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Leucine Regulation of the *ilvGEDA* Operon of *Serratia marcescens* by Attenuation Is Modulated by a Single Leucine Codon

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The effect of leucine limitation and of restricted leucine tRNA charging on the expression of the *ilvGEDA* operon of *Serratia marcescens* was examined. In this organism, the *ilv* leader region specifies a putative peptide containing only a single leucine codon that could be involved in leucine-mediated control by attenuation (E. Harms, J.-H. Hsu, C. S. Subrahmanyam, and H. E. Umbarger, J. Bacteriol. 164:207–216, 1985). A plasmid (pPU134) containing the DNA of the *S. marcescens ilv* control region and three of the associated structural genes was studied as a single chromosomal copy in an *Escherichia coli* strain auxotrophic for all three branched-chain amino acids. The *S. marcescens ilv* genes responded to a multivalent control similar to that found in other enteric organisms. Furthermore, the *S. marcescens ilv* genes were derepressed when the charging of leucine tRNA was restricted in a *leuS* derivative of *E. coli* that had been transformed with pPU134. It was concluded that ribosome stalling leading to deattenuation is not dependent on either tandem or a consecutive series of codons for the regulatory amino acid. However, the fact that the single leucine codon is a less frequently used codon (CUA) may be important. The procedure for obtaining the cloned *ilv* genes in single chromosomal copy exploited the dependence of ColE1 replicons on the *polA* gene. The cloning experiments also revealed a branched-chain amino acid–glutamate transaminase in *S. marcescens* that is different from transaminase B.

In the accompanying paper (16), it is shown that the leader region of the *ilvGEDA* operon of *Serratia marcescens* exhibited a striking difference from the leader regions of three other enteric bacteria, including *Escherichia coli*. The multivalent repression of the operon by valine, isoleucine, and leucine in *E. coli* could be readily attributed to the distribution of codons for these amino acids in one portion of the leader that seemed to be involved in either of two mutually exclusive structures, the protector and the preemptor (or antiterminator). These alternative structures either allow or prevent, respectively, formation of the terminator, which prevents transcription from continuing into the structural genes. In the model proposed for *E. coli* (20, 24), much importance was attributed to two tandem leucine codons in the early part of the coding region of the leader. Should ribosome stalling have occurred at either of these leucine codons, the base pairing in the protector would have been considerably weakened, and a preemptor structure would have been expected. In *S. marcescens*, these two leucine codons are missing. Other differences in the nucleotide sequence of the leader from that of *E. coli* indicated that the protector structure postulated for *E. coli* probably would not have been formed by the *S. marcescens* leader.

This difference between *E. coli* and *S. marcescens* was of interest in view of the report of Kisumi et al. (18), which revealed a multivalent control that, at least superficially, resembled that observed in *E. coli*. However, for leucine to participate in a multivalent repression through an attenuation mechanism in *S. marcescens*, it would have to be assumed that such a control could occur only if a single leucine codon would be sufficient to sample the level of leucyl tRNA in the cell. In the leader region of other amino acid biosynthetic operons that are controlled by attenuation mechanisms, the putative peptide contains runs of several residues of the regulatory amino acid(s). Even the leader of the biosynthetic operon for the relatively little used amino acid tryptophan contains tandem tryptophan codons (21). The single leucine codon in the *S. marcescens* leader is different from a corresponding one in the same position in the *E. coli* leader in that it is a CUA in *S. marcescens* rather than a CUG, as it is in *E. coli*. In *E. coli*, CUA is a rarely used codon, whereas CUG is commonly used (12, 14). Whether the same is true in *S. marcescens* is unknown, but codon frequency can be a factor in attenuation, as has been shown for the attenuation control of the *pyrE* gene of *E. coli* (5). Another factor that may render this codon important is its location immediately preceding a region in which 9 of 10 codons specified either valine or isoleucine.

