Point Mutations in the Regulatory Region of the *ilvGMEDA* Operon of *Escherichia coli* K-12

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The *ilvGMEDA* operon of *Escherichia coli* K-12 is preceded by a regulatory region containing a promoter, a leader, and an attenuator. This region has been extensively characterized biochemically. In this note point mutations of the regulatory region are reported. The effect of these mutations on expression from the *ilv* regulatory region supports the previous biochemical analysis.

The genes for biosynthesis of the branched-chain amino acids isoleucine, leucine, and valine are divided into several transcriptional units (26). The largest is the *ilvGMEDA* operon, which is multivalently regulated by all three amino acids (26). Limitation of the growth of either *Escherichia coli* K-12 or *Salmonella typhimurium* by deficiencies in any of the three amino acids results in derepression of the operon (6). Alternatively, the addition of all three amino acids to minimal medium results in repression of the operon (26). Eidlic and Neidhardt (5) made the first observation that *ilv* gene regulation is tRNA mediated by using an *E. coli* strain that contained a temperature-sensitive valyl-tRNA synthetase. Subsequent studies of mutant strains of *S. typhimurium* (4) and *E. coli* K-12 (13) with altered tRNA modification supported this conclusion. DNA sequence analysis suggested that the *ilvGMEDA* operon is preceded by a leader-attenuator. In both *E. coli* K-12 (14, 20) and *S. typhimurium* (25), the leader region encodes a putative 32-amino-acid polypeptide that contains 15 branched-chain amino acid residues (Fig. 1).

The isolation and characterization of mutant or variant strains assisted in elucidating the mechanisms of the regulation of gene expression. Such mutants provided much insight into the regulation by attenuation of the *trp* (24, 28), *his* (10), *thr* (16, 17), and *leu* (3) operons. Investigation of the regulation of the *ilvGMEDA* operon has been limited by the absence of mutations that alter the leader-attenuator. Comparison of the regulatory region of *S. typhimurium* with that of *E. coli* K-12 (25) indicated only seven nucleotide (nt) differences over more than 200 base pairs (bp). None of the observed nucleotide differences would be expected to affect leader-attenuator function. Harms et al. (7) also determined the DNA sequences of the regulatory regions of the *ilvGMEDA* operons of *E. coli* B, *Klebsiella aerogenes*, *Edwardsiella tarda*, and *Serratia marcescens*. The nucleotide sequences for *E. coli* B and *K. aerogenes* were found to be similar to those reported for *E. coli* K-12 and *S. typhimurium*; i.e., no nucleotide changes that should alter either the secondary structure of the leader RNA or the amino acid composition of the leader peptide were observed. The nucleotide sequences determined for both *Edwardsiella tarda* and *Serratia marcescens* were found to differ substantially from those for *E. coli* K-12. As a result, the RNA secondary structure should change and the deduced amino acid sequence of the leader peptide should also vary. Thus, regulation of the attenuation of the *ilvGMEDA* operon by leucine for *Edwardsiella tarda* and *Serratia marcescens* requires response to a single leucine codon (9). By altering the leucine

codon with oligonucleotide site-directed mutagenesis, Harms and Umbarger (8) demonstrated that the single leucine codon could account for leucine-specific regulation of the *ilvGMEDA* operon. Bennett and Umbarger (1) isolated a pair of deletions that extended through the *ilv* attenuator of *E. coli* K-12 by using a lambda bacteriophage containing an *ilvD-lacZ* fusion. They observed a 30-fold derepression of β-galactosidase relative to levels in the parental bacteriophage. Because of the size of the deletions, the observed extent of derepression must in part reflect deletion of the nonsense mutation (at bp 1,252) in the wild-type allele of *ilvG*, which results in reduced expression of the distal portion of the operon (12).

This note reports the first isolation of organisms carrying the point mutations that alter gene expression from the *ilvGMEDA* regulatory region of *E. coli* K-12. These mutants were isolated on the basis of observations of *E. coli* K-12 M152 (*galK2 recA3 rpsL200* IN[*rrnD-rrnE*]*1*) transformed with plasmid pRL137. This plasmid, constructed by using *Bam*HI and *Hind*III linkers to insert a 700-bp *Hae*III-*Alu*I restriction fragment into the *galK* expression vector pKO6 (21), extends from −50 to 350 bp beyond the sequence presented in Fig. 1 (i.e., to bp 645). The plasmid is constructed so that expression of galactokinase is dependent on the *ilv* promoter, leader, and attenuator. Transformants of strain M152 with pRL137 yielded white colonies on galactose McConkey agar (Table 1) and showed growth after 24 h on M63 minimal agar (19) with galactose as the sole carbon source (Table 1). Therefore, organisms with mutations in the *ilv* regulatory region that decreased galactokinase formation would fail to grow on minimal galactose, while organisms with mutations that increased expression from the *ilv* region would yield red colonies on galactose McConkey agar.

