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Role of Codon Choice in the Leader Region of the *ilvGMEDA* Operon of *Serratia marcescens*

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Leucine participates in multivalent repression of the *Serratia marcescens ilvGMEDA* operon by attenuation (J.-H. Hsu, E. Harms, and H. E. Umbarger, J. Bacteriol. 164:217–222, 1985), although there is only one single leucine codon that could be involved in this type of control. This leucine codon is the rarely used CUA. The contribution of this leucine codon to the control of transcription by attenuation was examined by replacing it with the commonly used leucine codon CUG and with a nonregulatory proline codon, CCG. These changes left intact the proposed secondary structure of the leader. The effects of the codon changes were assessed by placing the mutant leader regions upstream of the *ilvGME* structural genes or the *cat* gene and measuring acetohydroxy acid synthase II, transaminase B, or chloramphenicol acetyltransferase activities in cells grown under limiting and repressing conditions. The presence of the common leucine codon in place of the rare leucine codon reduced derepression by about 70%. Eliminating the leucine codon by converting it to proline abolished leucine control. Furthermore, a possible context effect of the adjacent upstream serine codon on leucine control was examined by changing it into a glycine codon.

Analysis of the nucleotide sequence of the regulatory region of the *ilvGMEDA* operon in *Serratia marcescens* revealed consecutive valine and isoleucine codons in the leader transcript but, in contrast to other enteric bacteria, only a single leucine codon in that portion of the leader involved in alternative secondary structures (11). This difference was of interest in view of a report by Kisumi et al. (17) showing multivalent control of the *ilv* operon of *S. marcescens* by all three branched-chain amino acids. That derepression could occur under conditions of limiting valine and isoleucine could readily be attributed to the distribution of multiple valine and isoleucine codons within the leader transcript, congruent with the model for control by attenuation of transcription (21, 25). However, for leucine to participate in multivalent repression by an attenuation mechanism, it would have to be mediated by ribosome stalling at a single leucine codon. That leucine participated in the control via the amount of charged leucyl tRNA was subsequently shown by Hsu et al. (14). The peptides specified by the leaders of all other amino acid biosynthetic operons that are controlled by attenuation of transcription contain several regulatory amino acids (19). Strikingly, the single leucine codon in the *S. marcescens* leader is the rarely used CUA (15).

That an effective detection system for the charging level of tRNA can be achieved by a single codon might also be due to the context in which the leucine codon is placed. Context effects due to tRNA-tRNA interactions at the A and P sites of the ribosome have been considered as a factor in translation efficiency (2). Less than optimal fitting of tRNA pairs at the A and P sites of the ribosome might result in a reduced affinity for the incoming acyl tRNA and an increased sensitivity to the charging level. It is of interest that the single leucine codon in the *ilv* leader is preceded by a serine codon, for which the tRNA has a large extra arm (34).

In this paper, we describe experiments designed to define the contribution of the single rare leucine codon CUA to control by attenuation of the *ilvGMEDA* operon of *S. marcescens*. In addition, the importance of the serine codon preceding the leucine codon has been examined by replacing it with one allowing the leucyl tRNA to be paired with a tRNA containing a small extra arm.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used in this study and their sources are listed in Table 1. Strain CU1436 was obtained by Mark Rudinski in our laboratory by transforming strain CU1318 (*recBC*) with linear pPU169, which carried the *ilvB2102*::Ω marker. This marker was transferred from strain CU1436 to strain CU1183 by P1 transduction and selection for spectinomycin-resistant colonies. Such a colony, which also required isoleucine, leucine, and valine for growth, was designated CU1458. The Ilv^- phenotype could be complemented by plasmid pPU5, carrying the *ilvGMEDAYC* sequences. Strain CU1460 was prepared by cotransduction of the *polA1* marker of strain CH1274 with Tn*10* into strain CU1458. The *polA1* marker was scored by the inability of the test strain to grow in the presence of 0.02% methyl methanesulfonate. The plasmids used are listed in Table 2.

Media and growth of cells. The media and methods used to grow cells under repressing and derepressing conditions were described by Gayda et al. (7). Repressing conditions were obtained by growing cells in minimal medium supplemented with 50 μg each of L-leucine and L-isoleucine and 100 μg of L-valine per ml. Derepressing conditions were obtained by transferring the cells from a repressed culture at 0.3 A_{660} to the same volume of fresh medium supplemented with two amino acids at the repressing concentration and one at a level 1/20 of the repressing concentration. The cells were harvested between 3 and 3.5 h later. This procedure should not be construed as a quantitative test for maximal derepressibility. The degree of derepression is some complex function of (i) the relative affinities of the several transport systems for the three branched-chain amino acids

TABLE 1. Strains used

Strain	Genotype	Source or reference
CU1183	*ΔilvGC2049 Δ(ara-leu) galT12 bglR*	Harms et al. (11)
CU1230	*ΔilvGE2130 ara Δ(pro-lac) thi recA56 hsdR*	Harms et al. (11)
CU1240	*dcm dam-3 metB1 galK2 galT22 lacY1 tsx-18 supE44* F'	R. Young, Texas A & M University, through J. Brenchley
CU1318	*leuB6 proA2 trpD9579 his-4 argEB thi-1 hsdR18 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 tonB56 supE44 recB21 recC22 sbcB15* λ^-	B. Bachmann
CU1436	*leuB6 proA2 trpB9579 his-4 argE-3 thi-1 hsdR18 ara-14 lacY1 galK2 xyl-5 mtl-1 rpsL31 tsx-33 tonB56 supE44 recB21 recC22 sbcB15 ilvB2102*::Ω λ^-	Transformation of CU1318 with linear pPU169 by M. Rudinski
CU1458	*ΔilvGC2049 Δ(ara-leu) galT12 bglR ilvB2102*::Ω	P1 transduction of CU1183 with CU1436 as donor
CU1460	*ΔilvGC2049 Δ(ara-leu) galT12 bglR ilvB2102*::Ω *zig-117*::Tn*10 polA1 metE*	P1 transduction of CU1458 with CH1274 as donor
CH1274	*trpA46 lysA xyl metE zig-117*::Tn*10 polA1 argH*	From C. W. Hill
JM103	F' *traD36 proAB*$^+$/*Δ(pro-lac) lacI supE44 thi lacZ* ΔM15 *strA endA1 sbcB15 hsdR4*	Yanisch-Perron et al. (38)

(28); (ii) the relative concentrations of the three amino acids competing for entry into the cell, which is changing very rapidly as the density of the culture approaches the limit set by the concentration of the limiting amino acid; (iii) the response of the deattenuation mechanism to the limiting amino acid; and (iv) the relative amount of the limiting amino acid required for the formation of the proteins specified by the derepressed operon.