In this paper, experiments are described that were designed to test the possibility of control by ribosome stalling at this single leucine codon or, indeed, whether the *S. marcescens ilvGEDA* operon was controlled by leucine or only by valine and isoleucine. This latter question was pertinent because *S. marcescens* was found to have a second branched-chain transaminase that might eliminate any selective advantage for derepression with limiting leucine.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* strains used were all derivatives of strain K-12. The *S. marcescens* strains were 8000, a wild-type strain, and GIHVLr6426, an isoleucine hydroxamate-resistant mutant of strain 8000 (19). Both were kindly supplied by Masahiko Kisumi. All bacterial strains used and their sources or derivations are listed in Table 1. The plasmids used and their sources or derivations are listed in Table 2.

Media and growth of cells. The media and the methods employed for growth of cells under repressing or derepressing conditions were described by Gayda et al. (10). For the investigation of the effect of restricted leucyl tRNA synthe-

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TABLE 1. Strains used

Strain	Genotype	Source or reference
CH1274	*trpA46 lysA xyl metE zig-117*::Tn*10 polA1 argH*	From C. W. Hill
CSH73	HfrH Δ*(ara-ilvHI) lac*	Cold Spring Harbor Laboratory (23)
CU1	F^+ wild-type K-12 λ^+	Laboratory collection
CU505	Δ*ilvGEDA YC2049 leu-455 galT12*	Watson et al. (35)
CU555	*ilvD513 leu-455 galT12*	Smith et al. (32)
CU873	*ilvD513 galT12*	P1 transduction of CU555 with CU1 as donor by F. J. Smith
CU925	Δ*ilvGE2130 ara* Δ*(pro-lac) thi*	Gayda et al. (10)
CU1112	F^- *ilvC44 leu-455 rbs-221 araC galT12* λ^-	Gayda et al. (10)
CU1113	F^- *ilvC44* Δ*(ara-ilvHI) rbs-221 galT12* λ^-	P1 transduction of CU1112 with CSH73 as donor by F. J. Smith
CU1115	F^- *ilvC44* Δ*(ara-ilvHI) rbs-221 galT12 bglR* λ^-	Selection of an arbutin-utilizing derivative of CU1113 by F. J. Smith
CU1165	F^- Δ*ilvGE2130* Δ*(ara-ilvHI) rbs-221 galT12 bglR*	P1 transduction of CU1115 with CU925 as donor by F. J. Smith
CU1213	*ilvA454 galT12 recA56 srlC*::ΔTn*10*	Harms et al. (16)
CU1214	Δ*ilvGE2130 ara* Δ*(pro-lac) thi recA srlC*::Tn*10*	Harms et al. (16)
CU1220	*ilvD513 galT12 recA56 srlC*::ΔTn*10*	Harms et al. (16)
CU1230	Δ*ilvGE2130 ara* Δ*(pro-lac) thi recA56 srlC*::Tn*10 hsdR*	Harms et al. (16)
CU1330	Δ*ilvGEDA YC2049 leu-455 metE zig-117*::Tn*10 polA1 galT12*	P1 transduction of CU505 with CH1274 as donor
CU1334	F^- Δ*ilvGEDA YC2049 leuS31 polA1 thy-35 rpsL120*	P1 transduction of CU1403 with Cu505 as donor
CU1403	F^- *leuS31 metE zig-117*::Tn*10 polA1 thy-35 rpsL120*	P1 transduction of KL231 with CH1274 as donor
CU1425	Δ*ilvGEDA YC2049 leu-455 metE zig-117*::Tn*10 polA1 galT12 ilvB2226*::pPU134	Transformation of CU1330 with pPU134
CU1426	F^-Δ*ilvGEDA YC2049 leuS31 polA1 thy-35 rpsL120 ilvB2227*::pPU134	Transformation of CU1334 with pPU134
KL231	F^- *leuS31 thy-35 str-120*	Low et al. (22)
8000	Wild-type strain of *S. marcescens* Sr41	Komatsubara et al. (19)
GIHVLr6426	Presumably *ilvGa ilvA* leading to derepression of operon and a feedback-insensitive threonine deaminase	Isoleucine hydroxamate-resistant mutant of 8000; Komatsubara et al. (19)

