Mutations in the Gene for a tRNA That Functions as a Regulator of a Transcriptional Attenuator in Bacillus subtilis

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ABSTRACT

It has been proposed that uncharged tRNA molecules may act as positive regulatory factors to control the expression of a number of operons in Bacillus subtilis and related bacteria by interacting with leader sequences to cause antitermination. In this study we report the isolation and characterization of regulatory mutations that modify one of the tRNA molecules predicted to have such a regulatory role. Three different alleles of the B. subtilis leucine tRNA gene leuG were found that resulted in higher expression of the ilv-leu biosynthetic operon. Each resulted in a base change in the D-loop of the leucine tRNA molecule with the anticodon 5'-GAG-3' (leucine tRNA<sub>AG</sub>). Experiments with strains that are diploid for mutant and wild-type alleles suggested that both charged and uncharged tRNA molecules may interact with leader sequences to control expression of the operon.

BACTERIA have evolved elaborate mechanisms to regulate gene expression in response to changes in physiological conditions and nutrient supply. The regulation of genes involved in amino acid biosynthesis in the gram-negative bacterium Escherichia coli has been studied in considerable detail. In recent years there has been increasing interest in the mechanisms that control the expression of corresponding genes in Bacillus subtilis, a gram-positive soil bacterium that occupies a very different ecological niche. In this report we describe mutations in a leucine tRNA gene that cause overexpression of the B. subtilis ilv-leu operon, which contains genes for branched-chain amino acid biosynthesis. Our analysis suggests that the operon is controlled by an attenuation mechanism in which a particular tRNA species helps to determine the frequency of transcriptional termination at a site in an untranscribed leader sequence.

The ilv-leu operon of B. subtilis contains seven genes required for the biosynthesis of leucine, isoleucine and valine (VANDYER 1987), preceded by a 482-bp untranslated leader sequence. Transcription of the operon is about 30-fold higher when cells are grown in limiting leucine than when they are grown in excess leucine (GRANDONI <i>et al.</i> 1993). Attenuation of transcripts within this leader accounts for most of the negative effect of excess leucine on expression of the operon (GRANDONI <i>et al.</i> 1992, 1993).

GRUNDY and HENKIN (1993, 1994) have proposed that a number of the aminoacyl-tRNA synthetase genes and amino acid biosynthetic operons of B. subtilis and related bacteria are regulated by a common mechanism in which uncharged tRNA molecules act as positive regulators by interacting directly with leader mRNA to promote transcriptional antitermination. Mutational analysis of the leader of the B. subtilis tyrosyl-tRNA synthetase gene (tyrS) led them to propose this novel model of transcriptional attenuation.

This proposed mechanism differs from the extensively studied mechanisms that regulate the amino acid biosynthetic operons of E. coli. Translation of upstream open reading frames is an essential component of the E. coli attenuation mechanisms (LANDICK and YANOFSKY 1987). In the GRUNDY and HENKIN model, the mechanism by which tRNA molecules influence transcriptional termination is independent of translation. Analysis of the ilv-leu operon leader sequence suggests that it is regulated by this mechanism (GRUNDY and HENKIN 1994; GRANDONI <i>et al.</i> 1993).

In the leader region of each of the operons that GRUNDY and HENKIN analyzed they identified a potential RNA secondary structure that could serve as a transcriptional terminator. Upstream of the terminator is a potential stem-loop structure that contains an unpaired bulge with a triplet sequence (the specifier) that they believe is a site of interaction with the anticodons of regulatory tRNA molecules (GRUNDY and HENKIN 1993). In the tyrS operon they showed that this site determines the specificity of the regulation. The tyrS leader has the tyrosine codon 5'-UAG-3' (UAC) at the specifier position. Changing the triplet to UUC, which codes for phenylalanine, altered the regulation so that transcription of the operon became dependent on starvation for phenylalanine instead of tyrosine. They noted in their papers that the leucyl-tRNA synthetase (leuS) and ilv-leu operons both have the specifier triplet CUC that codes for leucine. Their model predicts that tRNA molecules that recognize CUC control expression of both of the operons. In this paper we describe our analysis of mutations within a leucine tRNA<sub>GAG</sub> gene (leuG) of B. subtilis that cause overexpression of the ilv-leu operon.
The mutations in the leuG gene were isolated in a screen of B. subtilis mutants that overexpress the ilv-leu operon. The mutations were identified as suppressors of a phenotype associated with an insertion of a transposon in ilvN, the second gene of the ilv-leu operon. We isolated and characterized mutations that allow ilvN:Tn917-containing mutants that have point mutations within the leucine operon. Our analysis of the mutations supports the Grundy and Henkin model.