Our initial mutagenesis of plasmid pRL137 was done by the hydroxylamine hydrochloride procedure described by Busby et al. (2). pRL137 (50 µg) was treated with 1 M hydroxylamine hydrochloride for 2 h at 75°C. Plasmid DNA was recovered and digested with the restriction endonucleases *Bam*HI and *Hind*III. The 700-bp *ilv* insert was purified by polyacrylamide gel electrophoresis as described by Maxam and Gilbert (18). The *ilv* fragment was inserted into unmutagenized parental vector pKO6 and then transformed into strain M152. Transformants were selected on galactose McConkey agar containing ampicillin (100 µg/ml) and tested for growth on minimal galactose agar.

When this procedure is used, colonies produced from the initial transformation should represent unique events because, after mutagenesis, the hydroxylamine-treated DNA is

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         HaeIII                                               A(950)
         -50                      -25                          |            +1
         GGCCAAAAAA TATCTTGTAC TATTTACAAA ACCTATGGTA ACTCTTTAGG CATTCCTTCG


                                           C(952)
                              25             |                   50
         AACAAGATGC AAGAAAAGAC AAA ATG ACA GCC CTT CTA CGA GTG ATT AGC CTG
                                  Met Thr Ala Leu Leu Arg Val Ile Ser Leu


                              75                             100
         GTC GTG ATT AGC GTG  GTG GTG ATT ATT ATC  CCA CCG TGC GGG GCT GCA
         Val Val Ile Ser Val  Val Val Ile Ile Ile  Pro Pro Cys Gly Ala Ala


                                           Sau3AI
                         125                        150
         CTT GGA CGA GGA AAG  GCT  TAGAGATCAAGCCTTAACGAACTAAGACCCCCGCACCGA
         Leu Gly Arg Gly Lys  Ala  *


              A(951)
              175 |                      200                      225
         AAGGTCCGGGGGTTTTTTTTGACCTTAAAAACATAACCGAGGAGCAGACAATGAATAACAGCACA


                                                         ilvG
                         250                     275              289
         AAATTCTGTTTCTCAAGATTCAGGACGGGGAACTAACT  ATG AAT GGC GCA CAG TGG
                                                 Met Asn Gly Ala Gln Trp
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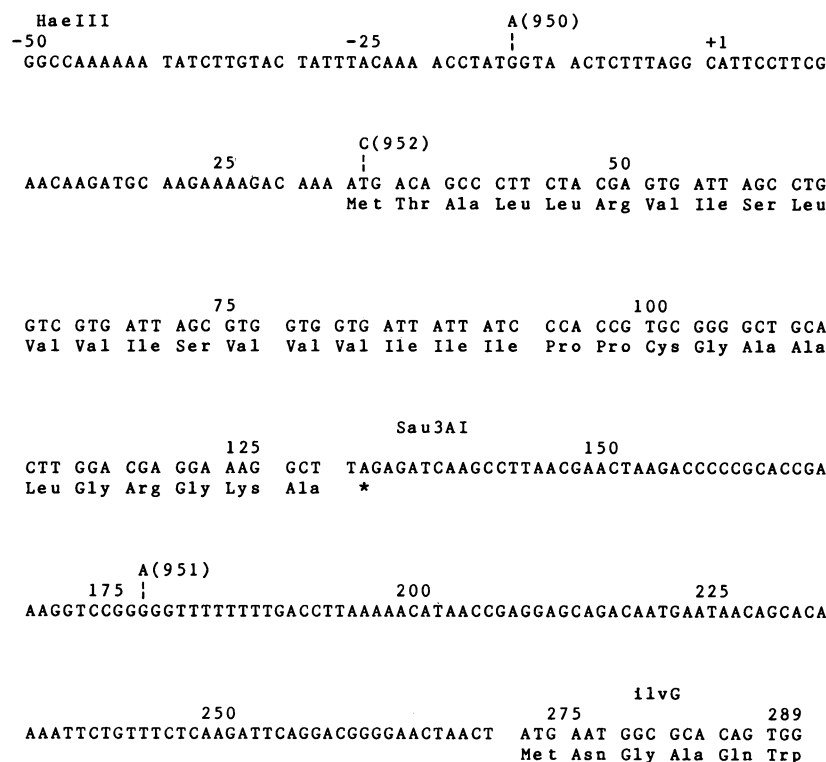
FIG. 1. 5'-Terminal sequence of the *ilvGMEDA* operon. The *Hae*III site that forms one end of the 700-bp *ilv* insert of pRL137 is indicated, as is the *Sau*3AI site that is the deletion point for pMO167. The sequence is numbered from the transcription initiation site (15), and the nucleotide changes of each mutant are indicated. The terminal nucleotides for the leader RNA correspond to bp 185 and 186.