tase activity on the expression of the *ilv* operon of *S. marcescens*, the strain carrying a temperature-sensitive leucyl tRNA synthetase and the control strain, with normal synthetase, were grown at 30°C in rich medium, L broth (2) supplied with thymidine at a final concentration of 50 μg/ml. Growth of the cultures was monitored by measuring the optical density at 660 nm in a Spectronic 88 spectrophotometer. When optical density at 660 nm was approximately 0.4, the cultures were shifted to the restrictive temperature, 37°C, and incubated for an additional 2.5 h. Ampicillin, when used, was used at a concentration of 50 μg/ml for rich medium and 35 μg/ml for minimal medium. Tetracycline was used at a concentration of 15 μg/ml. The *polA1* marker was scored by the inability of the test strain to grow in the presence of methyl methanesulfonate at a concentration of 0.02% (17).

Enzyme assays. The preparation of cell extracts and the assays of the isoleucine and valine biosynthetic enzyme activities were described earlier (31). Valine-resistant acetohydroxy acid synthase was measured in the presence of 1 mM valine. Protein was determined by the Bradford protein assay (Bio-Rad Laboratories; 6). Specific activities are expressed as nanomoles per milligram of protein per minute.

The effect of plasmids carrying the cloned genes for the two glutamate–branched-chain amino acid transaminases of *S. marcescens* on the substrate pattern of transamination was determined by the general method of Umbarger et al. (33). The reaction mixture contained, in a total volume of 1.0 ml, 100 μmol of potassium phosphate (pH 8.0), 0.1 μmol of pyridoxal phosphate, 20 μmol of [5-^{14}C]α-keto-glutarate (specific activity, 15.6 μCi/mmol), 0.2 ml of cell extract, and 20 μmol of the various amino acids, except that 1.25 μmol of tyrosine was used. The amino acid donor was omitted from the control tubes. After 15 min at 37°C, the reactions were stopped by adding 0.1 ml of 50% trichloroacetic acid. Subsequently, the reaction mixtures were subjected to centrifugation. Supernatant fluid (0.5 ml) was placed on small Dowex-50 columns (column volume, ca. 2 ml). After the column was washed with at least four volumes of H_2O, the amino acid fraction was eluted with two column volumes of 4 N NH_3. The eluate was made to a 4-ml volume, and the glutamate formed was determined in a scintillation counter.

Genetic procedures. Generalized transductions with P1*cm* were performed by the method of Rosner (27) as described by Gayda et al. (10). The *recA56* marker was transferred to strains by cotransduction with the *srlC300*::Tn*10* marker (7). UV sensitivity was scored by exposing one-half of a freshly streaked culture on an L-broth plate to a 12-s irradiation from a germicidal lamp at a flux of 50 ergs/mm^2 per s. In some cases, the tetracycline resistance marker was subsequently removed by the method of Bochner et al. (3).

Construction and analysis of plasmids. The methods used for the isolation and restriction analysis of plasmid DNA and for transformation of bacterial strains were those described by Watson et al. (35). The plasmids used are described in Table 2. The construction and isolation of two plasmids pPU133 and pPU134, deserve special comment.