### MATERIALS AND METHODS

**Media:** Bacterial strains were grown on plates of tryptose blood agar base (TBAB; Difco) or Spizizen minimal medium with 0.5% glucose, 20 μg/ml tryptophan, 1 μg/ml biotin, 10 μM MnCl₂, and 17 g/liter purified agar (Harwood and Cutting 1990). Additives included either 50 μg/ml ampicillin, 5 μg/ml chloramphenicol (CAM), 10 μg/ml neomycin, 40 μg/ml methionine, 100 μg/ml leucine, 70 μg/ml isoleucine, or 50 μg/ml valine. Plates for selecting for the MlsR phenotype contained 1 μg/ml erythromycin and 25 μg/ml lincomycin (MLS). 5-Bromo-4-chloro-3-indolyl β-D-galactosidase activity of strains carrying a transcriptional fusion of the operon with the lacZ gene of E. coli. Fusions were constructed as described by Perkins and Youngman (1986). Sequences containing the lacZ gene were inserted into a transposon located in ilvN, the second gene of the ilv-leu operon. Constructs with two different antibiotic markers were used. ilvN:Tn917lacZ-erm confers resistance to erythromycin and lincomycin. ilvN:Tn917lacZ-afl confers resistance to chloramphenicol. Both constructs are called ilvNlacZ below.

### TABLE 1

B. subtilis strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
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</thead>
<tbody>
<tr>
<td>CU1065</td>
<td>trpC2</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU1962*</td>
<td>[metB5] sup-3</td>
<td>This laboratory</td>
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<tr>
<td>CU4609</td>
<td>ilvNlacZerm leuB16 trpC2 (SBP)</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU4645</td>
<td>ilvNlacZerm leuB16 trpC2 ileuG1 (SBP)</td>
<td>This laboratory</td>
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<tr>
<td>CU4670</td>
<td>ilvNlacZerm leuB16 trpC2 ileuG2(SBP)</td>
<td>This laboratory</td>
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<td>CU4694</td>
<td>ilvNlacZerm leuB16 trpC2 ileuG5 (SBP)</td>
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<tr>
<td>CU4785</td>
<td>ilvNlacZerm leuB16 metB5 ileuG1 (SBP)</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU4831</td>
<td>metB5 (SBP c2 delZ::Tn917)</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU4938*</td>
<td>ilvNlacZerm leuB16 trpC2 ileuG1 (SBP[+3 leuF] ileuG1)]</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU4934*</td>
<td>[metB5] (SBP[sup-3 leuF ileuG])</td>
<td>This laboratory</td>
</tr>
<tr>
<td>CU4936</td>
<td>ilvNlacZerm leuB16 trpC2 (SBP[pCV-2])</td>
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<td>CU4937</td>
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<tr>
<td>CU5023*</td>
<td>ilvNlacZerm leuB16 ileuF1 ileuG1 (SBP[luf] AleuG-neo)]</td>
<td>This laboratory</td>
</tr>
</tbody>
</table>

Additional strains are listed in Table 4.

* The [metB5] mutation in CU1962, CU4934 and CU4949 is suppressed by the sup-3 suppressor, and the strains are prototrophic for methionine.

* These strains carry SBP prophages that contain the entire trnS operon.

### TABLE 2

**TABLE 2**

**Primer sequences**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Location</th>
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<tr>
<td>A5</td>
<td>5'-GGAGATCTACTAATCCCTCCTTCTCC-3'</td>
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</tr>
<tr>
<td>A7</td>
<td>5'-GGAGATCTCTTATTCCACCCACGACCGTGA-3'</td>
<td>2301</td>
</tr>
<tr>
<td>A16</td>
<td>5'-GGAGATCTACTCATGGA-3'</td>
<td>465</td>
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<tr>
<td>M₁</td>
<td>5'-GGAGATCTACTCATGGA-3'</td>
<td>1660</td>
</tr>
<tr>
<td>M₁₁</td>
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<tr>
<td>M₁₂</td>
<td>5'-GGAGATCTACTCATGGA-3'</td>
<td>1521</td>
</tr>
</tbody>
</table>

The approximate locations where primers match sequences in the trnS operon are indicated in Figure 2. The coordinates refer to positions in the previously published trnS sequence (Figure 3, Garrity and Zahler 1993). The 3' ends of the primers correspond to nucleotides in the published sequence at the indicated positions. Primers A5 and A7 contain BglII (AGATCT) sites added to their 5' ends. M₁ and M₁₁ matches in sequences in leuF excluding the leuF1 mutation, and has a BamHI (GGATCC) site at its 5' end. M₁ and M₁₁ are complementary at their 5' ends.
wered aerated at 37°C until turbid and plated. Washed cellswas used to inoculate 5-ml tubes of LB. Cultures relative to known genes resistant colonies were patched to TBAB containing X-gal, and blue (Lac+) colonies were chosen.