Site-specific mutagenesis. Basically, the procedures for site-specific mutagenesis with oligonucleotide primers and single-stranded templates were those of Zoller and Smith (39). The template was a bacteriophage M13mp10 derivative containing the 0.9-kilobase-pair (kb) *Pvu*I-*Hin*dIII *S. marcescens ilvGMEDA* leader fragment (see below). To obtain the mutations carried by pPU145 and pPU146 (Fig. 1), oligonucleotides, i.e., a 19-mer, 5'-CTAGGCCGATCACTT-GGGC-3', and a 21-mer, 5'-CTAATCACGCCCG-GGCTAATC-3', respectively, were used as primers for second-strand synthesis by Klenow fragment of DNA polymerase I. The oligonucleotides were synthesized with an ABI synthesizer (model 380A) by Philip Andrews, Biochemistry Department, Purdue University. To be able to trace newly synthesized closed circular molecules after separation on alkaline sucrose gradients, second-strand synthesis was initiated for 15 min in the presence of [^{32}P]dCTP (15 μCi, 800 Ci/mmole) and 2.5 μM dCTP and completed in the presence of 0.5 mM dCTP. The primer used to make the mutation in pPU144 (CTA to CTG) was a 550-base-pair (bp) *Eco*RII fragment beginning with the site specifying the serine codon of the *ilvGMEDA* leader and ending with that specifying amino acid 88 in the *ilvG* gene of *Escherichia coli* K-12. The fragment was isolated from pPU119 (1). The 3' recessed ends were filled in before the fragment was used as a primer with Klenow fragment of DNA polymerase I. All alterations resulted in the introduction of new restriction endonuclease cleavage sites into the modified plasmids so that the desired mutants were readily recognized in quick-screen assays of the transformants. Closed circular DNA molecules were isolated on alkaline sucrose gradients, and the pooled fractions were dialyzed and used to transform JM103. Infected clones were identified as colorless plaques and purified by single-colony isolation (5). Replicative-form DNA was isolated by the alkaline lysis procedure (24) and used for restriction analysis.

M13 phage and plasmid constructions. The template for site-specific mutagenesis was obtained by inserting a 0.9-kb *Pvu*I-*Hin*dIII fragment, after removing the 3' protruding end of the *Pvu*I site with T4 polymerase, into the *Sma*I-*Hin*dIII site of M13mp10 replicative form. This fragment was derived from pPU129 and contained the *ilvGMEDA* leader sequence of *S. marcescens* and a portion of the *ilvG* gene (11).

After mutagenesis and screening, the mutant leader fragments were fused to the contiguous *ilv'GME* region in two ways (Fig. 2). The mutant leader fragments were transferred to pUC8 by using the *Eco*RI and *Hin*dIII sites of both phage and plasmid to yield derivatives pPU144, pPU145, and pPU146. pPU147 (containing *S. marcescens-E. coli* hybrid *ilv* leader and *ilvG* gene and an *S. marcescens ilvE* gene) was obtained by inserting the 0.9-kb *Eco*RI-*Hin*dIII fragment of pPU144 into the same sites in pPU143. pPU143 is a pBR322

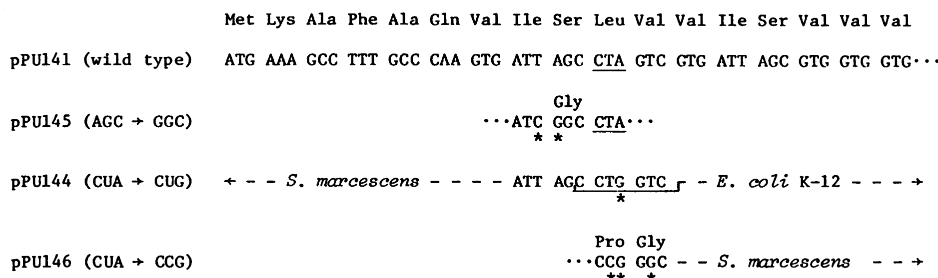

FIG. 1. *S. marcescens* attenuator mutants. A portion of the wild-type leader carried by pPU141 is shown. Only the bases in the vicinity of the base changes (*) for the mutants are shown. The hybrid leader (in pPU144) begins with bases from *S. marcescens* and continues with bases from *E. coli* from the sequence CCTGGTC onward.

