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Molecular Cloning and Expression of the *ilvGEDAY* Genes from *Salmonella typhimurium*

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The *ilvGEDAY* genes of *Salmonella typhimurium* were cloned in *Escherichia coli* K-12 by in vitro recombination techniques. A single species of recombinant plasmid, designated pDU1, was obtained by selecting for Val^r Amp^r transformants of strain SK1592. pDU1 was shown to contain a 14-kilobase *Eco*RI partial digestion product of the *S. typhimurium* chromosome inserted into the *Eco*RI site of the pVH2124 cloning vector. The *ilvGEDAY* genes were found to occupy a maximum length of 7.5 kilobases. Restriction endonuclease analysis of the *S. typhimurium ilv* gene cluster provided another demonstration of the gene order as well as established the location of *ilvY* between *ilvA* and *ilvC*. The presence of a ribosomal ribonucleic acid operon on the pDU1 insert, about 3 kilobases from the 5′ end of *ilvG*, was shown by Southern hybridization. The expression of the *ilvGEDA* operon from pDU1 was found to be elevated, reflecting the increased gene dosage of the multicopy plasmid. A polarity was observed with respect to *ilvEDA* expression which is discussed in terms of the possible translational effects of the two internal promoter sequences, one located proximal to *ilvE* and the other located proximal to *ilvD*.

Eight genes which encode proteins involved in the biosynthesis of the amino acids L-isoleucine and L-valine have been identified on the chromosome of *Salmonella typhimurium*. Six of these genes are located in a cluster at approximately 83 min on the standard linkage map (32); five of these genes (*ilvGEDAC*) encode the enzymes α-acetohydroxy acid synthase II, transaminase B, dihydroxy acid dehydrase, L-threonine deaminase, and acetohydroxy acid isomeroreductase, respectively (40). The remaining gene in this cluster, *ilvY*, encodes a *trans*-active regulatory protein required for the expression of the *ilvC* gene (4). Two additional genes, *ilvB* and the structural gene for transaminase C, also participate in isoleucine-valine biosynthesis. *ilvB* encodes α-acetohydroxy acid synthase I which can be distinguished from the isozyme specified by *ilvG* because its activity is inhibited by L-valine; *ilvB* lies between *rbsP* and *pyrE* close to *oriC* on the *S. typhimurium* chromosome, probably at a position similar to the area defined for *ilvB* in the *Escherichia coli* K-12 chromosome (R. Weinberg and R. O. Burns, unpublished observation) (29). The structural gene for transaminase C remains unmapped. The role of transaminase C in valine biosynthesis is recognized in *ilvE* mutant strains which are isoleucine auxotrophs; the loss of α-ketoisovalerate-alanine amino-transferase activity in a transaminase B-deficient strains imparts an L-valine auxotrophy (D. Blazey, D. Primerano, and R. O. Burns, unpublished observation).

As a continuation of our general interest in branched-chain amino acid biosynthesis in *S. typhimurium* we recently initiated a study of the major *ilv* gene cluster. Analysis of transposon-generated *ilv* deletion mutations has established the structural gene order *ilvG-E-D-A-C* (3). The *ilvGEDA* and *ilvC* genes are separate transcriptional units. The expression of *ilvGEDA* is multivalently regulated by isoleucine, valine, and leucine (11). Umbarger and colleagues (H. E. Umbarger, personal communication) have shown by nucleotide sequence analysis that a sequence corresponding to an attenuator structure precedes the *S. typhimurium ilvG* gene. This observation strongly suggests that the principal mechanism of regulation of the *ilvGEDA* gene cluster, as in *E. coli* K-12, involves a translational control of transcription (18, 28). An additional interesting feature of the *ilvGEDA* gene cluster is the presence of two internal, apparently constitutively acting promoters, one directing synthesis of an *ilvEDA* transcript and another an *ilvDA* transcript (3). The expression of *ilvC* in *S. typhimurium*, as in the case of *E.*

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coli K-12, is induced by a mechanism which involves the α-acetohydroxy acid biosynthetic intermediates and the *trans*-active *ilvY* gene product (14, 42).

We have applied recombinant DNA techniques to further characterize the *ilv* gene cluster in *S. typhimurium*. We report here the molecular cloning and preliminary characterization of a fragment of the *S. typhimurium* chromosome that contains the *ilvGEDAY* genes as well as closely linked ribosomal RNA (rRNA) and tRNA structural genes. In addition, the expression of the cloned *ilvGEDA* operon and several subcloned fragments of the operon are examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are listed in Table 1. The primary cloning vehicle was pVH2124, a spontaneously occurring deletion derivative of RSF2124 (35). pVH2124 retains the ampicillin resistance (Amp^r) determinant and a unique *Eco*RI site in the colicin E1 structural gene, but it is about 3 kilobases (kb) smaller than RSF2124 (V. Burdett, personal communication). Purified unmodified pVH2124 was provided by V. Burdett and was then transformed into DU650 for plasmid preparation. Purified unmodified pBR322 (5) was a gift of D. Bastia and was also maintained in strain DU650.

Media. Most of the media used have been described previously (3). The primary selection medium for *S. typhimurium ilv* recombinant plasmids was agar (Difco Laboratories) with Davis-Mingioli salts without citrate (10) and supplemented with 1 mg of L-valine per ml, 20 μg of ampicillin per ml, 10 μg of thiamine hydrochloride per ml, and 0.5% glucose. Subsequent transformations for complementation analysis and subcloning of fragments were performed on nutrient agar (Difco) containing 20 μg of ampicillin per ml. A Davis-Mingioli medium was also used for plasmid preparation, modified by the omission of citrate and supplemented with 0.5% Casamino Acids (Difco), 20 μg of tryptophan per ml, 10 μg of thiamine hydrochloride per ml, 20 μg of ampicillin per ml, and 0.4% glucose.

Enzymes. All restriction endonucleases were purchased from either New England Biolabs or Bethesda Research Laboratories, except for *Hin*dIII which was the generous gift of D. Bastia. T4 DNA ligase and alkaline phosphatase were obtained from Bethesda Research Laboratories. BAL-31 nuclease was prepared from *Alteromonas espejiani* BAL-31 as described previously (12). S1 nuclease was from Miles Laboratories.