Bacterial transformation: The method of Hanahan (1983) was used to transform E. coli strains. The method of Cutting and Vander Horn (1990) was used to transform B. subtilis strains.

Isolation of leucine-resistant mutants: Five-milliliter samples of LB were inoculated with isolated colonies of CU4609 trnN-lacZ leuBl6 trpC2 and aerated at 37°C until cultures were slightly turbid. Cells were pelleted, washed in saline-citrate (0.1 M NaCl, 0.01 M sodium citrate), and plated on minimal agar medium with 160 μg/ml leucine. Mutants leuG2 through leuG5 were obtained after treatment with the mutagen N-methyl-N'-nitro-N-nitrosoguanidine (MNNG; Aldridge). Pellets of the exponentially growing cells were resuspended in 100 μg/ml MNNG in saline-citrate, aerated for 30 min at 37°C and washed in saline-citrate. One tenth milliliter of washed cells was used to inoculate 5-ml tubes of LB. Cultures were aerated at 37°C until turbid and plated as before. Leucine-resistant colonies were patched to TBAB containing X-gal, and blue (Lac+) colonies were chosen.

Gene mapping: Leucine-resistant mutations were mapped relative to known genes on the B. subtilis chromosome using standard methods of transformation and transduction with phage PBS1 (Cutting and Vander Horn 1990). Five mutations were tightly linked to the sup-3 nonsense suppressor. Sequenc- ing polymerase chain reaction (PCR) products derived from the five mutant strains showed that each had a single base-pair change in a particular leucine tRNA gene located in the same operon as the sup-3 mutation. The operon has been named trnS (Garrity andTimer 1993). The leucine tRNA gene was named leuG; it codes for leucine tRNAeuc.

DNA sequencing: Plasmid DNA segments were sequenced using Sequenase kits (U.S. Biochemical Corp.). PCR products were sequenced using fentomole sequencing kits (Promega) after purification from low melt gels using Magic PCR preps (Promega).

DNA isolation: Small amounts of plasmid DNA (1-3 pg) were isolated using Magic Minipreps (Promega). Larger amounts (50-500 pg) were purified by CsCl gradient centrifugation (Manatis et al. 1982).

**ΔleuG**: The method described by Higuchi (1990) was used to amplify a DNA fragment from the trnS region in which 49 bases internal to the leuG gene, including the entire anticodon arm, were deleted. The deletion was named ΔleuG (Figure 1). The deletion creates an EcoRI restriction site within the re- mainder of the leuG gene. The fragment of about 1.1 kb was cloned into the BamHI site of pBluescript (Stratagene). Plasmid pDGD218 was obtained (Figure 2).

Primer sequences are indicated in Table 2. Approximate positions where primers hybridize to sequences in trnS are indicated in Figure 3. Details of the procedure can be found in (Garrity 1994). The amplified product included sequences between primers A3 and A7 with sequences between primers M6 and M12 deleted.

**ΔleuG-neo**: A 1.0-kb fragment of DNA including the neomycin-resistance gene (neo) of pUB110 (Bonn 1990) was ligated to a partial EcoRI digest of pDGD218. The resulting plasmid, pDGD224 (Figure 2), contains sequences derived from the trnS operon with the neo gene inserted into the EcoRI site located within ΔleuG. The neomycin gene is transcribed in the same direction as trnS.
A fragment of DNA from an XbaI and Scal digest of pDG224 was ligated to a DNA fragment from an XbaI and Scal digest of pDG213 (Figure 2). Plasmid pDG213 was described previously (GARRITY and ZAHLER 1993). It is a derivative of pBR322 with a 4.2-kb insert from the region of the trnS operon. The resulting plasmid, pDG234, has the ΔleuG-neo deletion inserted into the trnS-containing fragment of pDG213. CU4645 ilvN-lacZ leuB16 trpC2 was transformed with pDG234 linearized by Scal, and Neo colonies were isolated. A Neo transformant was designated CU5016 ilvN-lacZ leuB16 trpC2 ΔleuG-neo.

leuF1: Primer M1 is complementary to sequences in the leuF gene except that it has a base substitution to change the anticodon from UAG to GAG. A PCR product was obtained using primers A16 and M1, with template DNA from strain CU1065 trpC2. The product was cut with BamHI and Scal and ligated to pBluescript cut with EcoRV and BamHI. The resulting plasmid was designated pDG232 (Figure 4). A fragment of DNA derived from a digest of pDG224 cut with Scal and XbaI was ligated to pDG232 cut with BamHI and XbaI. Plasmid pDG235 was obtained. It contains the region of the trnS operon with ΔleuG-neo and the leuF1 mutation. CU4987 ilvN-lacZ leuB16 trpC2 ΔleuG-neo.