TABLE 2. Plasmids used

Plasmid	Genotype[a]	Description	Source or reference
pUC8	*bla*+ *lacZpZoZ'*	Primary cloning vector with multiple cloning sites	Vieira and Messing (37)
pKK232-8	*bla*+	Promoter selection vector	Brosius (3)
pHP45Ω	*bla*+ *aadA*+	pBR322 derivative containing Ω (*aadA* gene of the R100.1 plasmid)	Prentki and Krisch (27)
pPU129	pBR322 Ω(0.0 kb::*S. marcescens GEDA'* 9.5 kb)*74*	Carries *S. marcescens ilvGMEDA* genes inserted into *Eco*RI site of PBR322	Harms et al. (11)
pPU131	pUC13 Ω[0.428 kb::pPU43 *ilvBN* 3.4 kb(−)]*1*	*Hin*cII fragment of pPU43 carrying the *E. coli ilvBN* genes inserted in the *Hin*cII site of pUC13	Hsu et al. (14)
PU132	pUC8 Δ[0.399–0.415 kb]*1* Ω[0.400 kb::pPU131 *ilvB'* 0.43–2.05 kb(+)]*1*	1.62-kb *Hin*cII-*Hin*dIII fragment containing the proximal portion of the *ilvB* gene inserted into the multiple cloning sites of pUC8	Hsu et al. (14)
pPU134	pPU132 Ω[2.05 kb::pPU129 *ilvGMEDA'* 9.5 kb(+)]*1*	*S. marcescens ilvGMEDA'* genes from pPU129 inserted into the *Eco*RI site of pPU132	Hsu et al. (14)
pPU141	pUC8 Δ[0.280–0.400 kb]*2* Ω[0.280 kb:: *S. marcescens ilvGpG'* 0.89 kb(+)]*2*	Carries *S. marcescens ilvGMEDA* leader in the *lacZ'* region of pUC8	0.89-kb *Pvu*I-*Hin*dIII fragment carrying the *S. marcescens ilvGMEDA'* leader ligated with *Pvu*I (partial)-*Hin*dIII-digested pUC8, by J.-H. Hsu (Hsu, Ph.D. thesis)
pPU143	pBR322 Ω(0.029 kb::*S. marcescens ilv'GMED'* 3.7 kb)*82*	Contains the *S. marcescens ilv'GMED'* region inserted into the *Hin*dIII site of pBR322	3.7-kb *Hin*dIII fragment of *S. marcescens* ligated with *Hin*dIII-digested pBR322 (Hsu, Ph.D. thesis)
pPU144	pUC8 Δ(0.399–0.415 kb) Ω[0.406 kb:: *ilvGpG'* (*S. marcescens* 0.25 kb *E. coli* 0.55 kb *S. marcescens*::0.089 kb) 0.89 kb(+)]*9*	Contains an *ilv S. marcescens-E. coli* hybrid leader fragment inserted into the multiple cloning site of pUC8; the hybrid results in a CUA-to-CUG conversion at the regulatory Leu codon of the *S. marcescens* leader carried in pPU141	0.9-kb *Eco*RI-*Hin*dIII fragment of the M13mp10 phage carrying the *S. marcescens-E. coli* hybrid leader region (see text) was inserted into *Eco*RI-*Hin*dIII-digested pUC8
pPU145	pUC8 Δ(0.399–0.415 kb)*9* Ω[0.399 kb::*S. marcescens* mutant *ilvGpG* 0.89 kb (+)]*10*	Carries a mutant *S. marcescens* leader region with an AGC (Ser-9)[b]-to-GGC (Gly-9) change in the multiple cloning site of pUC8	0.9-kb *Pvu*I-*Hin*dIII *ilv* leader fragment from an M13mp10 derivative subjected to oligonucleotide-directed mutagenesis[c]
pPU146	pUC8 Δ(0.399–0.415 kb)*10* Ω(0.399 kb:: *S. marcescens* mutant *ilvGpG* 0.89 kb)	Carries a mutant *S. marcescens* leader region with a CTAGTC (Leu-10, Val-11)-to-CCGGGC (Pro-10, Gly-11) change in the multiple cloning site of pUC8	Construction as above from a different M13mp10-mutagenized derivative
pPU147	pPU143 Δ(0.0–0.02 kb)*1* Ω[4.362::pUC8 (34 bp *S. marcescens* 0.25 kb *E. coli* 55 bp *S. marcescens* 89 bp) *ilvGpG'* 0.92 kb(+)]*1*	Carries the hybrid *ilv* leader region with the CTA→CTG change and carried in pPU144 inserted into *Eco*RI-*Hin*dIII region of pPU143 to yield contiguous *ilvGMED'* DNA	0.92-kb *Eco*RI-*Hin*dIII fragment from pPU144 ligated into *Eco*RI-*Hin*dIII-digested pPU143
pPU151	pPU145 Ω[1.30 kb::pPU143 *ilv'GMED'* 3.7 kb(+)]*1*	Carries the *'GEMD'* fragment from pPU143 inserted into *Hin*dIII site of pPU145 to obtain contiguous *ilvGMED'* DNA controlled by the AGC (Ser-9)→GGC (Gly-9) mutant leader region	3.7-kb *Hin*dIII fragment of pPU143 ligated into *Hin*dIII-digested pPU145
pPU152	pPU146 Ω[1.30 kb::pPU143 *ilv'GMED'* 3.7 kb(+)]	Carries the *ilv'GMED'* fragment from pPU143 inserted into *Hin*dIII site of pPU145 to yield contiguous *ilvGMED'* DNA, controlled by the CCGGGC (Pro-10, Gly-11) mutant leader region	3.7-kb *Hin*dIII fragment of pPU143 ligated into *Hin*dIII site of pPU146
pPU160	pUC8 Δ(0.399–0.415 kb)*4* Ω[0.415 kb:: pPU131 *ilv'BN* 2.05–3.83 kb(−)]*4*	Carries the *E. coli ilv'BN* region inserted into the multiple cloning site of pUC8	1.78-kb *Hin*dIII-*Hin*cII fragment from pPU131 containing the promoter distal portion of *ilvB* and the adjacent *ilvN* gene ligated with *Hin*dIII-*Hin*cII-digested pUC8, by J.-H. Hsu
pPU169	pPU131 Ω(2.03 kb:: pHP45Ω 2.32–4.32 kb)*1*	Carries the *E. coli ilvBN* region with the Ω fragment of pHP 45Ω in *ilvB*	2-kb *Hin*dIII-flanked Ω fragment of pPU45 inserted into *Hin*dIII site of pPU131, by M. F. Rudinski
pPU187	pPU132 Δ(2.02–2.03 kb)*1* Ω[2.03 kb:: pPU147 *ilvGpGMED'* 4.9 kb(+)]*1*	Carries the *S. marcescens ilvGMED'* region under the control of the CTA→CTG leader region from	4.9-kb fragment of *Eco*RI-*Bam*HI fragment from pPU147 ligated with *Eco*RI-*Bam*HI-digested pPU132

Continued on following page

TABLE 2—*Continued*

Plasmid	Genotype[a]	Description	Source or reference
		pPU147 in the pUC8 multiple cloning site of pPU132 which carries the *E. coli ilv'BN* region	
pPU189	pPU132 Δ(2.03 kb-CC-)2[d] Ω[2.03 kb::pPU151 *ilvGpGMED'* 4.6 kb]2	Carries the *S. marcescens ilvGMED'* region from pPU151 under the control of the AGC (Ser-9)→GGC (Gly-9) mutant leader region in the pUC8 multiple cloning site of pPU132	4.6-kb *Eco*RI-*Hin*dIII fragment from pPU151 with a filled-in *Hin*dIII site and ligated with *Eco*RI-*Sma*I-digested pPU132
pPU190	pPU160 Δ(2.20 kb-CC-)*1* Ω(2.20 kb::pPU152 *ilvGpGMED'* 4.6 kb)*1*	Carries the *S. marcescens ilvGMED'* region from pPU152 under the control of the CCGGCC (Pro-10, Gly-11) mutant leader region in the pUC8 multiple cloning site of pPU160	4.6-kb *Eco*RI-*Hin*dIII fragment from pPU152 with a filled-in *Hin*dIII site ligated with *Eco*RI-*Sma*I-digested pPU160
pPU191	pKK232-8 Δ(0.182–0.186 kb)*1*[e] Ω[0.182 kb::pPU132 *ilvB'* 1.62 kb(−)]*1*	Carries the *E. coli ilvB'* region from pPU132 in the multiple cloning site of pKK232-8	1.62-kb *Bam*HI-*Hin*dIII *ilvB'* fragment of pPU132 with a filled-in *Hin*dIII site ligated with *Bam*HI-*Sma*I-digested pKK232-8
pPU193	pPU191 Ω[1.81 kb::pPU141 *ilvGpG'* 0.91 kb(+)]*1*	*cat* gene of pPU191 under control of *S. marcescens* wild-type *ilvG* leader	0.9-kb *Eco*RI-*Hin*dIII *ilvGpG'* fragment from pPU141, with *Eco*RI site filled in and ligated with *Bam*HI-*Hin*dIII-cut pPU191 after *Bam*HI site was filled in
pPU194	pPU191 Ω[1.81 kb::pPU144 *ilvGpG'* 0.91 kb(+)]*2*	*cat* gene of pPU191 under control of *S. marcescens* CTA→CTG mutant leader region	Prepared as pPU193 was, except *ilvGpG'* fragment derived from pPU144
pPU203	pPU191 Ω[1.81 kb::pPU145 *ilvGpG'* 0.91 kb(+)]*3*	*cat* gene of pPU191 under control of *S. marcescens* AGC (Ser-9)→GGC (Gly-9) mutant leader region	Prepared as pPU193 was, except *ilvGpG'* fragment derived from pPU145
pPU204	pPU191 Ω[1.81 kb::pPU146 *ilvGpG'* 0.91 kb(+)]*4*	*cat* gene of pPU191 under control of *S. marcescens* CCGGCC (Pro-10, Gly-11) mutant leader region	Prepared as pPU193 was, except *ilvGpG'* fragment derived from pPU146