DNA isolation. High-molecular-weight chromo-

TABLE 1. *Strain list*

Strain	Genotype	Source or reference
S. typhimurium LT2		
LT2	F ilv^+	Laboratory collection
409	F $hsd_{LT}R$ $hsd_{LT}M$ $T7^s$	(6)
DU702	F⁻ *ilvA ilvY*	Laboratory collection[a]
SA722	Hfr *serA15 pur268*	K. E. Sanderson (34)
E. coli K-12		
SK1592	F ilv^+ *hsdR4* $hsdM^+$ *gal thi endA sbcB15*	S. Kushner via V. Burdett
JA199	F⁻ ilv^+ *leuB5 hsdR* $hsdM^+$ Δ*trpE5*	J. Carbon via V. Burdett
DU650	F Δ(*ilvEDAC*) *leuB5 hsdR* $hsdM^+$ Δ*trpE5*	From JA199[b]
DU650(pDU450)	F′ $ilvGEDAYC^+$	SA722 × DU650[c]
CU1126	F⁻ *ilvB2102 ilvI2202* Δ(*pro-lac*)	H. E. Umbarger
CU503	F⁻ *ilvE12*	H. E. Umbarger
CU534	F⁻ *ilvD2017*	H. E. Umbarger
DU1008	F⁻ *ilvA454*	H. E. Umbarger
CU390	F⁻ *ilvC*	H. E. Umbarger
CGSC4349	F′ lac^+(F42) *thr-31 leu-5 thi-1 his-4 pro-42 lac-4*	*E. coli* Genetic Stock Center
CGSC4349(pDU1)	F′ lac^+, pDU1	Transformation of CGSC4349 to Amp^r with pDU1

[a] DU702 is similar to the original *S. typhimurium ilvY* mutant, DU701 (4), except that DU702 was derived from a different *ilvA*::Tn*10* insertion strain, TT69 (obtained from J. Roth). The genotype of DU702 is *ilvA ilvY* tet^s as the result of a Tn*10*-generated chromosomal alteration.

[b] DU650 was constructed in this laboratory from JA199ilv^+ by exploiting the close linkage of *rbs* to *ilv*. A UV-induced *rbs* mutant of JA199ilv^+ was obtained by penicillin selection, and the mutant was then transduced to Rbs^+ with P1 phage grown on CU505 rbs^+ Δ(*ilvEDAC*) (from H. E. Umbarger). A single Rbs^+ Ilv^- transductant was purified, and the inheritance of the Δ(*ilvEDAC*) genotype was confirmed by enzyme assay.

[c] A 30-min mating between SA722 and DU650 Δ(*ilvEDAC*) was performed essentially as described previously (26), and Ilv^+ transconjugants were obtained. A number of these was characterized, and in each case the Ilv^+ phenotype was sensitive to curing by acridine orange and could be transferred at high frequency to appropriate recipients. The F′ $ilvGEDAYC^+$ factor pDU450 is prototypical of this class of F′ factors which also transfer *metE* but not *rbsP*. Since SA722 transfers chromosomal markers in the order O-*ilv-rbsP-pyrE- · · · -metE-cya* (33), pDU450 and its related F′ factors must be the result of type II excisions.

somal DNA was prepared from *S. typhimurium* LT2 as described previously (34). Plasmid DNA was isolated from chloramphenicol (180 μg/ml)-treated cultures (8), and cleared lysates were prepared by the method of Katz et al. (16). The DNA was concentrated by precipitation with polyethylene glycol (15) and purified by cesium chloride-ethidium bromide density gradient centrifugation. Plasmid DNA was then extracted with *n*-butanol to remove ethidium bromide and dialyzed first against 50 mM Tris-hydrochloride (pH 8.0)–10 mM EDTA and then against 10 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA.

A rapid procedure for partial purification of recombinant plasmids (2) was performed on 1-ml Luria broth cultures. The plasmid DNA prepared in this manner could be used for restriction analysis and transformation.

Construction and characterization of recombinant DNAs. All restriction reactions were run under the standard conditions suggested by the manufacturer. For the initial cloning of the *S. typhimurium ilv* gene cluster, *S. typhimurium* chromosomal DNA was partially digested with *Eco*RI. pVH2124 was digested to completion with *Eco*RI and then treated with alkaline phosphatase as described previously (39). The ligation of 1 μg of phosphatase-treated pVH2124 and 4 μg of partially *Eco*RI-digested *S. typhimurium* chromosomal DNA was carried out in 100 μl of buffer containing 66 mM Tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM ATP (43), and 0.25 U of T4 ligase. The ligation reaction was incubated at 12°C for 24 h before the DNA was used in transformation. The subcloning of fragments from the parental chimeric plasmid, pDU1, into pBR322 was achieved with identical ligation conditions, except that a vector/fragment molar ratio of 1:3 was used.

Nuclease treatment of recombinant plasmids. BAL-31 exonuclease digestion at the *Xho*I site of pDU1 was performed under the low-salt conditions of Legerski et al. (20) in 20 mM Tris-hydrochloride (pH 8.1)–12.5 mM $CaCl_2$–12.5 mM $MgSO_4$–0.2 M NaCl–1 mM EDTA. In 20 μl of this buffer, *Xho*I-digested, linear pDU1 (0.43 μg) was incubated with approximately 0.2 U of BAL-31 nuclease at 30°C for 10 min. The reaction was stopped by the addition of 1.8 μl of 0.25 M EDTA (pH 8.0). After dilution to 100 μl in 0.05 M Tris (pH 8.0)–10 mM EDTA–0.5 M NaCl, the nuclease-treated DNA was extracted three times with phenol and two times with diethyl ether and then precipitated with 2.5 volumes of ethanol. The DNA was collected by centrifugation in an Eppendorf microcentrifuge and resuspended in 10 μl of T4 ligase buffer. Blunt end ligation was effected by adding 0.5 U of T4 ligase and incubating at room temperature for 3 to 4 h.

S1-digestion of *Kpn*I- or *Xho*I-digested pDU1 was performed in 100 μl of buffer containing 30 mM sodium acetate (pH 4.5), 300 mM NaCl, 4.5 mM $ZnCl_2$ (41), 1 μg of linearized pDU1, and 300 U of S1 nuclease. After a 1.5-h incubation at room temperature, 15 μl of 1 M Tris (pH 8.5) was added, and the DNA was prepared for blunt end ligation as before.

The nuclease-treated DNAs were used to transform SK1592 to Amp^r. Rapid screening of Amp^r clones was performed to identify those containing derivatives of pDU1 resistant to *Kpn*I or *Xho*I endonucleases (2).