Initially, pPU132 was constructed by inserting an *E. coli* 1.62-kilobase (kb) *Hin*cII-*Hin*dIII fragment from pPU131 into the multiple cloning region of pUC8 (34). The inserted fragment contained the promoter-proximal portion of the *ilvB* gene (25, 29). pPU134 was constructed from pPU132 by inserting the 9.5-kb *Eco*RI fragment from pPU129 into the *Eco*RI site that remained in the multiple cloning region. The selection was by complementation of the *ilvGE2130* deletion

TABLE 2. Plasmids used

Plasmid	Genotype[a]	Description	Source or reference
pBR322	*bla*$^+$ *tet*$^+$	Primary cloning vector	Bolivar et al. (4)
pUC8	*bla*$^+$ *lacZpZoZ'*	Primary cloning vector with multiple cloning sites	Vieira et al. (34)
pUC13	*bla*$^+$ *lacZpZoZ'*	Primary cloning vector with multiple cloning sites	Norrander et al. (26)
pPU38	pBR322 Ω[0.0 kb::K-12*uhp-ilvB* 17.5 kb(+)]*14*	*uhp-ilvB* region of K-12 inserted into *Eco*RI site of pBR322	Chromosomal *Eco*RI fragment of CU1165 ligated with an *Eco*RI-digested pBR322 by F. J. Smith
pPU43	pBR322 Δ[3.908–0.652 kb]*11* Ω[0.652 kb::pPU38*ilvB* 3.4 kb(+)]*15*	1.376-kb *Hin*cII region of pBR322 replaced by a 3.4-kb *Hin*cII fragment containing the entire *ilvB* gene from pPU38	*Hin*cII fragment of pPU38 ligated with a *Hin*cII-digested pBR322 by F. J. Smith
pPU129	pBR322 Ω[0.0 kb::*S. marcescens ilvGEDA'* 9.5 kb]*74*	Carries the *ilvGEDA'* genes of *S. marcescens* inserted into the *Eco*RI site of pBR322	Harms et al. (16)
pPU131	pUC13 Ω[0.811 kb::pPU43*ilvB* 3.4 kb]*1*	*Hin*cII fragment containing *ilvB* from pPU43 inserted into the *Hin*cII site of pUC13	3.4-kb *Hin*cII fragment of pPU43 ligated with a *Hin*cII-digested pUC13 by M. S. Rudinski
pPU132	pUC8 Δ[0.784–0.798 kb]1 Ω[0.784 kb::pPU131*ilvB'* 0.811–2.431 kb(+)]*1*	1.62-kb *Hin*cII-*Hin*dIII fragment containing the proximal portion of *ilvB* gene (1.62 kb) from pPU131 inserted into the multiple cloning sites of pUC8	1.62-kb *Hin*cII-*Hin*dIII fragment of pPU131 ligated with *Hin*cII-*Hin*dIII-digested pUC8
pPU133	pBR322 Ω[0.0 kb::*S. marcescens* ~11 kb]*75*	*S. marcescens* gene for a second glutamate–branched-chain amino acid transaminase inserted into the *Eco*RI site of pBR322	~11-kb*Eco*RI fragment of GIHVLr6426 chromosome inserted into *Eco*RI site of pBR322
pPU134	pPU132 Ω[2.42 kb::pPU129*ilvGEDA'* 9.5 kb]*1*	*S. marcescens ilvGEDA'* genes from pPU129 inserted into the *Eco*RI site of pPU132	9.5-kb *Eco*RI fragment of pPU129 ligated with *Eco*RI-digested pPU132

[a] The nomenclature employed is as used elsewhere (16). The unique convention is that (+) at the end of the insertion genotype indicates the orientation of the inserted genes relative to the way the genetic maps of the chromosome and plasmid are ordinarily drawn.

of the recipient strain (CU1230). The inserted 9.5-kb *Eco*RI fragment contained the intact *ilvG*, *ilvE*, and *ilvD* genes of *S. marcescens*. The plasmid was designed so that transcription from the *S. marcescens* 9.5-kb fragment would be independent of that from the *ilvB* 1.62-kb fragment.

pPU133 was obtained during the selection of two plasmids complementing the *ilvGE2130* deletion of strain CU1230. Both plasmids were constructed by inserting *Eco*RI fragments derived from the chromosomal DNA of *S. marcescens* GIHVLr6426 into the *Eco*RI site of pBR322. One of these plasmids contained an insert of 9.5 kb and carried the valine resistance marker attributed to *ilvG*. The insert thus appeared to be homologous to the 9.5-kb fragment cloned into pPU129 from the parent strain. In contrast, pPU133 contained an 11-kb insert and did not carry a valine resistance marker.