SPβ lysates: All SPβ phages in this study have the c2 temperature-sensitive repressor gene. Lysates were made by heat induction as previously described (WEINER 1986).

SPβ[pCV-2]: This is a recombinant SPβ phage that contains the vector used to construct SPβ[ΔleuG-leuF* ΔleuG*] (see below). CU4831 metB5 (SPβ[c2 Tn917]) was transformed with pCV-2 linearized with SsfI. Cam transformants were selected.

SPβ[ΔleuG-leuF*]: The deletion of a recombinant SPβ phage with a cloned insert including the trnS operon was previously reported (GARRITY and ZAHLER 1993). The insert carries the ΔleuG-leuF* mutation.

SPβ[ΔleuF leuG*]: Genetic recombination was used to isolate a derivative of SPβ[ΔleuF leuG*] in which the ΔleuF marker was replaced by its wild-type allele and the wild-type leuG allele was replaced by the leuG1 mutation. The construction was carried out by congenic [cotransformation of unlinked loci; see STREIPS (1991)].

Strain CU4949 ilvN-lacZ lys-3 metB5 (SPβ[ΔleuF leuG*]) is phenotypically Met' because the ΔleuF marker suppresses the metB5 nonsense mutation. This strain was transformed with excess DNA from CU4785 leuB16 metB5. Lys+ colonies were selected on minimal medium with leucine, isoleucine, valine, methionine, X-gal and chloramphenicol. Light blue colonies were selected on minimal agar plus leucine, isoleucine, valine, X-gal, and chloramphenicol and without methionine. A methionine
auxotroph was isolated. The new strain was called CU4987 ilvN-lacZ metB5 (SPβ[leuF+ leuG1]). The insert in the SPβ prophage had lost the sup-3 mutation and gained the leuG1 marker.

**SPβ[leuF1 ΔleuG-neo] and SPβ[leuF+ ΔleuG-neo]:** These are derivatives of SPβ[leuF+ leuG1] in which the leuG1 allele was replaced by ΔleuG-neo. The heterogeneate CU4987 ilvN-lacZ metB5 (SPβ[leuF+ leuG1]) was transformed with DNA extracted either from CU5016 ilvN-lacZ leuB16 trpC2 ΔleuG-neo, or from CU4993 ilvN-lacZ leuB16 trpC2 leuF1 ΔleuG-neo. Colonies were selected on TBAB with neomycin and X-gal.

The CU5016 transformation gave 104 blue colonies and 5 white colonies. The CU4993 transformation selecting for NeoR gave 1 blue transformant that acquire the deletion also received leuF1. The colonies were white whether the deletion disrupted the chromosomal or phage copy of leuG, because leuF1 suppresses leuG1. We mixed the white colonies together and made a single SPβ lysate. Some of the phase in this lysate transferred the neo gene to recipients. An SPβ lysate made from one of these neomycin-resistant strains was shown to transfer neomycin resistance to other strains along with other markers on the phage. The phage was designated SPβ[leuF1 ΔleuG-neo]. A bacterial strain carrying it is CU5023 ilvN-lacZ leuB16 trpC2 leuG1 (SPβ[leuF1 ΔleuG-neo]).

**Strain verification:** Sequencing of PCR products was used to confirm the genotypes of some of the strains constructed in this work. Details of the strain verification are available (GARRITY 1994). It was confirmed that strains CU4993 ilvN-lacZ leuB16 trpC2 leuF1 ΔleuG-neo and CU5023 ilvN-lacZ leuB16 trpC2 leuG1 (SPβ[leuF1 ΔleuG-neo]) both contain the leuF1 mutation and the ΔleuG-neo deletion. Strain CU5040 ilvN-lacZ leuB16 leuF+ leuG' (SPβ[leuF+ leuG1]) carries the leuG1 mutation.