Gene transfer of recombinant DNAs. The *E. coli* K-12 strains were transformed by the method of Mandel and Higa (24), with minor modifications. The transformation frequency in the initial cloning experiment was determined by plating appropriate dilutions on nutrient agar containing 20 μg of ampicillin per ml. Since the insertion of extraneous DNA into the *Eco*RI site of pVH2124 causes inactivation of colicin E1 expression, the frequency of recombinant transformants was estimated by determining the fraction of Amp^r transformants that were unable to elaborate colicin E1 (35). Mobilization of pDU1 by F′ *lac* into *S. typhimurium* strains was effected by standard mating techniques (26). The method of Lederberg and Cohen (19) was used to transform *S. typhimurium* strains with pBR322-ilv^+ subclones of pDU1. The plasmids were first transferred into strain 409, a readily transformable intermediate host. Partially purified plasmid DNA from strain 409 could then be used to transform other *S. typhimurium* strains at improved frequencies.

Agarose gel electrophoresis. Electrophoresis was generally performed in a vertical slab gel apparatus with 0.8 or 1% agarose gels and the E buffer of Loening (23). Gels were stained for 30 min in E buffer containing 1 μg of ethidium bromide per ml. The DNA bands were visualized with short-wave UV light and photographed with Polaroid type 665 film and a Wratten no. 5 red filter. Molecular weights of DNA fragments were determined by comparison of mobilities with standard λ (30) or pBR322 (37) restriction fragments.

Enzyme assays. The isoleucine-valine biosynthetic enzymes were assayed as described previously (3).

Hybridization. Ribosomes were prepared from *S. typhimurium* LT2 by the method of Takanami (38). Purified intact ribosomes were then phenol extracted, and the rRNA was precipitated with ethanol and collected by centrifugation. The rRNA precipitate was dissolved in 0.01 M Tris-hydrochloride (pH 7.2) and used in the labeling procedure described below. For the preparation of bulk tRNA, *S. typhimurium* LT2 was grown to late exponential growth phase at 37°C as described by Littauer and Kornberg (22). The tRNA was isolated from these cells by the method of Zubay (44). The ethanol precipitate was dissolved in 0.01 M Tris-hydrochloride (pH 7.2) containing 0.1 mM dithiothreitol and loaded into a DEAE column which had been equilibrated with the same buffer. The column was washed with five volumes of the Tris-hydrochloride buffer, and the tRNA was eluted with 1.0 M NaCl in the same buffer. The tRNA was precipitated with ethanol from the fractions with peak absorbancy at 260 nm and collected by centrifugation. The precipitated bulk tRNA was dissolved in 0.01 M Tris-hydrochloride for labeling.

The RNAs were ^{32}P labeled with polynucleotide kinase by the method of Richardson (31). rRNA (0.4 mg) and tRNA (0.02 mg) each were reacted with 0.12 nmol of [γ-^{32}P]ATP with a specific activity of 4,200 Ci/mmol. The [^{32}P]RNAs were separated from the nucleotide on a G-50 Sephadex column and then concentrated by ethanol precipitation.

The RNA-DNA hybridization reaction was per-

formed as described by Southern (36). Autoradiograms were made with Kodak X-Omat R X-ray film.

RESULTS

Molecular cloning of the *ilv* region of the *S. typhimurium* chromosome. The selection for *S. typhimurium ilv* recombinant plasmids depended upon the cryptic nature of *ilvG* in *E. coli* K-12. The *ilvG* gene, encoding an acetohydroxy acid synthase that is insensitive to feedback inhibition by valine, is not expressed in wild-type *E. coli* K-12 (40). The other two acetohydroxy acid synthases, the products of the *ilvB* and *ilvHI* genes, are sensitive to valine inhibition. Thus, the cryptic nature of *ilvG* makes *E. coli* K-12 sensitive to growth inhibition by exogenous valine. The *ilvG* gene of *S. typhimurium*, however, is normally expressed, and the growth of the organism is not inhibited by valine. It has been shown that the expression of *ilvG* from *S. typhimurium* F′ *ilv*$^+$ plasmids maintained in *E. coli* K-12 confers complete resistance to valine inhibition (D. Blazey, R. Kim, and R. O. Burns, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, H53, p. 112). Therefore, *E. coli* K-12 transformants harboring *S. typhimurium ilvG*$^+$ recombinant plasmids should manifest a valine-resistant (Val^r) phenotype. Since the spontaneous mutation of *E. coli* K-12 to Val^r may occur as frequently as the transformation to *ilvG*$^+$, recombinant transformants were selected for simultaneous acquisition of Val^r and Amp^r, conferred by the cloning vector pVH2124.

The *E. coli* K-12 strain SK1592 was transformed with the ligation products of *Eco*RI-digested pVH2124 and *S. typhimurium* chromosomal DNA as described above. Six Val^r Amp^r clones were obtained by direct selection from a population of 3,000 recombinant transformants. Digestion with *Eco*RI of the partially purified plasmids from these clones gave only one restriction pattern, indicating that each plasmid contained the same *S. typhimurium* chromosomal insert. One recombinant plasmid, designated pDU1, was selected for further analysis.

Characterization of the *S. typhimurium ilv*$^+$-pVH2124 chimera. The purified plasmids pVH2124 and pDU1 were compared by agarose gel electrophoresis (Fig. 1). The larger pDU1 contains an insert of 14 kb which represents a partial *Eco*RI digestion product of the *S. typhimurium* chromosome. A single internal *Eco*RI site divides the insert into two fragments of 2.3 and 12 kb. The 8.7-kb fragment of pDU1 corresponds to *Eco*RI-linearized pVH2124 vector.