[a] The genotype designations follow the conventions recommended by Novick et al. (26) and those in Instructions to Authors. They are similar to those followed in earlier publications. The convention for the designation of cleavage sites in plasmids for which the entire nucleotide sequence is known is that the first T in the *Eco*RI recognition site is designated base 1 in derivatives of pBR322 and the first T in the sequence TCGCGCGTTC is designated base 1 in derivatives of pUC plasmids. The position shown for restriction endonuclease cleavage sites is that of the 5' base at the site of cleavage. Thus, the *Eco*RI site is at position 4362 in pBR322 and at position 430 in pUC8. The sites of insertion or deletion in some previously described plasmids have been changed in accordance with these conventions. (+) indicates that the orientation of the insert is the same with respect to vector as the chromosome and vector maps are usually drawn. (−) indicates that the orientation of the insert with respect to the vector is different from the usual map respresentation. Thus, the orientation of both *ilv* inserts in pPU134 (Fig. 2) is (+).

[b] Amino acid residue refers to the putative 32-residue leader peptide.

[c] For mutant generation, see text.

[d] The deletion resulted from removal of two C · G base pairs in the multiple cloning site.

[e] kb designations of the multiple cloning site in pKK232-8 are deduced from Brosius (3).

derivative with a 3.7-kb insertion in the *Hin*dIII site carrying *ilv'GMED'* of *S. marcescens* (J.-H. Hsu, Ph.D. thesis, Purdue University, West Lafayette, Indiana, 1986). Recombinant plasmids were selected for by complementation of the *ilvGME'2130* deletion in strain CU1230 (growth on minimal medium containing ampicillin [25 μg/ml]). pPU187, a plasmid that would allow recombination with the *E. coli* chromosome outside of the *ilv* gene cluster, was made by inserting the 4.9-kb *Eco*RI-*Bam*HI (*ilvGMED'*) fragment of pPU147 into the *Eco*RI-*Bam*HI sites of pPU132 (Fig. 2). pPU132 is a pUC8 derivative that carries a 1.6-kb *Hin*cII-*Hin*dIII fragment of the *E. coli* K-12 *ilvB* gene in its multiple cloning site (14).

The two other leader mutants were carried through a different construction scheme. The 3.7-kb *Hin*dIII fragment of pPU143 (*S. marcescens ilv'GMED'*) was inserted into the *Hin*dIII site of pPU145 and pPU146 to obtain pPU151 and pPU152, respectively. The recombinant plasmids were transformed into strain CU1230 and selected for by complementation of Δ*ilvGME2130*. Finally, the 4.6-kb fragments obtained by *Eco*RI and limited *Hin*dIII digestion of pPU151 and pPU152, carrying *ilv* DNA, were inserted into the *Eco*RI-*Sma*I sites of pPU132 and pPU160. The recessed 3' ends left by *Hin*dIII cleavage were filled in by Klenow fragment before treatment with *Eco*RI. pPU160, a pUC8 derivative, contains a 1.8-kb *Hin*dIII-*Hin*cII fragment of *ilvB* from *E. coli* (Hsu, Ph.D. thesis). These final constructs, pPU189 and pPU190 (Fig. 2), were recombined with the *E. coli* chromosome as described below.

Construction of pKK232-8 derivatives. pPU191, a derivative of pKK232-8, a promoter selection vector described by J. Brosius (3), was constructed by introducing the 1.6-kb *Bam*HI-*Hin*dIII *ilvB* fragment of pPU132 into the *Bam*HI and *Sma*I sites of pKK232-8. The *Hin*dIII site was filled in. pPU191 was then cut with *Bam*HI, and its recessed 3' ends were filled in and then cut with *Hin*dIII. This linear plasmid was then ligated separately with the 0.9-kb *Eco*RI-*Hin*dIII mutant and wild-type leader fragments from pPU141, pPU144, pPU145, and pPU146. The *Eco*RI ends of each plasmid were filled in before treatment with *Hin*dIII. The inserts were thus flanked by a filled-in *Eco*RI/*Bam*HI hybrid site and a *Hin*dIII site. This construction scheme ensured that transcription from the *ilv* promoter proceeded toward the chloramphenicol acetyltransferase (*cat*) gene. Recombinant plasmids were transformed into strain CU1230 and selected for by the ability to grow in the presence of

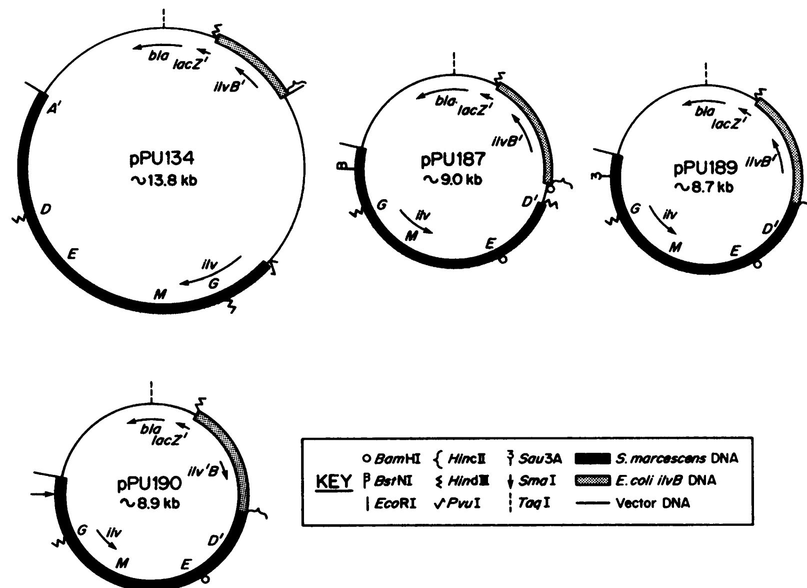

FIG. 2. Final plasmid constructs introduced into strains CU1330 and CU1460. The *ilvGMED'* genes are preceded by wild-type (pPU134) and mutant (pPU187, CUA→CUG; pPU189, AGC→GGC; pPU190, CUAGUC→CCGGGC) *ilv* regulatory regions.

chloramphenicol (30 μg/ml). The plasmids obtained were pPU193, pPU194, pPU203, and pPU204 (Fig. 3).