RESULTS

***S. marcescens* contains a second glutamate–branched-chain amino acid transaminase.** During the course of isolating pPU129, which is shown in the accompanying paper (16) to contain some of the *ilv* genes of *S. marcescens*, a plasmid (pPU133) was obtained that contained a single 11-kb *Eco*RI fragment that allowed the strain carrying the *ilvGE2130* deletion to grow in the absence of the branched-chain amino acids but not in the presence of valine; i.e., it complemented *ilvE* but not *ilvG*. Since the single *Eco*RI fragment in pPU129 was only 9.5 kb and complemented both genes, the DNA insert in pPU133 was clearly different from that in pPU129. Furthermore, pPU133 failed to hybridize with either the *ilv* leader region or *ilvE* probes from *E. coli*, whereas the insert in pPU129 did (data not shown).

Table 3 shows the transamination activity between [5-^{14}C] α-ketoglutarate and a variety of amino donors in extracts prepared from strain CU1230 (lacking tansaminase B) and from two derivatives. One derivative had been transformed with pPU129 containing the *S. marcescens ilvE* gene. The other derivative had been transformed with pPU133 containing the *S. marcescens* transaminase gene that seemed to be different from *ilvE*. Plasmid pPU129 strongly increased the amino donor activity of the branched-chain amino acids and, in addition, moderately increased amino donor activity of methionine and α-aminobutyrate. Plasmid pPU133 strongly

TABLE 3. Comparison of the effects of plasmids pPU129 and pPU133 on the utilization of several amino acids as amino donors in extracts of strain CU1230

Amino donor	Transaminase activity[a] (nmol of labeled glutamate formed per min/mg of protein) in:		
	CU1230	CU1230(pPU129)	CU1230(pPU133)
Glu	1,408	853	710
Ile	4	79	82
Val	17	66	168
Leu	17	101	305
Asp	246	231	215
Met	119	161	362
Phe	137	149	222
Tyr	120	101	99
α-NH_2 butyrate	2	32	184

[a] Cultures were grown in minimal medium supplemented with excess branched-chain amino acids. The medium for the plasmid-containing strains also contained ampicillin.

TABLE 4. Repression of valine-insensitive acetohydroxy acid synthase and transaminase B activities in strain CU1214 containing pPU129

Culture no.	Growth medium	Sp act (nmol/min per mg of protein)	
		Valine-insensitive acetohydroxy acid synthase	Transaminase B
1	Minimal[a]	11.8	78
2	As no. 1, plus isoleucine (0.5 mM) and valine (1.0 mM)	9.5	65
3	As no. 2, plus leucine (0.5 mM)	6.6	47

[a] Minimal medium contained 0.5 mM proline and 5 μg of thiamine per ml.

increased not only amino donor activity of the branched-chain amino acids, but also that for methionine and α-aminobutyrate. Another difference between the two plasmids was that pPU133 caused a very strong increase in phenylalanine-glutamate transaminase activity. Preliminary studies showed no indication of *ilv* control over this transaminase (data not shown). Because of the possibility that the *S. marcescens ilv* operon might not be affected by leucine, the transaminase gene cloned in pPU133 was of interest. For example, this transaminase might bypass the need for leucine control of the *ilvGEDA* operon in *S. marcescens* by catalyzing the conversion of valine to α-ketoisovalerate needed for leucine biosynthesis. If so, cells could form leucine even if the expression of the *ilvGEDA* operon was repressed by isoleucine and valine alone. It thus became of interest to examine the control of the *ilv* operon of *S. marcescens* in more detail.