The genetic composition of the cloned *S. typhimurium* chromosomal fragment was determined by complementation analysis with a series of *E. coli* K-12 *ilv* mutants transformed to Amp^r

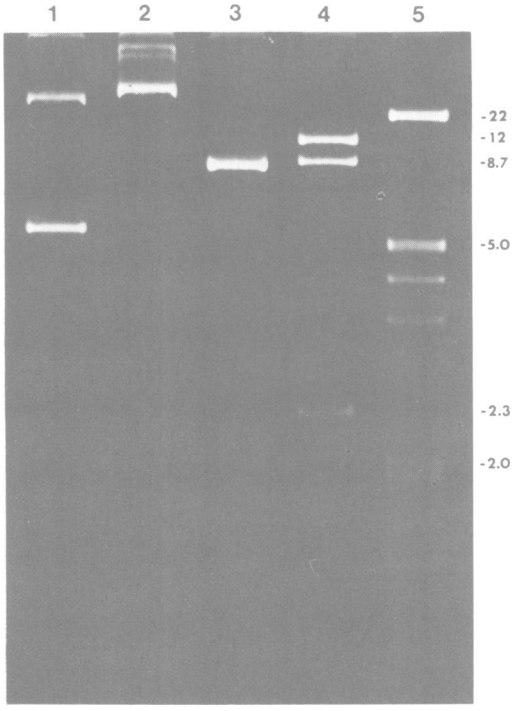

FIG. 1. *Agarose gel electrophoresis of pVH2124 and chimeric pDU1. Plasmids and restriction endonuclease digests were analyzed by electrophoresis on a 0.8% agarose gel as described in the text. The lengths in kb of specific restriction fragments are indicated. Lanes: 1, pVH2124; 2, pDU1; 3, pVH2124 plus EcoRI; 4, pDU1 plus EcoRI; 5, λ plus EcoRI plus HindIII.*

with pDU1. The pDU1 plasmid complemented the CU1126 *ilvB2102 ilvI2202*, CU503 *ilvE12*, CU534 *ilvD2017*, and CU1008 *ilvA454* mutants, and in each case complementation was associated with a Val^r phenotype. However, pDU1 failed to complement the CU390 *ilvC* or DU650 Δ(*ilvEDAC*) mutants. The pDU1 plasmid, therefore, must carry an intact *ilvGEDA* operon, but not the *ilvC* gene, indicating that one of the *Eco*RI termini must be between *ilvA* and the promoter distal end of *ilvC*. These results were confirmed by enzyme assay (see below).

The existence of the regulatory gene *ilvY* in *S. typhimurium* has been demonstrated by *cis-trans* analysis although *ilvY* could not be conclusively mapped with respect to *ilvA* and *ilvC* (4). The presence of *ilvY* on pDU1 was determined by F factor mobilization of the chimeric plasmid to the *S. typhimurium* strain DU702 *ilvA ilvY*. The interspecific mating between CGSC4349 (F′ *lac*, pDU1) and DU702 resulted in a transfer frequency of 10^{-4} Amp^r transconjugants per donor cell, and all 100 Amp^r trans-

conjugants examined were also Ilv$^+$. Enzyme assay of several DU702 (pDU1) transconjugants showed a return of *ilvC* activity, indicating the presence of a functional *ilvY* gene product. Therefore, pDU1 possesses an *ilvGEDAY*$^+$ genotype. This result also unequivocably demonstrates that the *S. typhimurium ilvY* gene is situated between *ilvA* and *ilvC*, as in *E. coli* K-12 (41), since pDU1 contains *ilvY* but not an intact *ilvC* gene.

Expression of the *ilvGEDA* operon from pDU1. The *ilv* enzymes encoded by pDU1 were assayed in the *E. coli* K-12 strain DU650 Δ(*ilvEDAC*) *leuB6* (Table 2). The *ilv* expression from a *S. typhimurium* F′ *ilvGEDAYC*$^+$ plasmid, pDU450, was also measured in the same background for comparison. Enzyme assay of DU650 (pDU1) confirmed the conclusions of the complementation analysis in that the *ilvGEDA*, but not *ilvC*, gene products were detected. Under repressing conditions, the expression of the *ilvGEDA* from pDU1 was 5- to 20-fold greater than that from pDU450. This is consistent with the expected difference in copy number between RSF2124, present in about 10 copies per chromosome (35), and F plasmids, which are maintained at 1 or 2 copies per chromosome (reviewed in reference 13).

The pDU1 *ilvGEDA* operon derepresses in response to valine limitation, and the *ilvG*, *ilvE*, and *ilvA* genes are expressed at elevated levels reflecting the expected gene dosage effect. The relatively poor expression of *ilvD* has been seen under a variety of conditions with both pDU1 and various subclones of pDU1. The apparent limit to *ilvD* expression could reflect the requirement of the dihydroxy acid dehydrase for an unknown cofactor or could be a manifestation of the relative instability of the enzyme. This problem is under investigation.

The responsiveness of the *ilvGEDA* genes to branched-chain amino acid limitation implies that the pDU1 *ilvGEDA* operon includes the major *ilv* promoter and the site of multivalent regulation, presumably the *ilv* attenuator. Of the four enzymes encoded by the *ilvGEDA* operon, the *ilvG*, *ilvE*, and *ilvA* gene products have been purified. The catalytic constants of the AHASII, transaminase B, and threonine deaminase, respectively, are 20 (D. Primerano and R. O. Burns, unpublished observation), 49 (9), and 700 (7) μmol of product formed per min per mg of protein. Based on the catalytic constants of the purified enzymes, it is estimated that under the condition of valine limitation the AHASII, transaminase B, and threonine deaminase enzymes constitute 6.5, 17, and 2.6%, respectively, of the total cellular protein of DU650 (pDU1). Thus, despite the parallel multivalent regulation of the *ilvGEDA* operon, there is a marked difference in the extent of expression of the *ilvG*, *ilvE*, and *ilvA* genes. The possible significance of this inequality will be discussed below.

Subcloning of *ilv* fragments from pDU1 and identification of specific restriction endonuclease sites within the *ilv* gene cluster. A partial restriction map of pDU1 was determined (Fig. 2), and the *ilv* genes were localized by subcloning of specific restriction fragments into the vector pBR322. The genetic composition of each fragment was determined by complementation analysis, and the genetic assignments were confirmed by enzyme assay. Thus, the *ilvGEDAY* genes are located from approximately 7 to 13.5 kb on the insert map.

An estimate of the physical length of the *ilvGEDAY* genes can be made by examining the *ilv* expression from two *SalI* fragment subclones of pDU1, pDU4, and pDU5. The pDU5 subclone, containing a 6.0-kb *SalI* fragment, possesses an *ilvEDAY*$^+$ genotype, and the pDU5 *ilvEDA* genes show essentially constitutive

TABLE 2. *Expression of S. typhimurium ilvGEDA operon from pDU1 and pDU450*

Strain	Medium[a]	Sp act (nmol/min/mg of protein)				
		Acetohydroxy acid synthase II (*ilvG*)[b]	Transaminase B (*ilvE*)	Dihydroxy acid dehydrase (*ilvD*)	Threonine deaminase (*ilvA*)	Acetohydroxy acid isomereroductase (*ilvC*)
DU650(pDU1)	Repressing	57	520	180	650	ND[c]
	Valine limited	1,300	8,100	1,900	18,000	ND
DU650(pDU450)	Repressing	2.7	86	22	84	24
	Leucine limited	210	2,200	1,400	3,800	320

[a] The media used have been described previously (3).