Integration of plasmids into the *E. coli* chromosome. Since the copy number of plasmids varies with different growth conditions, interpretation of results is often difficult. This problem was avoided by introducing the plasmids as single copies into the chromosome as described by Hsu et al. (14). To provide homology with the chromosome, either a 1.6-kb *Hin*cII-*Hin*dIII or a 1.8-kb *Hin*dIII-*Hin*cII fragment of the *ilvB* gene was inserted into the plasmids as described above. The plasmids were designed so that transcription of the *ilvGMEDA* leader and the *ilvB* fragment (when the 1.6-kb fragment was used) would proceed in opposite directions. The final plasmid constructs that were integrated into the chromosome are shown (Fig. 2 and 3). The recipient strains

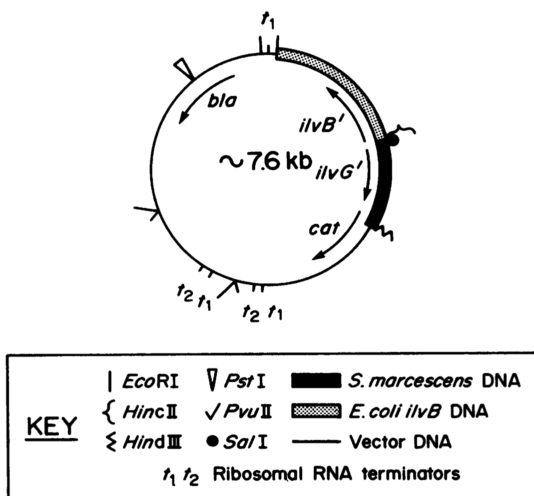

FIG. 3. Final plasmid construct of pKK232-8 derivatives pPU 193 (wild type), pPU194 (CUA→CUG), pPU203 (AGC→GGC), and pPU204 (CUAGUC→CCGGGC). These plasmids were transferred to strains CU1330 and CU1460.

CU1330 and CU1460 carry the *polA1* marker, which does not allow independent replication of plasmids that contain the ColE1 replication system (10). They also have the entire *ilv* cluster deleted. Furthermore, strain CU1460 contains a deletion of the *ilvIH* gene and an inactivated *ilvB* gene. Transformants of CU1330 and CU1460 were selected in the presence of low concentrations of ampicillin (10 μg/ml) or chloramphenicol (5 μg/ml) to minimize possible integration of multiple copies of the plasmids. Drug-resistant colonies were scored for the IlvE$^+$ phenotype (growth on minimal medium containing the α-keto acid analogs of isoleucine and valine) and for the *polA1* marker (no growth on L agar containing methyl methanesulfonate). As a preliminary test for integration of plasmid into the chromosome, DNA of the resulting strains was isolated and analyzed on ethidium bromide-stained agarose gels. Furthermore, the DNA was digested with the appropriate restriction enzyme and subjected to Southern blot analysis with a ^{32}P-labeled plasmid-derived probe.

Enzyme assays. Preparation of cell extracts and acetohydroxy acid synthase and transaminase B assays were described earlier (32). Valine-resistant acetohydroxy acid synthase was measured in the presence of 1 mM valine. CAT activity was measured as described by Gorman et al. (9) for mammalian cells with minor changes. Cultures (10 ml) were grown under repressing and derepressing conditions (7) and harvested by centrifugation. Cells were suspended in 500 μl of 0.25 mM Tris hydrochloride (pH 7.5) and disrupted by sonic oscillation. Cell debris was removed by centrifugation, and the supernatant fluid was assayed for enzyme activity. The reaction mixture contained 0.15 μCi of [^{14}C]chloramphenicol (45.5 mCi/mmole), 1 mM acetylcoenzyme A, and 0.11 M Tris hydrochloride (pH 7.5) in a final volume of 100 μl. After 15 min of incubation at 37°C, the chloramphenicol was extracted twice with 1 ml and 0.4 ml of cold ethyl acetate, dried, and suspended in 15 μl of ethyl acetate. In some experiments, different amounts of extract or incubation times were used. The samples were subjected to ascending chromatography on silica gel thin-layer plates (Eastman Kodak Co., Rochester, N.Y.) in chloroform-methanol

(95:5). After autoradiography, the areas containing separated [^{14}C]chloramphenicol and acetylated chloramphenicol were removed, and the radioactivity was determined in a scintillation counter.

Protein was determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, Calif.).

DNA preparation and characterization and nucleotide sequence determination. Chromosomal DNA was isolated by the method of Schleif and Wensik (30), and plasmid DNA was isolated by the method of Sidikaro and Nomura (31). Cells were transformed with hybrid plasmids as described by Lederberg and Cohen (22). For restriction enzyme digestions and ligation reactions, the procedures suggested by the suppliers were followed. T4 polymerase was used to remove 3′ protruding ends from DNA fragments, and Klenow fragment was used to fill in 3′ recessed ends, as described by Maniatis et al. (24). DNA fragments were isolated from agarose gels by the method of Maniatis et al. The integration of a single copy of plasmids into the chromosome was examined by the membrane transfer technique of Southern (33). Probes were radiolabeled by nick translation as described earlier (11). Nucleotide sequences were analyzed by the dideoxy chain-termination method of Sanger et al. (29) to verify the mutations obtained. In pPU144, the nucleotide sequence of the *S. marcescens-E. coli* K-12 hybrid junctions within the leader and within *ilvG* were determined to ensure the retention of the *ilvG* reading frame.

RNA secondary structure analysis. The computer programs FISTEM and STEMER, kindly provided by P. T. Gilham, Department of Biological Sciences, Purdue University, were used to derive secondary structures for wild-type and mutant leader transcripts.

Chemicals and molecular biological reagents. Amino acids and thiamine hydrochloride were purchased from Sigma Chemical Co., St. Louis, Mo., and Calbiochem-Behring, La Jolla, Calif. T4 DNA ligase and restriction endonucleases were obtained from Bethesda Research Laboratories, Gaithersburg, Md., Boehringer Mannheim Biochemicals, Indianapolis, Ind., and New England BioLabs, Beverly, Mass. Klenow fragment and 5-bromo-4-chloro-3-indolyl-β-D-galactoside were obtained from Boehringer Mannheim, and T4 polymerase was obtained from New England BioLabs. The M13 nucleotide sequence analysis kit and isopropyl-β-D-thio-galactopyranoside were purchased from Bethesda Research Laboratories. [^{32}P]dATP, [^{32}P]dCTP, and the nick translation kit were obtained from Amersham Corp., Arlington Heights, Ill. [^{14}C]chloramphenicol was bought from New England Nuclear Corp., Boston, Mass. The vector pKK232-8 was obtained from Pharmacia, Inc., Piscataway, N.J.

