Effects of Mutations in the Pseudomonas putida miaA Gene: 
Regulation of the trpE and trpGDC Operons in P. putida by Attenuation

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Tn5 insertion mutants defective in regulation of the Pseudomonas putida trpE and trpGDC operons by tryptophan were found to contain insertions in the P. putida miaA gene, whose product (in Escherichia coli) modifies tRNA^{Trp} and is required for attenuation. Nucleotide sequences upstream of trpE and trpG encode putative leader peptides similar in sequence to leader peptides found in other bacterial species, and the phenotypes of the mutants strongly suggest that transcription of these operons is regulated solely by attenuation.

The arrangements of trp genes and their patterns of regulation differ widely among prokaryotes (8). The Escherichia coli trp operon, which consists of five contiguous genes—trpE, trp(GD), trpC(F), trpB, and trpA—encoding seven enzymatic activities, is negatively regulated by TrpR in the presence of tryptophan and by the trp attenuator in the presence of acylated tRNA^{Trp} (28). However, in Pseudomonas putida, Pseudomonas aeruginosa, and Pseudomonas syringae, the trpB and trpA genes, which encode the subunits of tryptophan synthase, comprise a separate operon that is positively regulated. In the presence of indoleglycerol phosphate (InGP), a substrate for tryptophan synthase, the trpBA operon is activated by the product of a gene, trpI, that is unique to these three species (1, 6, 19). Furthermore, trpE and trpGDC are transcribed independently (13, 14).

Isolation of unlinked mutations causing constitutive expression of P. putida and P. aeruginosa trpE, trpG, trpD, and trpC suggested that these genes, like their E. coli counterparts, were regulated by a TrpR-like repressor (5, 20). However, we have isolated phenotypically similar mutants induced by Tn5 insertion; the mutations all disrupt the P. putida miaA homolog, whose product (in E. coli) modifies tRNA^{Trp} and is required for attenuation (12, 18, 30). Assays of trp enzyme synthesis in the mutant strains strongly suggest that transcription of the trpE and trpGDC operons is regulated by attenuation and not by repression.

Selection of P. putida 5-MT* mutants. P. putida strain M (22) was mutated by transposition of a mini-Tn5 lacZ1 transposon from the suicide plasmid pUT/mini-Tn5lacZ1, which encodes kanamycin resistance (11). Mutants were selected at 30°C on M9 minimal medium containing 200 µg of 5-methyl-DL-tryptophan (5-MT)/ml, 20 µg of kanamycin/ml, and 100 µg of rifampin (to which P. putida, but not E. coli, is resistant)/ml.

Five Kanr 5-MT* mutants (MTR1 through MTR5), chosen randomly from among 19 mutants that remained after a preliminary screening of more than 100 original isolates from several experiments, were assayed for expression of anthranilate synthase II (AS II) (trpG gene product); in the presence of exogenous tryptophan, all of the mutants were derepressed (data not shown). Strains MTR1 through MTR5 were also defective in utilization of phenylalanine (0.1%) as the sole carbon and energy source. Thus, the insertions appeared to cause defects in one or more general regulatory functions, rather than in a single function specific for trp gene expression, but the defect in phenylalanine utilization was not explored further.

Strains MTR1 through MTR5 contain Tn5 insertions in the P. putida miaA gene. PsI-cut DNA from strains MTR1 through MTR5 was ligated into pUC18 (25), and plasmids containing the Tn5 insertion were identified. Restriction site analysis revealed that all five strains contained inserts at either of two sites within a single chromosomal 1.49-kb PsI1 fragment (data not shown). Southern hybridization to a mutant PsI1 fragment was used to screen a plasmid library containing PsI1 fragments isolated from wild-type cells; the screen identified two plasmids, pT301 and pT302, that contained the wild-type PsI1 fragments in opposite orientations. The nucleotide sequences of the pT301 and pT302 inserts and the corresponding MTR1 and MTR4 PsI1 fragments (GenBank accession no. AF016312) were identical except for the Tn5 insertion. An open reading frame that spans the region delineated by the mini-Tn5 insertions in MTR1 through MTR5 is 65% identical in nucleotide sequence and 62% identical in amino acid sequence to the E. coli miaA gene and its product, respectively (7). The P. putida open reading frame is homologous to the Agrobacterium tumefaciens miaA gene and the Saccharomyces cerevisiae mod5 gene (16, 21), each of which encodes a protein with a known tRNA-isopentenyladenine transferase activity. In E. coli, absence of the MiaA-modified modification of tRNA^{Trp} prevents attenuation in the trp operon and promotes attenuation in the tna (tryptophanase) operon, in each case by affecting the translational efficiency of the tRNA (15, 30). The
plates showed that transformants containing pTR301 and (23); formation of red colonies on MacConkey agar-lactose confirmed by complementation in E. coli miaA and 4-fold (Table 1, lines 2 and 3). Furthermore, in the two separately. In AS I, the product of the trpE gene, which is transcribed separately, is not upstream of the trp-tRNA gene, which is transcribed sequentially to the (UGA) trpC gene, which is transcribed sequentially to the trp-tRNA gene. Experiments 1 through 3, extracts were dialyzed for AS I assays and undialyzed for PRT and InGPS assays; for experiments 4 and 5, all extracts were dialyzed.