[b] The acetohydroxy acid synthase was assayed in the presence of 1 mM L-valine. The specific activity of the acetohydroxy acid synthase, therefore, reflects essentially the presence of the *ilvG*-encoded AHASII isozyme, which is resistant to feedback inhibition by L-valine.

[c] ND, Not detectable.

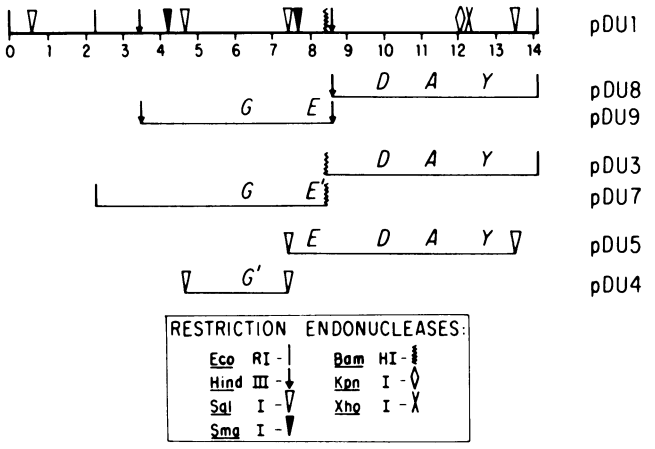

FIG. 2. *Restriction endonuclease map of the pDU1 S. typhimurium chromosomal insert. The map units are 1 kb. The approximate locations of the ilvGEDAY genes are indicated on the fragments carried by pBR322 subclones of pDU1. The G' and E' designations indicate that the one terminus of the subcloned fragments lies within the ilvG and ilvE genes, respectively.*

expression. The lack of multivalent regulation of the pDU5 *ilvEDA* genes implies that the genes are dissociated from the major *ilv* promoter and are solely expressed from the internal promoters. Therefore, the *Sal*I site at 7.4 map units on the pDU1 insert is between the major *ilv* promoter and *ilvEDA*. The contiguous 2.9-kb *Sal*I fragment, subcloned in pDU4, confers a Val^r Ilv$^+$ phenotype to CU1126, indicating expression of *ilvG*. However, the CU1126(pDU4) grows poorly on minimal medium, and enzyme assay shows a low level of multivalently regulated AHASII activity which is only about 1% of the activity previously seen with the pDU450 F' *ilv*$^+$. This suggests that the 2.9-kb *Sal*I fragment does not contain an intact *ilvG* gene and that the coterminal *Sal*I site at 7.4 map units must lie within the *ilvG* gene. The purified *ilvG* gene product has a subunit molecular weight of 55,000 (D. Primerano and R. O. Burns, unpublished observation), which could be encoded by a DNA sequence of approximately 1.5 kb. If it is assumed that the *Sal*I site at 7.4 map units is located close to the carboxy terminal end of *ilvG* and that the pDU5 6.0-kb *Sal*I fragment contains only the *ilvEDAY* information, then the maximum length of the *ilvGEDAY* region is 7.5 kb.

Two unique restriction sites exist within the 3' end of the *ilvGEDAY* region, one (recognized by the *Kpn*I endonuclease) at 12.0 kb and the other (recognized by *Xho*I) at 12.2 kb on the insert map. Since these sites are toward the distal end of *ilvGEDAY* cluster, it was of interest to determine their location with respect to the *ilvA* and *ilvY* genes. This was accomplished in part by digesting the parental chimera pDU1 with either the *Kpn*I or *Xho*I endonucleases and removing the staggered ends created by these enzymes with S1 nuclease. This treatment should introduce four base pair deletions at either the *Kpn*I or *Xho*I site, rendering the deletion derivatives resistant to redigestion with the appropriate restriction endonuclease. Two deletion derivatives of pDU1 were constructed in this manner, one resistant to *Kpn*I (designated pDU21) and the other lacking the *Xho*I site (designated pDU22). The relative sensitivities to *Kpn*I and *Xho* I of pDU1 and its deletion derivatives pDU21 and pDU22 are shown in Fig. 3. The identical *Eco*RI restriction patterns of these plasmids suggests that the S1 nuclease digestion was probably limited to the single-stranded tails of the linearized plasmids; no differences between these plasmids could be discerned with a number of other restriction endonucleases. The genetic effects of deleting the *Kpn*I and *Xho*I sites were determined by complementation studies and enzyme assay. The *Kpn*I-resistant derivative, pDU21, failed to complement *ilvA* mutants, and no threonine deaminase activity could be detected when the plasmid was introduced into DU650. Therefore, the *Kpn*I site must lie within the *ilvA* structural gene. The *Xho*I-resistant plasmid, pDU22, could not be distinguished from pDU1 by complementation studies or enzyme assay, indicating that the *Xho*I site is in neither *ilvA* nor *ilvY*.

The relative location of the *Xho*I site was determined more precisely by introducing larger deletions into pDU1 with BAL-31 nuclease. The BAL-31 nuclease degrades linear duplex DNA in a quasi-processive manner from both ends (12, 20). A series of deletion derivatives of pDU1