RESULTS

Mutations in the *S. marcescens ilv* regulatory region. If derepression of the *ilvGMEDA* operon of *S. marcescens* by limiting leucine is as efficient as it is because the single leucine codon in the protector stem of the leader is the rarely used CUA, then its replacement by CUG should abolish or reduce that derepression. Such a replacement was conveniently accomplished by constructing an *S. marcescens-E. coli* hybrid leader (Fig. 1). The *E. coli* K-12 *ilv* leader contains an *Eco*RII restriction endonuclease cleavage site spanning its leucine codon at that position, which is CUG. A 550-bp *Eco*RII fragment was isolated from pPU119 (1) grown in the *dam dcm* strain, CU1240, and the 3′ recessed ends were filled in with Klenow fragment of DNA polymerase I.

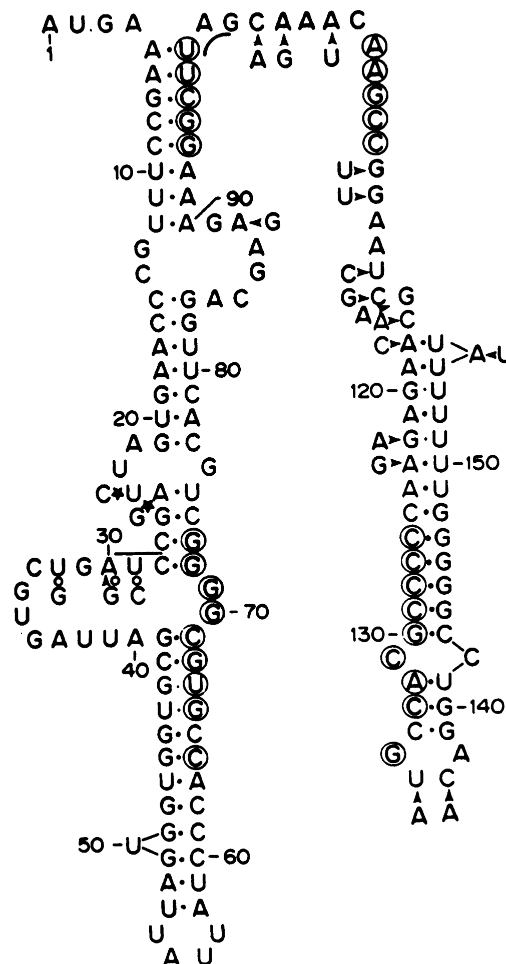

FIG. 4. Postulated secondary structure of *S. marcescens ilvGMEDA* operon regulatory region. The base changes introduced into the mutant leader transcripts are indicated. Symbols: ➤ pPU144 (CUA→CUG, *S. marcescens-E. coli* hybrid leader transcript; ★, pPU145 (AGC→GGC, Ser→Gly); ○, pPU146 (CUAGUC→CCGGGC, Leu Val→Pro Gly). In both pPU144 and pPU146, the adenine at position 30 is changed to a guanine. Bases capable of pairing to yield the preemptor are circled, as are bases capable of forming the pre-preemptor. The leucine and stop codons are indicated by black bars.

This fragment was used as a primer for second-strand synthesis of single-stranded phage M13 template with a 0.9-kb *Pvu*I-*Hin*dIII DNA fragment insertion containing the *S. marcescens* leader and part of the *ilvG* gene. After isolation of closed circular molecules on alkaline sucrose gradients, dialysis, and transformation of JM103, plaques were tested for the M13 recombinant replicative form containing a new *Bst*N1 (an isochizomer of *Eco*RII able to restrict methylated DNA) restriction endonuclease recognition site. Such a recombinant phage was chosen for further investigation. This mutagenesis procedure resulted in an *ilv* leader and an *ilvG* gene that were both hybrids between *S. marcescens* and *E. coli* K-12. The DNA nucleotide sequences of the hybrid junction areas were determined to verify the CTA→CTG conversion in the leader and to ascertain the nature of the junction within *ilvG*. This analysis confirmed the A-to-G change at the nucleotide position 30 (Fig. 4) in the leader. At the hybrid junction within *ilvG*,

however, the nucleotide sequence was expected to be GTC CTG GC (Val Leu Gly), which includes the *Eco*RII recognition sequence (CCTGG) that was carried at the end of the *Eco*RII primer fragment (and derived from *E. coli*) which was used for mutagenesis. Instead, GTC CTC GGC was found, maintaining the Leu codon but changing its third base to C, as found in *S. marcescens* (Hsu, Ph.D. thesis). This inversion might have occurred through incomplete hybridization, the presence of contaminating exonuclease during fragment isolation, or a mismatch repair, since the priming DNA used was propagated in a *dcm dam E. coli* strain. The reading frame of *ilvG* was maintained. The mutant leader was placed back into its natural environment upstream of *ilvG* by using a variety of plasmid constructions, as described in Materials and Methods (Fig. 2). The plasmids were transferred to strain CU1230 (Δ*GME2130*) by selecting transformants for drug resistance and scoring for complementation of the *ilvGME* deletion (growth on minimal medium containing valine). The *E. coli ilvG* DNA used in these constructions carries an *ilvO* mutation which compensates for the frameshift that occurs in the K-12 *ilvG* gene and thus allows formation of acetohydroxy acid synthase II activity (11, 20).

Two more *S. marcescens* leader mutants were created to aid in this analysis (Fig. 1). In one, pPU146, the CUA leucine and the adjacent downstream GUC valine codons were changed to CCG (proline) and GGC (glycine) codons. Therefore, one would predict the loss of leucine-mediated control by attenuation with this mutant leader. The mutation in pPU145 converted the AGC serine codon upstream and adjacent to the rare leucine codon into a GGC-glycine codon. This modification was designed to examine whether the presence of peptidyl seryl tRNA with its larger extra arm made it more difficult for a leucyl tRNA to bind to the A site. If such were to occur, the sensing of low leucyl tRNA levels by a single codon might be facilitated. In addition, these mutations introduced new restriction sites into the *S. marcescens* leader, a *Sma*I and *Sau*3A site, respectively (Fig. 2). These sites were used in screening for the mutant *ilv* leader sequences. The mutations were verified by nucleotide sequence analysis and then used to replace the parental type leader region in plasmids containing the *ilvGME* genes (Fig. 2). The mutant plasmids were introduced into *polA* strains (to force chromosomal integration [10]) for comparison with the parental type leader.

Construction of the pKK232-8 derivatives is described in Materials and Methods and shown in Fig. 3.