**Effects of miaA insertions on trp gene expression.** Enzyme assays were used to investigate the effects of P. putida miaA mutations on the production of phosphoribosyltransferase (PRT) (trpD product) and InGP synthase (InGPS) (trpC gene product), which are encoded in the trpGDC operon, and AS I, the product of the trpE gene, which is transcribed separately. In trp+ miaA+ P. putida (Table 1, line 1), enzyme levels are low in the absence of tryptophan and are not repressed even by 500 μg of tryptophan/ml. The absence of repression of trp gene expression in miaA+ trp prototrophs, which has been observed previously (9, 20), is due to the inability to starve for tryptophan sufficiently to relieve attenuation (17). However, in the presence of tryptophan, miaA mutations increase expression of trpE between 10- and 30-fold and of trpGDC between 3- and 4-fold (Table 1, lines 2 and 3). Furthermore, in the two miaA mutant strains, trp gene expression is roughly the same in the presence and in the absence of tryptophan. The unexpectedly high level of AS I activity in the presence of tryptophan in the miaA1 mutant most likely reflects the extreme variability of the assay. If we assume that the phenotype of the miaA mutants is evidence for attenuation, these data suggest that trpE and trpGDC transcription is regulated only by attenuation, since AS I, PRT, and InGPS levels are either unaffected or decreased by at most 40% when miaA mutant cultures are incubated in the presence of tryptophan. This small decrease could be due to differences in culture conditions caused by pleiotropic effects of the mutations or to residual trp-tRNA activity (and translation of the leader peptide) in the absence of MiaA. In any case, it is insignificant compared to the 10- to 100-fold effects of trpR in E. coli (29).

Enzyme activity was also assayed in a trp auxotroph, trpB9 (22), in which endogenous tryptophan levels are lower, making cells more sensitive to the concentration of tryptophan in the culture medium (Table 1, line 4). As expected, excess tryptophan reduces expression of trpE by approximately 20-fold and of trpGDC by 2- 7-fold. The miaA1 insertion (Table 1, line 5) completely relieves tryptophan-mediated regulation and increases expression of trpE, trpD, and trpC in the presence of excess tryptophan to approximately the levels attained in the miaA+ strain in medium containing limiting tryptophan. These results indicate that tryptophan-mediated repression can be completely relieved by the miaA insertion and therefore that the trpE and trpGDC operons are regulated solely by attenuation (or by some other mechanism requiring the translational activity of tRNA\textsuperscript{Trp}).

**Effects of miaA1 on transcription of trpE.** Published nucleotide sequences (10, 13, 14) reveal highly conserved sequences that may encode 14-amino-acid polypeptides upstream of trpE in P. putida, P. aeruginosa, and P. syringae and upstream of trpG in P. putida (Table 2). There is a putative leader peptide, but no methionine initiator codon, upstream of trpG in P. aeruginosa, which suggests loss of the ability to be regulated by attenuation. A comparison to leader peptides from other species (Table 2) reveals limited sequence similarity between groups of related sequences. The Pseudomonas sequences are highly positively charged relative to the others. An unusual feature of the upstream region of Pseudomonas trpE is that the putative leader peptide is the carboxy terminus of a reading frame homologous to phosphoglycerate phosphatase (Gph) of E. coli (13, 14; GenBank accession no. AB030825), although in the E. coli genome gph is not upstream of trpE (4).

Possible promoters for P. putida and P. aeruginosa trpE and trpG were identified upstream of the putative leader sequences (Fig. 1). Reverse transcriptase mapping using a primer complementary to the P. putida trpE coding region confirmed both in vivo and in vitro the location of the transcription start site schematized in Fig. 1 (Fig. 2). Synthesis of this transcript in

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**Table 1. Effects of miaA mutations on P. putida trp gene expression**

| Expt | Strain          | [Trp] (μg/ml) | AS I (trpE) | PRT (trpD) | InGPS (trpC) |
|------|-----------------|--------------|------------|------------|-------------|
| 1    | M (wild type)   | 500          | 18.6 ± 0.1 | 6.0 ± 0.5  | 6.5 ± 1.8   |
|      |                 | 0            | 17.4 ± 3.3 | 7.4 ± 0.4  | 10.8 ± 1.2  |
| 2    | MTR1 miaA1::Tn5 | 500          | 523 ± 122  | 24.2 ± 1.5 | 28.3 ± 4.4  |
|      |                 | 0            | 229 ± 89   | 27.2 ± 6.2 | 39.9 ± 2.9  |
| 3    | MTR4 miaA2::Tn5 | 500          | 228 ± 12   | 18.0 ± 1.8 | 45.9 ± 1.5  |
|      |                 | 0            | 215 ± 75   | 25.0 ± 5.0 | 78.9 ± 2.7  |
| 4    | M9 trpB9        | 50           | 7.9 ± 0.1  | 3.1 ± 0.7  | 3.2 ± 1.0   |
|      |                 | 2            | 155 ± 38   | 6.7 ± 0.0  | 21.8 ± 4.0  |
| 5    | M19 trpB9 miaA1::Tn5 | 50 | 184 ± 2.7 | 11.7 ± 2.6 | 10.5 ± 0.8 |
|      |                 | 2            | 167 ± 47   | 10.8 ± 0.1 | 15.6 ± 0.1 |