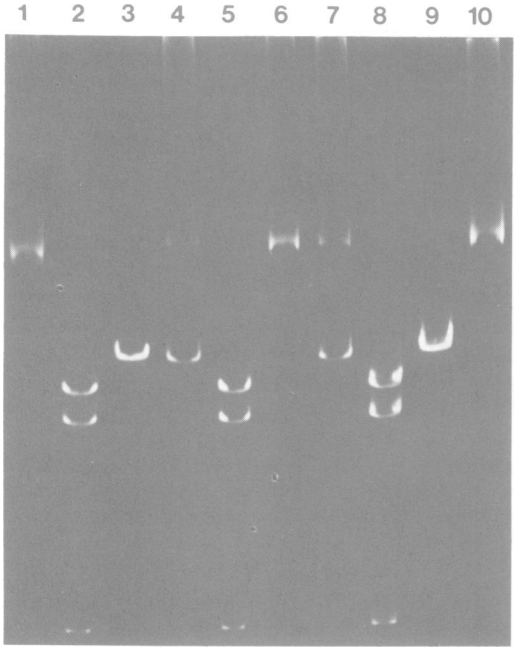

FIG. 3. *Comparison of KpnI⁻ and XhoI⁻ plasmids pDU21 and pDU22 with pDU1. The plasmids and restriction digests was analyzed by electrophoresis on a 1% agarose gel. Lanes: 1, pDU1; 2, pDU1 plus EcoRI; 3, pDU1 plus KpnI; 4, pDU1 plus XhoI; 5, pDU21 plus EcoRI; 6, pDU21 plus KpnI; 7, pDU21 plus XhoI; 8, pDU22 plus EcoRI; 9, pDU22 plus KpnI; 10, pDU22 plus XhoI.*

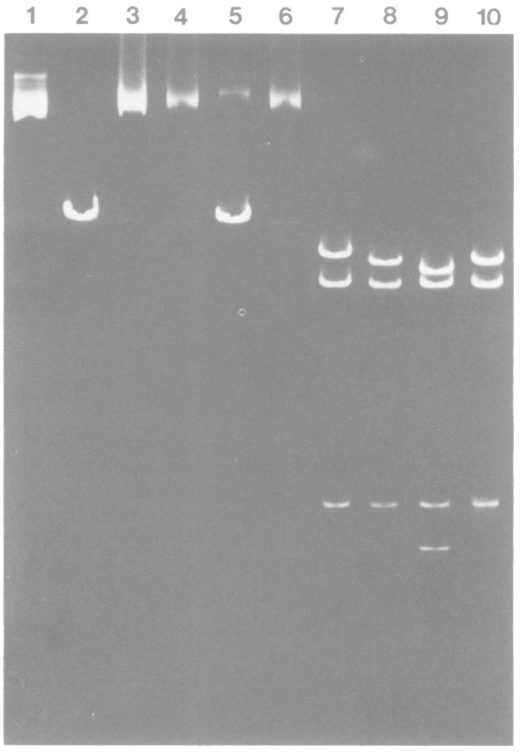

FIG. 4. *Agarose gel electrophoresis of pDU1 and its BAL-31 nuclease-treated derivative, pDU2. The samples were analyzed by electrophoresis on a 1% agarose gel. Lanes: 1, pDU1; 2, pDU1 plus KpnI; 3, pDU2; 4, pDU2 plus KpnI; 5, pDU1 plus XhoI; 6, pDU2 plus XhoI; 7, pDU1 plus EcoRI; 8, pDU2 plus EcoRI; 9, pDU1 plus EcoRI plus XhoI; 10, pDU2 plus EcoRI plus XhoI. The faint bands that are seen in lanes 1, 3, 4, and 5 represent linearized plasmid that is formed upon long-term storage of pDU1 and pDU2.*

was generated by BAL-31 nuclease digestion of *Xho*I-linearized pDU1; a single plasmid (designated pDU2) was identified which lacked *ilvA* gene activity but retained *ilvY* function. Restriction analysis (Fig. 4) showed that the deletion in pDU2 was approximately 1 kb in length and included the *Kpn*I site, previously shown to be within the *ilvA* gene. Thus, BAL-31 nuclease digestion from the *Xho*I site into the *ilvA* gene affects only *ilvA* activity, implying that the *Xho*I site must lie between *ilvA* and *ilvY*. Since the *Sal*I fragment ending at 13.5 map units, subcloned in pDU5, contains an intact *ilvY* gene (Table 3), the *ilvY* gene must lie within the 1.3-kb region defined by *Xho*I and *Sal*I. The subunit molecular weight of the *E. coli* K-12 *ilvY* gene product has been estimated to be 35,000 (42), a size which requires a coding sequence of 0.95 kb. If the *S. typhimurium ilvY* gene were of similar size, then the deletion in pDU2 could extend to the right of the *Xho*I site a maximum of 0.35 kb without affecting *ilvY*.

The region from 13.5 to 14.1 kb on the pDU1 insert must represent a portion of the *ilvC* gene, which should be 1.6 kb in length to encode the 57,000-dalton subunit of its gene product, the acetohydroxy acid isomeroreductase (14). The *cis*-active *ilvC* regulatory elements must be present in the pDU1 insert if *ilvC* is transcribed in

TABLE 3. *Expression of ilvGE in DU650(pDU9) and DU650(pDU10)*

Strain	Medium	Sp act (nmol/min/mg of protein)	
		Acetohydroxy acid synthase II[a] (*ilvG*)	Transaminase B (*ilvE*)
DU650(pDU9)	Repressing	560	880
	Valine limited	5,400	9,500
DU650(pDU10)	Repressing	530	1,000
	Valine limited	6,200	8,200

[a] The acetohydroxy acid synthase activity was assayed in the presence of 1 mM L-valine and reflects essentially the activity of the valine-resistant AHASII isozyme.

the same direction as *ilvGEDA*, as is the case in *E. coli* K-12 (40).

Differential amplifaction of *ilvG* expression from subclone pDU9. The subcloning of specific *S. typhimurium ilv* genes into pBR322 permits a further study of the effects of increased gene dosage on *ilv* gene expression since pBR322 exists in 20 to 30 copies per chromosome (5). The pDU9 plasmid, consisting of the 5.2-kb *Hin*dIII fragment of pDU1 inserted into pBR322, directs the multivalently regulated synthesis of both the *ilvG* and *ilvE* gene products (Table 3). The derepressed level of the pDU9-encoded transaminase B is slightly higher than that seen previously with pDU1, constituting 19% of the total cellular protein. Despite this modest increase in the activity of the *ilvE* gene, the pDU9 *ilvG* gene expression is over fourfold greater than from pDU1. This represents an increase in the amount of AHASII from 6.5 to 27% of the total cellular protein. The same result was obtained with pDU10, which is a pBR322 subclone carrying the 5.2-kb *Hin*dIII fragment in the opposite orientation, decreasing the possibility of some effect of plasmid sequences on *ilvE* expression. In addition, the activity of the pDU9-encoded transaminase B was not improved when the culture was grown on the presence of 20 μg of pyridoxine per ml, so that the formation of active transaminase B was not limited by a paucity of its cofactor, pyridoxal-5′-mono-phosphate (data not shown). Therefore, an increase in the gene dosage of *ilvGE* is associated with differential amplification of *ilvG* expression. The relationship between relative *ilvG* expression and gene dosage is extended by the data for pDU450 F′ *ilv*$^+$ which show that AHASII and transaminase B constitute 1 and 4.5%, respectively, of the total cellular protein upon derepression (Table 2). The low-copy F′ *ilv*$^+$ plasmid thus provides the lowest expression of *ilvG* with respect to *ilvE*. This effect of gene dosage on relative *ilvG* expression could involve differential formation of *ilvG* mRNA compared with formation of *ilvGE* mRNA as is discussed below.