Expression of *ilvG*, *ilvM*, and *ilvE* genes under control of mutant leader regions. Plasmid-derived valine-resistant acetohydroxy acid synthase and transaminase B activities were determined under conditions of excess and limiting branched-chain amino acids in strains CU1330 and CU1460 (Table 3). The construction of pPU134 (carrying the wild-type *S. marcescens ilv* leader and *GMEDA'* genes) and its integration into CU1330 was done by Jung-Hsin Hsu in our laboratory and was described previously (14). On separately limiting for valine, isoleucine, or leucine, both acetohydroxy acid synthase and transaminase B activities were derepressed. The derepression accompanying valine or isoleucine limitation was readily explained since the *S. marcescens* leader contained a distribution of isoleucine and valine codons identical to that in the *E. coli* leader. It had been concluded that the derepression obtained with limiting leucine was also due to deattenuation and resulted from ribosomes stalling at the single leucine codon in the leader sequence (14). In pPU187, the CUA was changed to CUG.

TABLE 3. Expression of *ilvGM* and *E* genes of *S. marcescens* under control of mutant regulatory regions

Plasmids and growth conditions	Activity (nmol/min per mg of protein) for strain:		
	CU1330[a]		CU1460[b]
	AHAS[c]	TRB[d]	AHAS
pPU134 (wild type)			
Repressing	1.3	1.1	4.2
Limiting valine	18.6	62.9	44.1
Limiting isoleucine	19.0	84.0	38.7
Limiting leucine	13.2	87.5	36.9
pPU189 (AGC→GGC)			
Repressing	1.1	0.7	4.0
Limiting valine	15.1	31.4	22.3
Limiting isoleucine	20.9	32.0	42.4
Limiting leucine	15.5	37.3	24.5
pPU187 (CUA→CUG)			
Repressing	1.3	0.5	2.8
Limiting valine	15.4	56.6	31.8
Limiting isoleucine	17.8	52.9	36.7
Limiting leucine	3.6	7.8	5.6
pPU190 (CUAGUC→CCGGGC)			
Repressing	1.0	3.1	6.0
Limiting valine	8.6	39.4	18.3
Limiting isoleucine	9.4	37.5	21.0
Limiting leucine	2.8	4.2	4.8

[a] Plasmids were integrated into host strain CU1330 Δ(*ilvGC2049 leu455 galT12 metE zig117*::Tn*10 polA*).

[b] Host strain CU1460 Δ[*ilvG-C2049* Δ(*ara-leu*) *ilvB2102*::Ω *bglR metE galT12 zig117*::Tn*10 polA*].

[c] Valine-resistant acetohydroxy acid synthase activity.

[d] Transaminase B activity.

Growth with limiting valine or isoleucine resulted in derepression of the *ilvG* and *ilvE* genes comparable to that in the wild type. Under conditions of limiting leucine, there was derepression, but it was reduced 3.6-fold for acetohydroxy acid synthase and 5-fold for transaminase B from that in pPU134 (wild type). (It should be noted that the *ilvG* gene of pPU187 is a hybrid gene with the promoter proximal 420 bp derived from *E. coli* K-12 and the rest from *S. marcescens*.)

In pPU190, the leucine codon was changed to a proline codon; thus, no leucine codon is located in that part of the leader thought to be important in generating a response to amino acid limitation. Surprisingly, the derepression of acetohydroxy acid synthase activity under conditions of limiting leucine was the same as that observed with the leader with the commonly used leucine codon (pPU187). Transaminase B activity, however, exhibited significantly less activity upon leucine limitation with pPU190 than it did with pPU187, with an apparent derepression well within experimental error, as was to be expected if leucine control was abolished. Since the apparent response of acetohydroxy acid synthase activity to leucine limitation might have been due to incomplete inhibition of the valine-sensitive acetohydroxy acid synthase activities which would have been derepressed by limiting leucine, all the plasmids were introduced into another *polA1* strain, CU1460. This strain contained deletions removing the genes for acetohydroxy acid synthase II and III and an inactivating insertion in the *ilvB* gene that prevented synthase I formation.

The behavior of strain CU1460 was unexpected, since the valine-resistant acetohydroxy acid synthase activity under repressing conditions in most transformed derivatives, as well as in the parental strain itself, was actually higher than

that in strain CU1330. Tests with the untransformed strain demonstrated that this activity was essentially unaffected by branched-chain amino acid supplements and from about 30 to 50% inhibited by valine (data not shown). Thus, the activity is clearly independent of the three major isozymes. Although this low activity may be present in all strains and is masked by even the repressed levels of the other acetohydroxy acid synthases, it is more likely that the activity is the result of some compensatory mutation in this background lacking the three major isozymes. Nevertheless, even with this basal level of nonrepressible and partially valine-resistant acetohydroxy acid synthase activity, the results are in accord with those obtained with the same plasmids in strain CU1330. With pPU190, the plasmid lacking a leucine regulatory codon, there appeared to be a complete loss of leucine control.

pPU189 carries the rare leucine codon but contains a glycine instead of a serine codon adjacent to it. This change was introduced to determine whether the ease of entrance of leucyl tRNA into the A site of a ribosome was reduced by the presence at the P site of a peptidyl tRNA that has a large extra arm (34). Such a reduced affinity for an amino acyl tRNA might result in an increased sensitivity in the process of sensing the charging level of that tRNA. Substitution of the serine codon by a glycine codon would result in a peptidyl tRNA in which the extra arm was small. Comparison of enzyme activities with pPU134 (wild type) in either strain CU1330 or CU1460 did not reveal a significant difference under the conditions tested. Thus, a context effect on attenuation based simply on the size of the extra arm of tRNA can probably be eliminated.

Expression of the *cat* gene under the control of mutant leader regions. Because of the complication arising from the basal level of acetohydroxy acid synthases I and III in strain CU1330 and the apparently new activity in strain CU1460, an alternative method to evaluate the effect of mutant leaders on downstream gene expression was used. The method chosen was to place them upstream of the *cat* gene in vector pKK232-8 (3). Transcriptional terminators upstream of the insertion sites prevent any transcription into the *cat* gene except that initiated at the inserted promoter region. Expression of the *cat* gene in pPU193 under control of the wild-type leader region responded to conditions of excess and limiting branched-chain amino acids, as does *ilv* operon expression. The finding confirms that the *cat* gene is regulated by the *ilv* control region (Table 4). Derepressed enzyme levels were comparable under limiting valine, isoleucine, or leucine conditions. However, pPU194 (CUA to CUG) exhibited only one-sixth of the derepression of the CAT activity that the wild-type strain exhibited when leucine was limiting. The response to limiting leucine by the strain carrying pPU204, which completely lacks a leucine codon in the critical position, seemed to be negligible. On the other hand, regulation by either valine or isoleucine of expression of the *cat* gene was retained in strains carrying the mutant regulatory regions. These data fully support the results shown in Table 3. The Ser-to-Gly leader mutation in pPU203 did not seem to have an effect on its regulatory properties and is in accord with the results obtained with pPU189 (Table 3).