*Values are averages ± standard deviations for two experiments. Cell extracts were prepared by sonication, and assays for enzyme activity and protein concentration were performed as described by Crawford and Gunsalus (9). For experiments 1 through 3, extracts were dialyzed for AS I assays and undialyzed for PRT and InGPS assays; for experiments 4 and 5, all extracts were dialyzed.

*Enzyme levels appear to be unregulated in prototrophs due to the presence of endogenous tryptophan.

*Double mutants were constructed by transducing miaA1 into P. putida trpB9. All three independent Kan’ transductants tested exhibited increased levels of trp enzyme activity, confirming that this phenotype is due to the Tn5 insertion.
vivo is regulated by tryptophan in a trpB9 miaA strain but not in a trpB9 miaA strain (Fig. 2, lanes 6 to 9), although the ratio of the observed trpE RNA level in the absence of tryptophan to that in the presence of tryptophan in the miaA strain was only 6.6, which was substantially lower than the corresponding ratio of enzyme activities (Table 1). We were unable to detect trpE RNA by reverse transcriptase mapping using a primer complementary to the putative leader transcript, possibly because of a secondary structure in the leader. We also attempted to identify a terminated transcript in vitro. Because the trpE promoter is weak, we mutagenized the presumed 210 region (TAACGT) to TATAAT in order to increase the level of transcription. Although the phenotype of the mutant in vitro confirmed the location of the promoter (data not shown), we did not detect a terminated transcript.

Regulation of trp genes in fluorescent pseudomonads. Five 5-MTr mutants contained Tn5 insertions in the P. putida miaA homolog, and no insertion was detected in a trpR-like gene. The phenotypes of miaA insertion mutants strongly suggest that the trp genes are regulated by attenuation rather than by repression, since trp enzyme levels in the mutants were approximately the same as the “derepressed” levels in miaA strains. Furthermore, the pattern of regulation of the trpG and trpEDC operons in fluorescent pseudomonads (5, 9) resembles that described for the E. coli trp operon in that regulation of attenuation, in contrast to repression, by tryptophan is detected only

### Table 2. Leader peptide sequences for trpE and trpG

| Gene                        | Leader peptide sequence | Reference or GenBank accession no. | Charge | Groupa |
|-----------------------------|-------------------------|------------------------------------|--------|--------|
| Pseudomonas syringae trpE   | MKVIKA-LARRWRRA         | 10                                 | +5     | γ-3    |
| Pseudomonas putida trpE     | MKVIKA-LARRWRRA         | 13                                 | +5     | γ-3    |
| Pseudomonas aeruginosa trpE | MKVIKA-LARRWRRA         | 14                                 | +5     | γ-3    |
| Pseudomonas putida trpG     | MRVIKA-HARRWRRA         | 13                                 | +5     | γ-3    |
| Pseudomonas aeruginosa trpG | TS LIKA-FARRWRRA        | 14                                 | +4     | γ-3    |

*a* For gram-negative organisms, the designation indicates the applicable subdivision of the purple bacteria. GP, gram positive.
Several patterns of trp gene regulation in gram-negative bacteria have been reported. First, the entire E. coli trp operon is subject to both repression and attenuation. Second, in P. putida, P. aeruginosa, and P. syringae, the trpG and trpEDC transcripts are distinct and appear to be subject only to attenuation while the trpBA operon is activated by the TrpI protein, which is unique to these three species. And third, in Rhizobium meliloti (3), only the trpE(G) gene is regulated (by attenuation). TrpI-mediated regulation is logically similar to regulation in species in which trpB and trpA are repressed by TrpR. InGP is produced and the trpF promoter is activated when trp genes whose products function earlier in the pathway are expressed. In a prototroph, endogenous tryptophan is sufficient to reduce expression of both trpE and trpGDC nearly to basal levels. Thus, addition of tryptophan causes only a small decrease in expression of these genes. Exogenous tryptophan down-regulates trpBA transcription to a much greater extent (~10-fold), most likely through feedback inhibition of AS I, since inhibiting the conversion of chorismate to anthranilate would prevent synthesis of InGP.

Gram-positive bacteria have evolved different mechanisms for achieving similar results. In Bacillus subtilis, attenuation is mediated by an RNA-binding protein (TRAP), and an operon containing 6 of the 7 trp genes (not including trpG) is part of a 13-gene “supra-operon” whose transcription can be initiated at the upstream aroF promoter as well as at the trpE promoter (for a review, see reference 27).

This work was supported in part by NIH grant GM50577 to G.N.G. We thank Susan Brown for technical assistance and S. Plattner for help in preparing Fig. 2.

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