Demonstration of an rRNA operon closely linked to the *S. typhimurium ilv* gene cluster. In *E. coli* K-12, the rRNA operon *rrnC* is located near *ilv* (17) with a distance of about 5 kb between the 3′ end of the 5S rRNA sequence and the *ilvE* gene (25). If a homologous rRNA operon exists in *S. typhimurium*, then it should be included on the pDU1 insert. This was demonstrated by the Southern hybridization technique (36), using a ^{32}P-labeled *S. typhimurium* rRNA probe. The rRNA probe hybridized to *Eco*RI-*Hin*dIII restriction fragments of pDU1 that span the region from 0 to 3.4 kb on the insert map (Fig. 5). Very weak hybridization to the 5.2-kb *Hin*dIII fragment (3.4 to 8.6 kb) was noted with prolonged exposure times, suggesting that this fragment contains a short length of the rRNA operon.

The *E. coli* K-12 *rrnC* operon is transcribed in the same direction as *ilvEDA* (25, 27), and it is known that the transcriptional unit includes the tRNA^{Asp}- and tRNA^{Trp}-transcribing genes at the 3′ end (27). The presence of tRNA genes on the 5.2-kb *Hin*dIII fragment of pDU1 was also demonstrated by Southern hybridization with a ^{32}P-labeled bulk tRNA probe; therefore, the 3′ end of the rRNA operon must be located a short distance beyond 3.4 map units on the 5.2-kb *Hin*dIII fragment. This fragment also includes intact *ilvG* and *ilvE* genes (subcloned in pDU9), requiring approximately 2.4 kb of coding sequence based upon the subunit molecular weight (55,000) of the *ilvG* gene product and the *ilvE* product transaminase B (31,500) (21). Accordingly, the 3′ end of this rRNA operon is a maximum distance of 2.8 kb from *ilvG*.

DISCUSSION

The molecular cloning of the *S. typhimurium ilvGEDAY* genes provides an additional demonstration of the gene order of the multivalently regulated *ilvGEDA* operon and establishes the location of the regulatory gene *ilvY* between *ilvA* and *ilvC*. The maximum length of the *S. typhimurium ilvGEDAY* cluster is 7.5 kb. This is similar to the 6.5-kb length of the *E. coli* K-12 system described by Umbarger and co-workers (25), assuming that the cryptic *ilvG* gene in that species is equivalent in size to the *S. typhimurium* gene. The *S. typhimurium ilvDAY* region has a maximum length of 4.9 kb, as defined by a *Hin*dIII-*Sal*I fragment with termini at 8.6 and 13.5 pDU1 map units, respectively. The homologous *E. coli* K-12 region is 4.2 kb in length (25). In view of the present uncertainties concerning the precise termini of the *ilvG* and *ilvC* genes, the *S. typhimurium* lengths are maximum estimates, and it is apparent that the *ilv* gene clusters of both species are comparable in size. An additional homology between these closely related genes is demonstrated by the presence of an rRNA operon on pDU1, located about 4 kb from the 5′ end of *ilvE*. The *E. coli* K-12 *rrnC* operon is situated about 5 kb from the 5′ end of *ilvE* (25).

The selection for *S. typhimurium ilv* recombinant plasmids depended upon a major difference between *Salmonella* and *E. coli* K-12, namely the cryptic nature of *ilvG* in *E. coli* K-12 and the corresponding sensitivity of that strain to growth inhibition by valine. The pDU4 *ilvG′* subclone demonstrates that the amount of

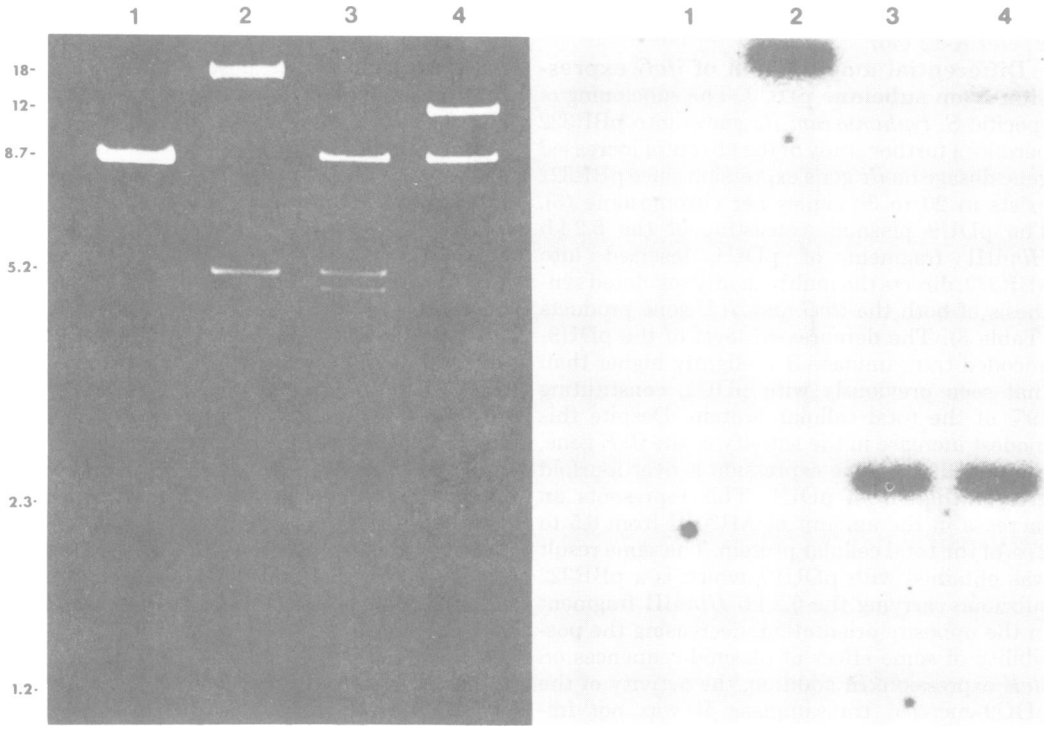

FIG. 5. *An rRNA operon on the pDU1 insert. The left half of the figure shows restriction digests of pVH2124 and pDU1 after electrophoresis on a 0.8% agarose gel, and the lengths of the restriction fragments are given in kilobases. The right half of the figure is an autoradiograph of the Southern transfer of the gel to which [^{32}P]rRNA has hybridized. Lanes: 1, pVH2124 plus EcoRI; 2, pDU1 plus HindIII; 3, pDU1 plus EcoRI plus HindIII; 4, pDU1 plus EcoRI.*