Characterization of plasmid pPU129. The derivative of pBR322 that contained a 9.5-kb *Eco*RI fragment of *S. marcescens* DNA, pPU129, is shown in the accompanying paper to carry the *ilv* leader region and part of the *ilvGEDA* operon (16). This plasmid complemented a strain carrying the *ilvGE2130* deletion and an *ilvD* strain, but not a strain carrying an *ilvA* lesion. Hybridization with several probes (data not shown) supported the complementation tests and showed that the cloned DNA extended well into but not through the *ilvA* gene. Table 4 shows the levels of acetohydroxy acid synthase and transaminase B activities in *E. coli* CU1214 containing pPU129. The host strain, with the *ilvGE2130* deletion, lacked both the valine-insensitive acetohydroxy synthase and transaminase B. Thus, these two activities were due to the pPU129 plasmid. Although the plasmid is present in multiple copies in the cell, expression of its *ilv* genes appeared to be repressed by the presence of the three branched-chain amino acids. So that a more critical analysis of this repression could be made, a strain of *E. coli* was prepared that contained only a single copy of the *S. marcescens ilv* genes and could be grown under derepressing conditions.

Effect of branched-chain amino acid restriction on the expression of the *ilv* operon of *S. marcescens* in *E. coli*. It had been anticipated that it would be possible to introduce pPU129 as a single copy into the chromosome of *E. coli* by using the procedure of Greener and Hill (13). This procedure takes advantage of the fact that plasmids containing a colE1 replication system cannot be replicated in a *polA1* strain, and a drug resistance marker carried by the plasmid can be maintained only by a *rec-dependent* integration into a region of homology between chromosome and plasmid. Even though a cross-hybridization between the *ilv* regions of *E. coli* and *S. marcescens* had been observed (16), we were unable to obtain integration of pPU129 into the *polA1* strain used (CU1330), since its entire *ilv* region had been deleted (18). Therefore, the *S. marcescens* DNA carried by pPU129 was inserted into pPU132, which carried an arbitrarily chosen 1.62-kb *Hin*cII-*Hin*dIII fragment of *E. coli* that contained the major portion of the *ilvB* gene (26, 29). This plasmid was readily transferred to strain CU1330 by selecting the transformants for ampicillin resistance and scoring for IlvE$^+$ (growth on minimal medium containing the α-keto acid analogs of isoleucine and valine). The resulting strain was designated CU1425 and was presumed to carry pPU134 in a fragment of the *ilvB* gene for which it was diploid.

Since strain CU1425 (Δ*ilvGEDA YC2049*) was auxotrophic for all three branched-chain amino acids even after integration of pPU134 DNA (which does not carry *ilvA*, *Y*, or *C*), it was possible to examine the expression of the *ilvG* (acetohydroxy acid synthase activity in the presence of valine) and *ilvE* (transaminase B activity) genes under conditions of derepression. Both activities were derepressed not only by limiting valine and isoleucine, but by limiting leucine as well (Table 5).

The derepression by limiting isoleucine and valine was expected in view of the arrangement and number of codons for these amino acids in a presumed critical area of the transcript. The derepression by limiting leucine was in accord with the repression pattern shown in Table 4 as well as with the results of Kisumi et al. (18). However, it remained unclear whether this derepression occurred by attenuation or by the presence of some hitherto undetected leucine repressor carried in *E. coli*. To examine the question further, a *leuS* strain carrying the *polA1* marker as well as the deletion of the *ilv* region was transformed with pPU134 to yield strain CU1426.