**RESULTS**

The **leuG1-leuG5 mutations:** B. subtilis strains that carry the ilvN-lacZ insertion are auxotrophic for isoleucine and valine when grown in the presence of excess leucine (VANDEYAR 1987). We selected mutations that permitted growth in the presence of leucine and caused overexpression of the ilv-leu operon, indicated by the formation of blue colonies on media with excess leucine and X-gal. Five mutations (about 300 that were tested) that affect the regulation of the ilv-leu operon were closely linked to the trnS operon (GARRITY and ZAHLER 1993). Each of the mutants had a base substitution in the leuG gene. The predicted sequence of the leucine tRNA<sub>ΔleuG</sub> molecule coded by the leuG gene is shown in Figure 1. Each of the mutations was a transition in the D-loop of the tRNA. Three of the mutations (leuG1, leuG3 and leuG4) were identical.

All of the mutations allow B. subtilis strains with the ilvN-lacZ insertion to grow on minimal medium plates with
excess leucine. Strains with the leuG mutations and the ilvN-lacZ transcriptional fusion produce blue colonies on TBAB (complex) plates with X-gal. Strains wild-type for expression of the operon. The expression of the leucine than it is in the minimal and in complex media. The produce small colonies on TBAB plates. of the greatest effect. Expression of the strains with the excess leucine. Strains with the They affect the growth rate of the cells, which was significantly reduced in the mutants that had the greatest effect on the activity measured for CU4609. residues that are highly conserved among tRNA genes that the conserved pseudouridine loop to stabilize the three-termined for some molecule to be non-functional. The fact that the conserved DNA with the entire trnS operon might disrupt the structure of the tRNA and cause the part of the tRNA gene has been determined for some tRNA genes it has been suggested that the conserved G residues interact with bases in the pseudouridine loop to stabilize the three-dimensional structure of the tRNA. These mutations might disrupt the structure of the tRNA and cause the molecule to be non-functional. The fact that the leuG5 mutation has a much less severe phenotype than leuG1 suggests, at least for leuG5, that the mutant tRNA is partly functional.

The leuG* allele is partly dominant over leuG1: The trnS operon was cloned onto a recombinant SPβ phage (GARRITY and ZAHLER 1993). Phage SPβ[sp-3 leuF+ leuG*] (previously called SPβS) contains a fragment of DNA with the entire trnS operon. It has the nonsense suppressor mutation sp-3 in a lysine tRNA gene of trnS and the wild-type leuF and leuG alleles.

Lysogens formed by lysogenizing strains with the recombinant SPβ phages are diploid for the trnS operon. We lysogenized CU4645 ilvN-lacZ leuB16 trpC2 leuG1 with SPβ[sp-3 leuF* leuG*]. The resulting heterogenote strain in the diploid strain with the sup-3 allele partly complements the leuG1 mutation. To be certain that the presence of sp-3 in CU4933 was not responsible for the decrease in ilv-leu expression, we constructed a heterogenote with the genotype leuF* leuG*/SPβ[sp-3 leuF* leuG1]. We lysogenized B. subtilis strains carrying the ilvN-lacZ reporter gene with phage SPβ[leuF* leuG1]. When we lysogenized a leuG1 strain we got lysogens that formed dark blue colonies on TBAB X-gal plates (Table 4). This suggested that the leuG* allele partly complemented the leuG1 mutation.

We did a similar light blue phenotype when we plated either of the reciprocal leuG*/leuG1 diploid strains on TBAB X-gal plates: CU4645 lysogenized with SPβ[sp-3 leuF* leuG*], or CU4609 lysogenized with SPβ[leuF* leuG1] (CU4933 and CU4938, Table 4). This shows that the wild-type leuG* allele partly complements the leuG1 mutation. We assayed the β-galactosidase activity of CU4933 ilvN-lacZ leuB16 trpC2 leuG1 (SPβ[sp-3 leuF* leuG*]) grown in minimal medium with leucine, isoleucine and valine to compare ilv-leu operon expression in the diploid strain with the leuG* and leuG1 haploid strains (Table 3). As stated before, a leuG1 strain was shown to express the ilv-leu operon 2-fold higher than a leuG* strain. CU4933, the leuG*/leuG1 diploid strain, expressed the operon only 2-fold higher than the wild type. As a control we constructed a leuG1 strain that was lysogenic for a recombinant SPβ phage that does not have the extra copy of trnS. This strain, CU4937, expressed the ilv-leu operon 15-fold higher than the wild-type strain.

