 5. *Analysis of restriction fragments bearing RNA polymerase binding sites. pGMM7 was digested with HindIII and PvuII. The mixture was incubated with RNA polymerase at various polymerase/DNA ratios. Reaction mixtures were filtered through nitrocellulose filters, and the fragments retained on the filter were analyzed by agarose electrophoresis. Control refers to an equal amount of restriction digest analyzed directly (see text for details). Fragment designations are as in Fig. 4.*

the insert that lies upstream of the presumed *ilvG* promoter.

Of some additional interest is the fact that the 1.1-kb *Pvu*II-*Hin*dIII fragment (Fig. 5E) that contains all or part of the *ilvO* portion of *ilvG* exhibited detectable RNA polymerase binding only at the highest concentration of RNA polymerase used. Since this fragment would be expected to bind polymerase if there were a functional *ilvEDA* operon, the possibility of an *ilv*-controlled promoter proximal to *ilvE* seems unlikely. In other experiments (not shown), the effect of a higher KCl concentration (0.15 M) was examined, as was the effect of including three and the four nucleotide triphosphates in the incubation mixture. Neither modification appeared to make polymerase binding to the 1.1-kb fragment any stronger.

The strong promoter on the 1.7-kb *Pvu*II fragment was further localized by cleaving the purified fragment with *Hin*fI. When binding studies were performed with these fragments, only the 0.5-kb fragment (H_2) was bound by the polymerase (Fig. 6).

DISCUSSION

Several kinds of experimental evidence reported from this laboratory have pointed to the possibility that transcription of the *ilvE*, *ilvD*, and *ilvA* genes in *E. coli* was not initiated from a promoter immediately preceding the *ilvE* gene but from a promoter that preceded the "silent" *ilvG* gene. This evidence included the finding

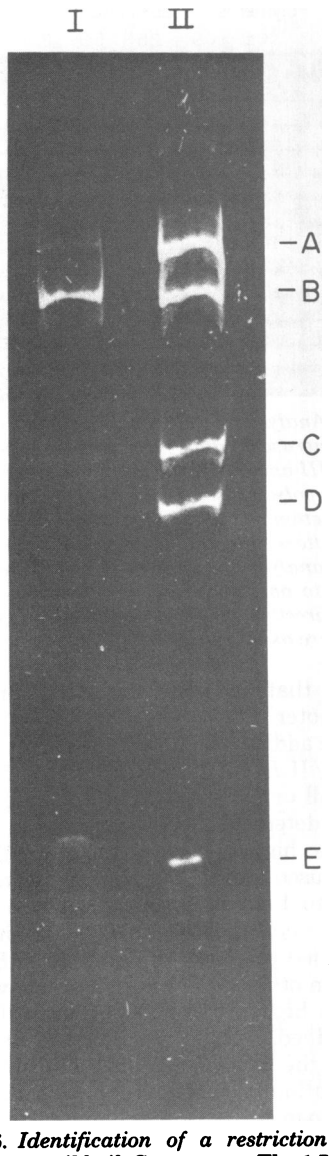

FIG. 6. *Identification of a restriction fragment bearing a possible ilvG promoter. The 1.7-kb PvuIII fragment isolated from pGMM7 (fragment C in Fig. 4) was digested with HinfI. After incubation, heparin-resistant retention of DNA fragments in RNA polymerase was analyzed on a 5% acrylamide gel. I, Polymerase-bound fragment. II, Control mixture. Letters A through E refer to H_1 through H_5, respectively, in Fig. 4.*