Effect of restricted leucyl tRNA synthetase activity on the expression of the *ilv* operon of *S. marcescens*. The *leuS31* marker carried by strain CU1426, described by Low et al. (22), exhibits a temperature-sensitive phenotype and, even at 30°C, exhibits a growth requirement for leucine when isoleucine and valine are present. Thus, it might be expected that a strain bearing this lesion might always have a reduced level of leucyl tRNA even at 30°C in a rich medium

As expected, the activities of both *ilvG* (measured as acetolactate formed in the presence of valine) and *ilvE* (transaminase B) expression were higher in the *leuS* strain than in the *leuS*$^+$ strain even at the permissive temperature (Table 6). More striking, however, was the approximately threefold derepression of both activities that was observed with the *leuS* strain after a 3-h incubation at 37°C. In

TABLE 5. Expression of the *ilvG* and *ilvE* genes of *S. marcescens* carried on the chromosome of *E. coli* CU1425[a]

Growth condition	Sp act (nmol/min per mg of protein)	
	Valine-resistant acetohydroxy acid synthase	Transaminase B
Repressing	4.3	7.2
Limiting isoleucine	21.8	170
Limiting leucine	18.9	192
Limiting valine	24.9	150

[a] Strain CU1425, a *polA1* strain, carried plasmid pPU134 as a single copy integrated into its chromosome. It lacked the *ilv* gene cluster.

TABLE 6. Effect of restricted leucyl tRNA formation on *S. marcescens ilv* genes in *polA1* strains carrying pPU134[a]

Strain	Growth time and temp	Sp act (nmol/min per mg of protein)	
		Valine-resistant acetohydroxy acid synthase	Transaminase B
CU1425 (*leuS*$^+$)	30°C	3.3	1.4
	3 h, 37°C	3.6	2.2
CU1426 (*leuS31*)	30°C	24.8	51.1
	3 h, 37°C	80.5	139.5

[a] Both strains were grown in L broth.

contrast, the temperature shift did not affect these activities in the *leuS*$^+$ strain.

DISCUSSION

The finding that there is a striking degree of difference between the leader regions of the *S. marcescens* and *E. coli ilv* operons led to this examination of the regulation of *S. marcescens ilvGEDA* operon expression in *E. coli*. The absence from the leader sequence of *S. marcescens* of the tandem leucine codons that had been found in three other members of the enteric group raised the possibility that leucine might not participate in the regulation of the *ilvGEDA S. marcescens* operon. This suspicion was increased when a unique branched-chain amino acid–glutamate transaminase was found. Such a transaminase might eliminate any selective pressure or need for leucine control over the *ilvGEDA* operon. On the other hand, it had appeared from the studies of Kisumi et al. (18) with an *ilv* mutant of *S. marcescens* that regulation of the isoleucine and valine biosynthetic enzymes was essentially the same as that found in *E. coli*. Indeed, in this study, it was found that repression of these enzymes specified by a multicopy plasmid carrying the *S. marcescens ilv* genes was greater when all three branched-chain amino acids were present than when only isoleucine and valine were present. This result supports the idea that leucine participates in the multivalent repression of the *S. marcescens ilv* genes in *E. coli* cytoplasm.

However, a more dependable assessment of multivalent repression can be made with auxotrophic strains that allow derepression when any one of the three branched-chain amino acids is limiting and in which the complication of multiple copies of the genes in question is absent. Therefore, we took advantage of the fact that pBR322 derivatives are unable to replicate autonomously in *polA1* strains but can be carried in such strains following integration by homologous recombination (13). This procedure previously has been employed to find the chromosomal location of cloned fragments of uncertain origin (9, 13, 30) and to replace normal genes with sequences altered in vitro (15). The same procedure should be a useful one for the study of single chromosomal copies of other genes cloned in plasmids containing colE1 replication systems. A potential pitfall would be the selection of a strain containing multiple copies of the integrated plasmid, as described by Gutterson and Koshland (15). In the experiments reported here, probing restriction enzyme digests of the chromosomal DNA assured us that the strains used contained only single copies of the plasmid (data not shown).