not replicated until it is inserted into unmutagenized pKO6 and transformed into M152. Selection and screening of several hundred transformants yielded approximately 60 colonies with the selected phenotypes. The plasmids from these colonies were isolated by miniscreen or rapid-screen isolation, and the plasmid structure was analyzed by restriction endonuclease digestion. After repeated retransformation and reisolation (a total of three times), 25 plasmids which yielded transformants with either of the two pheno-

TABLE 1. Characteristics of *ilv* wild-type and mutant plasmids

| Plasmid | Mutation | Phenotype | | Galacto-kinase[a] |
		Galactose McConkey agar[b]	Minimal galactose agar[c]	
pKO6	Parental vector	W	−	1.0
pRL137	Wild type	W	+	8.0
pRL201	*ilvGp2950* (bp −15, G→A)[d]	W	−	0.69
pDM287	*ilvGa951* (bp 177, G→A)[d]	R	+	24
pMO167	Δ*ilvGa*	R	+	25
pMW333	*ilvGe952* (bp 34, T→C)[d]	W	−	1.4

[a] Galactokinase assays utilized extracts of M152 transformed with the indicated plasmid. Each variant was grown on M63 glucose medium and assayed as described elsewhere (21). Galactokinase units are nanomoles of galactose-1-phosphate formed per minute per milligram of protein per femtomole of plasmid.

[b] W, White colony at 15 to 18 h; R, red colony.

[c] Growth (+) after or lack of growth (−) after 24 h on M63 (19) with galactose as the sole carbon source.

[d] Nucleotide change indicated in Fig. 1.

types described were isolated. Plasmid DNA was prepared, and the DNA sequence (18) of the *ilv* promoter-leader-attenuator region was determined. Subsequently, the remainder of the *ilv* insert was sequenced for the plasmids found to contain mutations within the regulatory region. This stringent analysis yielded seven plasmids belonging to either of two classes. The small number (both in type and quantity) of mutants recovered probably reflect several factors: (i) mutagenesis with hydroxylamine yields only G · C-to-A · T transitions, (ii) *ilvGp2* is already a weak or poor promoter, and (iii) the selection and screening criteria used by us were highly stringent.

All the plasmids uniquely altered in the *ilv* regulatory region belonged to two classes, represented by the mutations *ilvGp2950* and *ilvGa951*. The first group (four isolates) yielded white colonies on galactose McConkey agar and failed to grow on minimal galactose agar (Table 1, pRL201). Galactokinase expression was assayed as described previously (21). These mutant plasmids yielded an approximately 10-fold-lower expression of galactokinase (0.69 nmol/mg of protein per fmol of pRL201 versus 8.0 nmol/mg of protein per fmol of pRL137). DNA sequence determination indicated that there is a G-to-A transition at −15 bp (Fig. 1) relative to the transcription initiation site (defined by analysis both in vivo and in vitro; 15). This mutation is consistent with similar mutations previously identified as down mutations in the promoters of bacteriophage lambda (23) and of the *arg* operon (22). As such, they confirm that the sequence TAACTCT (−13 to −7) corresponds to the −10 region of other bacterial promoters (14).

The second group of mutants (three isolates) yielded red colonies on galactose McConkey agar and grew on minimal

galactose agar. This phenotype is represented by pDM287 (Table 1), in which there has been a G-to-A transition at bp177 (Fig. 1, *ilvGa951*). The change of this base pair would reduce the stability of the RNA stem-loop structure formed from bp 151 to 180, which is required for transcription termination (15). Calculation of the free energy of formation of the RNA stem-loop structure for the wild-type *ilv* operon yields a value of -14.0 kcal (ca. -5.9×10^{-4}J), while that for the *ilvGa951* mutation is -7.4 kcal (ca. -3.1×10^{-4}J). Consistent with this mutation resulting in reduced transcription termination at the attenuator is the finding that the level of galactokinase observed for pDM287 (24 U; Table 1) is similar to that observed for plasmid pMO167 (25 U; Table 1), in which the attenuator is deleted by insertion of the restriction fragment from -50 (*Hae*III) to $+133$ (*Sau*3A) bp. This group of mutations corresponds to similar mutations in the attenuator observed in other amino acid biosynthetic systems regulated by a leader-attenuator (reviewed by Landick and Yanofsky [11]).