that RNA hybridizable to *ilvG* was formed when the *ilvEDA* cluster was derepressed (25) and the finding that a phage carrying *ilv* DNA extending only to the region before *ilvE* (*ilvO*) did not exhibit *ilv*-specific control, whereas one carrying *ilv* DNA from a region preceding the *ilvG* gene did exhibit *ilv*-specific control (9). Furthermore, deletion of the *ilvO* region did not result in a loss of *ilv* control over *ilvEDA* expression but, rather, an enhanced level of *ilvEDA* expression (9). Thus the possibility was raised that *ilvO* might be a partially polar mutation that prevented *ilvG* expression and reduced *ilvEDA* expression (9). Thus, *ilvO* mutations, which led to valine resistance, were viewed as mutations that abolished this polarity and restored *ilvG* expression. In this study, it has been shown that an insertion element in *ilvG* of an *ilvO* plasmid results in loss of *ilvG* function and a very strong polar effect on the *ilvE*, *ilvD*, and *ilvA* genes. That the insertion element was in the *ilvG* gene was demonstrated by the fact that it had been inserted onto the 4.5-kb *Hin*dIII fragment shown earlier to carry both the *ilvG* gene and the *ilvO* determinant (25).

It is interesting that the very low level of *ilvEDA* expression observed with the *ilv* plasmid carrying the Tn*10* insertion was not affected by an *ilv*-specific control. In contrast, the expression in *ilvO*$^+$ cells is considerably higher and responds to *ilv* control. The unregulated escape from absolute polarity by the Tn*10* insertion is probably dependent upon a rather weak promoter that precedes the *ilvE* gene but which is not related to *ilv*-controlled expression.

Physical identification of both the strong promoter thought to be the site of *ilv*-specific transcription and the weak, unregulated promoter thought to account for the low level expression with the Tn*10*-containing plasmid may have been made in the RNA polymerase binding experiments. The fragment containing the region of the *ilvO* determinant and the beginning of the *ilvE* gene bound RNA polymerase only weakly. In contrast, the region preceding the *ilvG* gene bound RNA polymerase strongly. Only the fragment containing the *amp* promoter on the pPU15 plasmid bound polymerase more strongly. (The recent results of Blazey and Burns [3] imply that the promoter preceding the *ilvE* gene of *Salmonella typhimurium* is much stronger than that in *E. coli* K-12.)

In the earlier studies involving the *lac* genes fused to the *ilvD* gene, it was found that *ilv* control over β-galactosidase formation did not occur if the *ilv* DNA carried by the *ilv-lac* phage did not extend through the entire *ilvG* gene (9). There was *ilv* control if the region preceding the *ilvG* gene was present. However, in these studies, it was possible for the phage carrying the fusion to exhibit *ilv*-specific control only if there was a deletion that removed the *ilvO* region. Thus, one could not be certain that the *ilv*-specific control was, in fact, completely independent of the *ilvO* region. For this reason, the plasmids

constructed and used in this study were important. With these plasmids it could be shown that *ilv*-specific control was obtained when the region preceding *ilvG* was present with *ilvO*, but only if it was contiguous with *ilvO*. In other words, the region preceding *ilvG* is a *cis*-acting regulatory element. It was possible to use both kinds of plasmids in the presence and absence of the *ilvA* gene. No effect on gene expression was apparent that could be attributed to the presence or absence of the *ilvA* gene.

In addition to the *ilv*-specific, multivalent control of *ilv* gene expression, there is another control that appears to be independent of the *ilv*-specific control. This control is encountered most strikingly when a rich medium is employed and results in a level of enzyme activity much lower than that obtained with a supplement of the branched-chain amino acids (9). Since this control was exhibited by plasmid pPU36 as well as by plasmids pPU35 and pPU34, it is clear that this reduction in gene expression, whether transcriptional or translational, is independent of the *ilv*-specific control site preceding *ilvG*.

These studies and other studies from this laboratory make it seem quite likely that the control site for the multivalent control of the *ilvE*, *ilvD*, and *ilvA* genes lies before the *ilvG* gene, and that the operon is an *ilvGEDA* operon. That this multivalent control is one involving attenuation of transcription affected, in turn, by the translation of a leader transcript is supported by the nucleotide sequence studies recently reported by Nargang et al. (19) and by Lawther and Hatfield (13). The absence of an analogous control site in the region between *ilvE* and *ilvG* is also in accord with these results (14).

ACKNOWLEDGMENTS

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