### Table 3

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Dt in minutes</th>
<th>Activity</th>
<th>Fold expression</th>
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<tr>
<td>CU4933</td>
<td>leuF* leuG1 (SPβ[sp-3 leuF* leuG+])</td>
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<td>59.4</td>
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*The effects of leuF and leuG mutations on expression of the ilv-leu operon. All of the strains contain the ilvN::Tn917[lacZ-erm] reporter gene. They also have the trpC2 and leuB16 mutations. Different leuF, leuG and SPβ alleles are indicated. Strains were grown in minimal medium with tryptophan, glutamate, isoleucine, valine and leucine.

1) The doubling time Dt is in minutes.

2) β-Galactosidase activity is reported in Miller units. Fold expression was determined by dividing the β-galactosidase activity of each strain by the activity measured for CU4609.
The *leuG* gene is not essential: We constructed a plasmid containing *trnS* in which 49 bp of the *leuG* gene were deleted and replaced with a *neo* gene (*ΔleuG-neo*). We initially expected that the gene would be essential. No other gene that codes for leucine tRNA_{GAC} has been reported for *B. subtilis* (Green and Vold 1993). However, we found that we could transfer the deletion directly from the plasmid to the *trnS* haploid strain by transformation. PCR and Southern transfer experiments verified that the resulting strains are haploid for the region around *leuG* and carry only *ΔleuG-neo*. We conclude that the *leuG* gene is not essential for *B. subtilis*. However, haploid strains carrying *ΔleuG-neo* grow more slowly than *LeuG+* strains on all media. The doubling time of strain CU5016 *ilvN-1acZ leuB16 trpC2* had a doubling time of 49 min. We conclude that there could be another tRNA gene in *B. subtilis* that codes for a tRNA that can translate 5'-CUC-3' and 5'-CUU-3' codons. The lower growth rate of strains with the *ΔleuG-neo* deletion indicates that the gene for the other tRNA is probably not highly expressed. The growth rate of the strain with *ΔleuG-neo* in the minimal medium is similar to the growth rate of the *leuGI*-containing strain (compare CU5016 and CU4645 in Table 3). It is probable that the growth defect of the *leuGI*-containing strains is caused by a reduced ability to translate 5'-CUC-3' and 5'-CUU-3' codons.

Haploid strains that contain the *ΔleuG-neo* deletion and the *ilvN-lacZ* reporter gene do not have the overexpression phenotype that we saw with the *leuG* transition mutations. Strain CU5016 *ilvN-lacZ leuB16 trpC2 ΔleuG-neo* forms small white colonies on TBAB X-gal plates. Lyso genizing CU5016 with SPP[*leuF*+ *leuGI*] produced colonies that were blue on TBAB with X-gal. The *ΔleuG-neo* deletion is transferred onto the SPP phages that carry *trnS*. Transformation of CU4987 *ilvN-lacZ leuB16 metB5* (SPP[*leuF* leuGI]) with linearized plasmid DNA containing the *ΔleuG-neo* deletion produced transformants in which the *neo* gene was inserted into either the chromosomal or plasmage location of *trnS*. Phage with the *ΔleuG-neo* deletion made lysates that could be used to transduce the deletion to other strains. Using two *ΔleuG-neo*-containing plasmids, one with *leuF1* and the other with *leuF2*, we were able to make two new SPP phages, SPP[*leuF1 ΔleuG-neo*] and SPP[*leuF2 ΔleuG-neo*]. Infection of CU4645 *ilvN-lacZ leuB16 trpC2 leuG1* with SPP[*leuF1 ΔleuG-neo*] produced a heterologene that contains both the *leuF1* and *leuG1* mutations. The strain produced white colonies on TBAB.
X-gal plates (CU5023, Table 4). This again indicates that the _leuF1_ mutation partly suppresses the _leuG1_ mutation. As a control we lysogenized CU4645 _ilvN-lacZ leuB16 trpC2 leuG1_ with SPB[ _leuF_ ^*ΔleuG-neo_]. The lysogen (CU5022) produced blue colonies on TBAB X-gal plates. We measured the expression of the _ilv-leu_ operon in these two strains using the β-galactosidase assay (Table 3). The _leuF_ ^*ΔleuG-neo_ _ilv-leu_ operon expressed 2-fold higher than wild type. When _B. subtilis_ cells are starved for leucine, they overexpress the _ilv-leu_ operon by reading through the transcriptional terminator in the untranslated leader ( _GRANDONI et al._ 1992, 1995). Analysis of the _ilv-leu_ leader led us to believe that trans-acting factors are involved in its regulation. We isolated and characterized mutations unlinked to the _ilv-leu_ operon that caused overexpression of _ilv-leu_. The mutations were mapped to two loci: near _leuS_ described by _VANDER HORN and ZAHLER_ (1992) and near _sup-3_ (present work). All of the mutations near _sup-3_ that have been tested have base substitutions in the _leuG_ tRNA gene. The phenotypes of both classes of mutations can be explained by a model in which tRNA molecules control the _ilv-leu_ attenuator independently of either trans-acting regulatory proteins or the translation of an upstream open reading frame.