DISCUSSION

Our experiments with the *ilvGMEDA* leader sequence of *S. marcescens* lead to the conclusion that the effectiveness of a single leucine codon in mediating control by attenuation is due at least in part to the fact that it is a rare codon. Its substitution by a single, commonly used one reduces derepression to less than 30% of that observed with the parental leader. All other amino acid biosynthetic operons known to be regulated by an attenuation mechanism contain two or more codons of the regulatory amino acids in critical positions. The question arises whether attenuation is the sole mechanism by which leucine controls the expression of the *S. marcescens ilv* operon. Several experiments support the idea that it is. (i) Hsu et al. (14) demonstrated that derepression was obtained on raising the temperature of a *leuS* mutant of *E. coli* that carried the *S. marcescens ilv* genes in a single copy. (ii) An *S. marcescens ilv* leader mutant in which the terminator stem had lost one G · C pair responded neither with repression to the presence of excess branched-chain amino acids nor with derepression if any one of the three was limiting (Hsu, Ph.D. thesis). (iii) If the leucine codon was replaced by one specifying a nonregulatory amino acid (pPU190), derepression by limiting leucine was negligible.

These results do not justify postulating any additional end product-specific control mechanisms. There have been reports of derepression of *ilv* gene expression by mutations that seem unlikely to have affected attenuation (6, 16, 18). These mutations may have resulted in modified proteins that do interfere with *ilv* transcription in vivo, but such interference is not modulated by the levels of the three branched-chain amino acids. Lynn et al. (23) showed that regulation of the *thr* operon of *E. coli* by threonine and isoleucine is abolished in mutants missing threonine and isoleucine codons in the leader peptide. Thus, with this operon, too, the derepression resulting from the mutation described by Johnson and Somerville (16) does not seem to have affected an end product-specific regulatory mechanism.

The formation of mutually exclusive secondary structures of the leader transcript in response to the progression of transcription and ribosome movement is crucial in exerting control by attenuation. The mutations introduced during this

TABLE 4. Expression of *cat* gene under control of mutant regulatory regions

Plasmids[a] and growth conditions	CAT activity[b]
pPU193 (wild type)	
Repressing	0.4
Limiting valine	29.2
Limiting isoleucine	25.7
Limiting leucine	24.7
pPU203 (AGC→GGC)	
Repressing	0.9
Limiting valine	21.5
Limiting isoleucine	20.6
Limiting leucine	18.0
pPU194 (CUA→CUG)	
Repressing	0.7
Limiting valine	20.1
Limiting isoleucine	26.3
Limiting leucine	7.3
pPU204 (CUAGUC→CCGGGC)	
Repressing	0.6
Limiting valine	4.0
Limiting isoleucine	4.0
Limiting leucine	0.8

[a] Plasmids were integrated into strain CU1330 Δ*ilvGC2049 leu455 galT12 metE zig117*::Tn*10 polA1*.

[b] Percent chloramphenicol converted to acetylchloramphenicol per 15 min per microgram of protein.

study did not result in major changes in secondary structure formation but in some cases led to a slight increase in overall stability of the protector structure. For example, in pPU204 and pPU190 (Leu-Val to Pro-Gly) a G · C base pair was added to the protector structure (Fig. 4), with a resulting reduction in free energy of the protector of −3.8 kcal (ca. −15.9 kJ). This protector stem may also play a role in the selection of the pause site, which in the *E. coli* leader region is at base 85 (Fig. 4) and which by analogy may be the same in the *S. marcescens* leader region. Therefore, it may be that the increased stability of the protector stem and loop in plasmids pPU190 (Table 3) and pPU204 (Table 4) might have led to an increase in polymerase pause time and the reduced derepression observed on valine and isoleucine limitation with these plasmids.

Although changes in pPU189 and pPU203 also resulted in an added G · C pair to the protector, the increase in stem stability was less due to an A · U-to-G · U pair change as well. In contrast, in the leader of pPU187 and pPU194 (CUA to CUG), a hybrid between *S. marcescens* and *E. coli*, there was no base change in the protector structure. The other differences between the hybrid and parental *S. marcescens* leaders are outside of base pairing regions or consist of a switch of G · U and A · U pairs in the oligo(U) portion of the terminator pairing that probably does not occur until the attenuated transcript is released from the polymerase.

pPU187 and pPU194 (both CUA to CUG) exhibit a 3- to 15-fold derepression of the *ilv* and *cat* genes when leucine is limiting, although the critical portion of the regulatory region contains only the commonly used leucine codon CUG. It is conceivable that the position of the *leu* regulatory codon within the *S. marcescens* leader, its distance from the RNA polymerase pause site, the codon choice, and the strength of the pause site have evolved to allow a particularly efficient response to leucine limitation. In contrast, the leucine operon leader region of *Salmonella typhimurium* contains four consecutive regulatory codons, of which three are CUA codons (8). Carter et al. (4) showed a decreased response of *leu* operon expression in *Salmonella typhimurium* to excess and limiting leucine when the three CUA codons of the regulatory region were changed to CUG codons.

Studies done by J.-W. Chen in our laboratory with the *E. coli* K-12 *ilv* leader have demonstrated that starvation for the nonregulatory amino acids serine and arginine does not result in derepression. This result is of interest, since single, commonly used serine and arginine codons are located in positions presumably critical for secondary structures formed by the transcript (11, 21, 25). The location of consecutive codons for the nonregulatory amino acids alanine and threonine within the translated region of the *ilvB* leader of *E. coli* prompted studies on their involvement in the regulation of *ilvB* expression that have led to apparently contradictory results. Hauser and Hatfield (12) reported that starvation for either of these amino acids led to increased amounts of postattenuator mRNA. On the other hand, Tsui and Freundlich (36), measuring enzyme activity, failed to find evidence for derepression upon starving for the same amino acids. The basis for the different results is unexplained. Stroynowski and Yanofsky (35) reported a three- to fourfold increase in tryptophan operon expression of *S. marcescens* when cells were starved for histidine and arginine. Single, commonly used codons for these amino acids are located in RNA segments involved in secondary structure formation. The apparent differences in the way single codons can affect attenuation emphasize the dynamic nature of this type of regulation. The relative rates of transcription and translation, in turn dependent on the amount of charged tRNA, codon selection and location, RNA secondary structure formation, and polymerase pause sites, are involved in determining whether and to what extent transcription continues into the operon. This dynamic, functional dependency makes it difficult to assess the contribution of each of the factors. The fact that a single leucine codon can modulate the expression of the *ilvGMEDA* operon of *S. marcescens* might point to a subtle mechanism that allows a cell to respond specifically to limitation of a regulatory amino acid.

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