ilvG expression necessary to confer Val^r is very small. The AHASII activity provided by this plasmid, which carries a *SalI* fragment ending in the promoter distal region of *ilvG*, is only about 1% of the *ilvG* expression for F′ *ilv*$^+$ plasmids under derepressing conditions. The pDU4 plasmid confers Val^r to SK1592 and yet effects little change in the total acetohydroxy acid synthase activity or the fraction of activity resistant to feedback inhibition by valine. Thus, the valine-insensitive nature of the pDU4-encoded AHASII is clearly more important than its low activity in confirming Val^r. This qualitative change in acetohydroxy acid synthase activity is not the only mechanism by which a Val^r phenotype can occur. For instance, valine resistance in *ilvG* strains can occur by elevation of the level of AHASI either by mutation or by introduction of a multicopy plasmid bearing *ilvB* (R. Weinberg, D. Blazey, and R. O. Burns, unpublished observations).

The subclones obtained from pDU1 demonstrate the ability of the internal promoters (*pEDA* and *pDA*) to function in tbe absence of the major, multivalently regulated *pGEDA* promoter. Although the low constitutive expression from the *pDA* promoter under conditions of excess isoleucine, valine, and leucine may be physiologically insignificant, an advantage can be ascribed to expression of *ilvE* under the same conditions. It is known that transaminase B (*ilvE*) participates with transaminase C to insure the production of the pantothenate acid precursor α-ketoisovalerate from valine (D. Blazey, D. Primerano, and R. O. Burns, unpublished observations).

An alternative view of the internal promoters is that they are vestigial and that the nucleotide sequences which accompany them, including the ribosome binding sites, participate in intraoperon translational control within the major *ilvGEDA* mRNA. This view is supported by the observation that a marked translational polarity exists between *ilvGE* and *ilvDA* expression from pDU1; upon valine limitation transaminase B constitutes 17% of the total cellular protein while threonine deaminase constitutes 2.6%. The actual length of the *ilvE-ilvD* intercistronic region has not been determined, but it is known that the *Bam*HI site at 8.45 kb on the pDU1 insert

map lies very near the 3′ end of *ilvE*, and that the *Hin*dIII site at 8.6 kb lies between *ilvE* and *ilvD*, a distance of about 150 base pairs as judged by the mobility of the *Bam*HI-*Hin*dIII fragment produced by endonuclease cleavage at these sites. Thus, a significant distance separates *ilvE* and *ilvD* which could cause a strong polar effect on translation of *ilvDA*. The termination of translation in this region coupled with the relative inefficiency of the ribosome binding site which precedes *ilvDA* explains the differential expression of the *ilvGE-ilvDA* gene pairs. Additional complexity of this region is shown by recent results which demonstrate that at least some of P_{DA} lies within the *ilvE* structural gene (D. Blazey and R. O. Burns, Fed. Proc., p. 1649, 1981).

The regulatory significance of the strong polarity exerted between the *ilvGE* and *ilvDA* translational units is suggested by the relative catalytic activity of the *ilv* biosynthetic enzymes. AHASII and transaminase B have low catalytic constants and are produced in much greater amounts than threonine deaminase (and presumably dihydroxyacid dehydrase), which has a high catalytic constant. Therefore, by imposing a lower translational efficiency the ribosome binding site associated with *ilvDA* provides a mechanism for maximizing the efficiency of the *ilv* biosynthetic pathway which includes enzymes with widely different catalytic rates.

The converse situation apparently exists regarding *ilvG* and *ilvE* expression. It was noted that derepression of the pDU1 *ilvGEDA* operon results in 6% of the total cellular protein as the *ilvG* gene product compared with 17% as the *ilvE* encoded transaminase B. This could reflect a greater strength of the *pE* ribosome binding site or greater accessibility of ribosomes to that sequence as a result of differences in the secondary structure of the mRNA.

The major multivalently regulated transcript is assumed to be the full-length *ilvGEDA* mRNA, which is consistent with the parallel control of the individual genes. However, it may be possible to obtain significant amounts of a shorter *ilv* mRNA under at least one condition. The relative expression of *ilvG* with respect to *ilvE* was observed to increase with increasing gene dosage. For instance, the expression of *ilvG* from the high-copy-number pDU9 subclone was over fourfold greater than that seen with pDU1 despite a negligible change in *ilvE* expression. This could result from transcription termination between the *ilvG* and *ilvE* genes when *ilvGE* is present in very high copy number. The possibility that a transcriptional termination sequence exists between *ilvG* and *ilvE*, the activity of which could be modulated by a chromosomally determined antitermination factor, will be explored by fine structure analysis of the *ilvG-ilvE* intercistronic region. In addition, the individual species of *ilv* mRNA's present with respect to gene copy can be identified to further define the structural and regulatory features of the *ilvGEDA* operon.

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Addendum in Proof

Recent work indicates that the expression of *ilvEDA* from the *Sal*I subclone pDU5, previously thought to result from the internal promoters, is dependent on the tetracycline resistance promoter of pBR322. When the insert in pDU5 is inverted in orientation, the expression of *ilvEDA* is only about 1% of that seen with pDU5. Futhermore, if the tetracycline resistance promoter of pBR322 is removed by BAL-31 digestion, the expression of *ilvEDA* from pDU5 falls to the same low level. We conclude that most of the *ilvEDA* expression is the result of transcription from the pBR322 promoter. The residual expression of *ilvEDA* in pDU5 which lacks the major *ilv* promoter is much lower than would be expected from previous results with appropriate *ilv*::Tn10 insertion strains (3). These results suggest that the activity of the internal promoters is not entirely independent of the major *ilv* promoter.

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