The similarity of features found in the leaders of the _ilv-leu_ and _tyrS_ operons suggests that the _ilv-leu_ operon is regulated by a mechanism analogous to that proposed by _GRUNDY and HENKIN_ (1993) for the _tyrS_ operon. Analysis of mutations in the _tyrS_ leader led them to conclude that uncharged tyrosine tRNA that recognizes the codon UAC can interact with structures in the _tyrS_ leader RNA to promote antitermination. Similarly, features in the _ilv-leu_ leader suggest that an uncharged leucine tRNA that recognizes CUC promotes antitermination ( _GRUNDY and HENKIN_ 1993). The model predicts that any mutation that increases the level of uncharged leucine tRNA^GAG_ in the cell will lead to overexpression of the _ilv-leu_ operon. The leucine-resistant mutations located in the _leuS_ and _leuG_ genes could all be of this class.

Leucyl-tRNA synthetase, the gene product of _leuS_, is the enzyme that charges leucine tRNAs. The point mutation _leuS1_ results in increased expression of the _ilv-leu_ operon ( _VANDER HORN and ZAHLER_ 1992). Any kind of change in the enzyme that reduced its charging ability might lead to the accumulation of uncharged leucine tRNAs (including leucine tRNA^GAG_).

The mutations in _leuG_ that cause increased _ilv-leu_ expression alter the D-loop of leucine tRNA^GAG_. The phenotypes of the _leuG_ mutations could be the consequence of either the accumulation of uncharged leucine tRNA^GAG_ molecules due to a defect in charging, or the loss of function of the tRNAs in some other process in the cell. The G residues changed in the _leuG_ mutations are highly conserved in all tRNAs. Crystal structure studies of other tRNA molecules suggest that two of these residues may interact with residues in the pseudouridine loop to stabilize the RNA structure ( _Kim_ 1979). Our _leuG_ mutations might alter the tRNA three-dimensional structure, which might in turn affect its ability to be charged by its synthetase. Alternatively, some other function of the tRNA could be affected, such as its role in the translation of CUC and CUU codons.

The failure of the _leuG_ null mutant to overexpress the _ilv-leu_ operon appreciably supports a model in which the leucine-resistant phenotype conferred by the _leuG_ point mutations did not reflect a reduced function of the tRNA in a process such as translation. Our ability to construct null mutant strains suggests that there is another tRNA that also recognizes CUC and CUU codons, although a gene for such a tRNA has not been reported yet ( _GREEN and VOLD_ 1993).

The codominance of _leuG1_ and _leuG^*_ suggests a previously unrecognized aspect of the tRNA-directed antitermination mechanism. In a minimal medium with leucine, isoleucine, and valine, a strain carrying the _leuG1_ mutation expressed the _ilv-leu_ operon about 19-fold higher than a strain with the wild-type allele of _leuG_ (CU4645 and CU4609 respectively, Table 3). A diploid strain carrying both the _leuG1_ and _leuG^*_ alleles had only 2-fold higher expression than the haploid strain with _leuG^*_ (CU4933 and CU4609, respectively, Table 3). Having an extra copy of the operon with a wild-type copy of the _leuG_ gene reduces _ilv-leu_ overexpression greatly. We conclude that the _leuG^*_ allele is partly dominant over the _leuG1_ mutation.

The phenotype of the _leuG^*_/ _leuG1_ strains raises some interesting questions about the interaction of these tRNA genes with the _ilv-leu_ leader. If our hypothesis is correct and the _leuG1_ allele produces mutant leucine tRNA^GAG_ that is not efficiently charged, we predict that it should increase the intracellular level of uncharged leucine tRNA^GAG_ both in _trnS_ haploid and diploid strains. There is no obvious mechanism by which the presence of the wild-type copy of the _leuG_ gene could improve the charging of _leuG1_ mutant leucine tRNA^GAG_ molecules. It is possible that the _leuG^*_ allele could lower the level of uncharged leucine tRNA^GAG_ in the cell by some effect on the expression or turnover of the mutant _leuG1_ leucine tRNA^GAG_, but we think that this is unlikely. We will discuss our reasoning for this below.