An additional complication was encountered in attempting to obtain integration of the originally isolated plasmid, pPU129, presumably because of limited homology between the *S. marcescens* insert which contained the *ilvG*, *ilvE*, and *ilvD* genes and some upstream flanking DNA and that of the *polA* host from which all of the *ilv* gene cluster had been deleted (8). However, when the same cloned fragment was inserted into a plasmid that already had a small fragment of *E. coli* DNA to yield pPU134, the integration was successful. The resulting strain, CU1426, with only part of its deletion complemented by the plasmid, still requires the three branched-chain amino acids for growth and could be used to examine the effect of amino acid limitation. That limiting leucine as well as limiting isoleucine and limiting valine caused the derepression of the *ilvG* and *ilvE* genes of *S. marcescens* clearly indicated that expression of the *ilvGEDA* operon is under leucine control in *S. marcescens* and is, at least superficially, like the multivalent control found in *E. coli*.

There are two possible mechanisms that might account for the leucine control over the *ilvGEDA* operon: (i) an attenuation mechanism analogous to that proposed for *E. coli* (20, 25) but dependent upon the leading ribosome stalling at the single leucine codon when the supply of leucyl tRNA is restricted and (ii) the existence of a leucine-specific repressor for which no evidence has ever been reported. The hallmark for attenuation control of amino acid biosynthetic operons is a repression dependent upon efficient charging of the cognate tRNA. Evidence that a leucine-controlled attenuation did occur was obtained when derepression was observed upon raising the temperature of a *leuS* mutant of *E. coli* that carries the *S. marcescens ilv* genes in single copy. Thus it is clear that these genes do respond to leucine limitation by an attenuation mechanism.

The experiments with the *leuS* strain provide proof of an attenuation mechanism but do not eliminate the possibility of a repressor dependent on any one of the branched-chain amino acids. However, in a parallel study, we have constructed a plasmid in which part of the leader sequence involved in the formation of the terminator has been deleted. We have shown that there is neither repression in the presence of excess isoleucine, leucine, and valine nor derepression when any of the three is limiting in a strain carrying a single copy of this plasmid (unpublished results). Thus, it appears that attenuation is the only end product-specific mechanism regulating the expression of the *ilv* operon of *S. marcescens*.

It is of further interest that the attenuation that does occur is modulated by whether the ribosome stalls or continues at a single leucine codon. This single leucine codon in the *S. marcescens ilv* leader is one that is rarely used in *E. coli* and other bacteria and may also be rarely used in *S. marcescens*. Experiments are in progress to convert the single CUA in the *S. marcescens* leader to the more common CUG and to learn whether this change will modify the response of the *S. marcescens ilv* genes to limiting leucine in an *E. coli* background.

In the accompanying paper, it was pointed out that the secondary structure which was postulated for the *E. coli* leader and which placed the tandem leucine codons in critical positions was unlikely to be formed by the *S. marcescens* leader (16). However, the structure which was postulated for the *S. marcescens* leader and which placed the single leucine codon in a critical position could probably be formed as well by the *E. coli* leader. Such a structure, however, would make it difficult to postulate any role for the tandem leucine codons. Clearly there is need for a further analysis of the *ilv* leaders in the enteric bacteria to

determine which of the postulated structures do occur and the temporal relationships between polymerase progress along the leader region and ribosome progress along the leader transcript.

In another study in this laboratory, the effects of "nonregulatory" amino acids on attenuation of the *ilvGEDA* operon in *E. coli* were investigated. Starvation for these amino acids for which codons were located at presumably critical positions in the leader sequence did not cause derepression (J.-W. Chen and H. E. Umbarger, manuscript in preparation). This failure was initially attributed to the possibility that to cause derepression, tandem or a cluster of codons for the same amino acid are required for ribosome stalling at those specific codons. However, from the demonstration that a single leucine codon can modulate the attenuation of the *ilvGEDA* operon of *S. marcescens*, it seems that there may be a subtle mechanism, beyond our current understanding of attenuation, that allows cells to respond specifically to the limitation of "regulatory" amino acids.

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