As described above, the several hundred transformants isolated after hydroxylamine mutagenesis yielded some half-dozen mutations within the *ilv* regulatory region. These mutations were either down promoter mutations at -15 bp or attenuator mutations at 177 bp. It had been expected that mutations would also be obtained at the initiating AUG for translation of the leader peptide, mutations similar to those described for the *trp* operon by Zurawski et al. (28). To construct such a mutation, an oligonucleotide (RL36; GGGCTGTCGTTTTGTCT) was synthesized to change bp 34 from T · A to C · G, thus changing the codon from AUG (Met) to ACG (Thr). To accomplish this, the 700-bp *ilv* fragment from pRL137 was transferred to the single-stranded phage MP8 and the virus was mutagenized with RL36 as described by Zoller and Smith (27). Phage lysates were screened by differential hybridization of the mutagenic oligonucleotide and by DNA sequence analysis (27). The mutagenized *ilv* fragment was inserted into pKO6, yielding pMW333. A five- to sixfold decrease in the expression of galactokinase resulted from altering this base pair (1.4 versus 8.0 U) (Table 1).

The phenotypes of these three mutants are consistent with the results of previous analyses (15, 26). Transcription in vitro was performed to further analyze the effects of these mutations (Fig. 2). Transcription in vitro of pRL137 yields two short products (21), the 108-nt RNA from the replication region of the plasmid and the 186-nt RNA from *ilvGp2* into the *ilv* attenuator (Fig. 2, lane 1). Lanes 2, 3, and 4 in Fig. 2 show the products of transcription in vitro of pRL201, pDM287, and pMW333, respectively. Altering either the promoter or the attenuator resulted in reduced formation of the 186-nt transcript. However, alteration of the translation initiation triplet appeared to have no effect on formation of the 186-nt RNA (pMW333; Fig. 2, lane 4).

Quantitation of the transcription products by densitometric analysis further supported the effect of these mutations on transcription. The molar ratios of the 186- to 108-nt RNAs for pRL137 and pMW133 are 1.54 and 1.57, respectively, while for pRL201 and pDM287 the ratios are 0.210 and 0.303, respectively. These data are again consistent with the *ilv-950* mutation affecting expression from *ilvGp2* and with the *ilv-951* mutation affecting termination. Furthermore, analysis of the transcription of purified restriction fragments is consistent with the transcription of the plasmids (data not shown).

The point mutations described in this note are the first described for the regulatory region of the *ilvGMEDA* operon
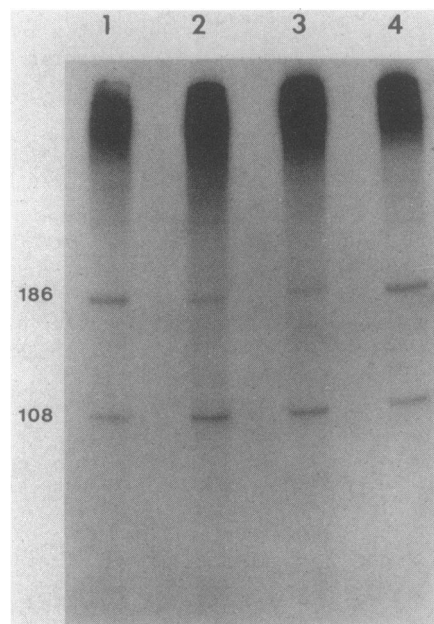

FIG. 2. Products of transcription in vitro of wild-type and mutant plasmids (lane 1, pRL137; lanes 2 to 4, pRL201, pDM287, and pMW333, respectively). Transcription reactions were performed as described previously (21), and the products were resolved on a 7.3 M urea–6% polyacrylamide gel. The 186- and 108-nt RNA products are indicated.

of *E. coli* K-12. They confirm previous conclusions about the structure of the *ilv* regulatory region that were based on biochemical analysis. The mutation *ilvGp2950* reduces both the expression in vivo and the transcription in vitro of galactokinase, in agreement with the proposed location of *ilvGp2* (14, 15). Our analysis of mutation *ilvGe952* is consistent with the site of translation initiation of the proposed *ilv* leader peptide. The characteristics of mutation *ilvGa951* confirm the site for formation of the stem-loop required for transcription termination. Together, these mutations minimize any uncertainty in our previous analysis (15).

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