If uncharged leucine tRNA^GAG_ levels are elevated to the same extent in _leuG1_ haploid and _leuG^*_/ _leuG1_ diploid strains, there should be at least as many uncharged tRNA^GAG_ molecules available to interact with the _ilv-leu_
leader in the diploid as in the haploid. The GRUNDY and HENKIN model predicts that only uncharged tRNA causes antitermination. The fact that ilv-leu expression in the leuG*/leuG1 diploid strain is much lower than in the leuG1 haploid strain suggests that both charged and uncharged leucine tRNA<sub>G</sub> molecules can interact with the ilv-leu leader, and perhaps compete for binding. If both charged and uncharged leucine tRNA<sub>G</sub> molecules are able to interact with the ilv-leu leader, higher levels of the charged tRNAs could compete with the uncharged tRNAs and reduce their ability to cause antitermination at the attenuator.

As part of our analysis of the leuG1 mutation, we constructed a mutation in another leucine tRNA gene that suppressed the mutant phenotype. The trnS operon has two adjacent leucine tRNA genes. The leuG gene codes for leucine tRNA<sub>AG</sub>. The leuF gene is one of two known identical leucine tRNA<sub>AG</sub> genes. We introduced into a leuG1 strain a leuF allele (leuF1) with a UAG to GAG anticodon change, which allows recognition of the CUC specifier codon in the ilv-leu leader. If charged wild-type leucine tRNA<sub>AG</sub> molecules compete with uncharged leuG1 mutant tRNAs for interaction with the ilv-leu leader, we thought that other charged tRNAs that can recognize the specifier codon in the leader might also compete with the uncharged leuG1 tRNA.

Strains carrying the leuF1, leuG1 and ilvN-lacZ reporter genes were plated on indicator plates to determine the effect of the mutations on expression of the ilv-leu operon. Strains with the leuF1 and leuG1 mutations produced white colonies. Colonies of isogenic strains without the leuF1 mutation were blue. We conclude that the leuF1 mutation suppresses the leuG1 mutation. This result suggests that charged tRNA molecules that recognize the CUC specifier triplet can compete with uncharged leucine tRNA<sub>AG</sub> for interaction with the ilv-leu leader.

We would like to address the possibility that either the leuG<sup>+</sup> allele or the leuF1 mutation might influence the leuG1 phenotype through some effect on the expression or stability of leuG1 mutant leucine tRNA<sub>AG</sub> molecules. If this were true, the conclusions based on the dominance and suppression results would not be valid. One might argue that creating strains diploid for trnS had some effect on its expression. For example, the cells might have some mechanism that can down-regulate the operon when its copy number is increased. The lower expression of ilv-leu in the leuG*/leuG1 diploid strains could then be explained not as a competition between the two alleles but as a reduction in leuG1 expression caused by the down-regulation. A similar explanation could apply to our observations with diploid strains carrying the leuF1 mutation.

To address this question we compared the phenotypes of two strains, CU5022 and CU5023. Each has the chromosomal leuG1 mutation and the ilvN-lacZ reporter gene. Each is lysogenic for an SPβ phage with a cloned insert of DNA including the trnS operon. In CU5022 the phage has the leuF<sup>+</sup> allele and ΔleuG-neo. In CU5023 the phage has the leuF1 mutation and ΔleuG-neo. In CU5022 ilv-leu expression was 12 times higher than in a leuG<sup>+</sup> haploid strain. In CU5023 the ilv-leu operon was expressed only twice as much as in the wild-type (Table 3). (A haploid leuG1 strain expressed the ilvN-lacZ fusion 19-fold more than the wild type.) The expression of the ilv-leu operon is much higher in CU5022 than in CU5023. Both strains are diploid for trnS, so having a second copy of the operon does not appear to cause a major reduction in the level of leuG1 mutant tRNA in the cell. We conclude that the leuF1 mutation suppresses the leuG1 phenotype by a mechanism that does not involve a reduction in the intracellular level of the leuG1 mutant tRNA.

Our results are in general agreement with the model of GRUNDY and HENKINS. Conditions that are likely to lead to increased quantities of uncharged leucine tRNA<sub>AG</sub> in the cell lead to overexpression of the ilv-leu operon. We strongly suspect that the uncharged tRNA interacts directly with the leader of ilv-leu to increase readthrough at the terminator. We also present evidence suggesting that charged leucine tRNA<sub>AG</sub> competes with uncharged leucine tRNA<sub>AG</sub> for binding to the leader. We have no evidence concerning proteins that may interact with the ilv-leu leader to affect attenuation.

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