Regulation of an anthranilate synthase gene in *Streptomyces venezuelae* by a *trp* attenuator

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The nucleotide sequence of a 2.4 kb *Bam*HI–*Sal*I fragment of *Streptomyces venezuelae* ISP5230 DNA that complements *trpE* and *trpG* mutations in *Escherichia coli* contains two ORFs. The larger of these (ORF2) encodes a 624 amino acid sequence similar to the overall sequence of the two subunits of anthranilate synthase. The two-thirds nearest the amino terminus resembles the aminase subunit; the remaining one-third resembles the glutamine amidotransferase subunit. Upstream of ORF2 is a small ORF encoding 18 amino acids that include three adjacent Trp residues; in addition the ORF contains inverted repeats with sequence and positional similarity to the products of attenuator (*trpL*) regions that regulate tryptophan biosynthesis in other bacteria. In cultures of a *trpC* mutant of *S. venezuelae*, increasing the concentration of exogenous tryptophan decreased the formation of anthranilate synthase; similar evidence of endproduct repression was obtained in a *trpCER* mutant of *E. coli* transformed with a vector containing the cloned DNA fragment from *S. venezuelae*. The anthranilate synthase activity in *S. venezuelae* cell extracts was inhibited by tryptophan, although only at high concentrations of the amino acid. A two-base deletion introduced into the cloned *S. venezuelae* DNA fragment prevented complementation of a *trpE* mutation in *E. coli*. However, *S. venezuelae* transformants in which the two-base deletion had been introduced by replacement of homologous chromosomal DNA did not exhibit a Trp[–] phenotype. The result implies that *S. venezuelae* has one or more additional genes for anthranilate synthase. In alignments with anthranilate synthase genes from other organisms, ORF2 from *S. venezuelae* most closely resembled genes for phenazine biosynthesis in *Pseudomonas*. The results bear on the function of the gene in *S. venezuelae*.

Keywords: anthranilate synthase genes, attenuator, gene disruption, nucleotide sequence

INTRODUCTION

Anthranilate synthase (AS) is the first enzyme in the branch of the aromatic biosynthesis pathway leading specifically to tryptophan. In most organisms, the

enzyme has two non-identical subunits; the larger one (ASI), encoded by *trpE*, can by itself synthesize anthranilate from chorismate in the presence of a relatively large concentration of ammonia. The smaller subunit (ASII), encoded by *trpG*, is a glutamine amidotransferase, and can generate the amino group of anthranilate from moderate concentrations of glutamine, thereby avoiding pH effects due to high cellular levels of ammonia (Crawford, 1989). The subunits of AS are most commonly monofunctional proteins, although in some organisms a subunit protein catalyses more than one of the activities required for tryptophan synthesis [e.g. Trp(G)D in *Escherichia coli* and *Salmonella typhimurium*; Huang & Zalkin, 1971].

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Abbreviation: AS, anthranilate synthase.

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The flow of metabolic intermediates to tryptophan is regulated by controlling transcription, as well as by feedback inhibition of AS activity. The latter is a highly conserved feature in all micro-organisms (Bae & Crawford, 1990), but transcriptional controls are more diverse. In *E. coli* and some other bacteria, *trp* genes constitute a single operon regulated by both repression and attenuation, with the region responsible for attenuation located immediately upstream of *trpE*. Although the regulation of bacterial tryptophan biosynthesis has received a lot of attention, it has not been investigated in streptomycetes. *Streptomyces venezuelae*, in which the chromosomal organization of *trp* genes is known to differ from that in other bacteria, contains AS genes separated from a *trpCBA* cluster, while *trpD* and *trpF* are at a third locus (Paradkar *et al.*, 1993). Whether genes at the three loci are coordinately regulated has not been investigated. There is some evidence for attenuation as a mechanism controlling gene expression in streptomycetes (cited in Potter & Baumberg, 1996) but as yet there is no fully documented example of an attenuator with a role in regulating a primary biosynthetic pathway. The cloning from *S. venezuelae* of a DNA fragment encoding AS activity (Paradkar *et al.*, 1991a) provided us with an opportunity to examine the organization, regulation and evolutionary relationships of AS genes in this species. The biosynthesis of chorismate-derived metabolites is of particular interest in *S. venezuelae* since the antibiotic chloramphenicol is one of the endproducts of the aromatic pathway (Vining & Stuttard, 1994).

METHODS

Bacteria, plasmids and culture media. The micro-organisms and plasmids used are described in Table 1. Strains of *S. venezuelae* were maintained on MYM agar (Stuttard, 1982). The minimal medium (MM) used for *S. venezuelae* cultures grown on agar was the maltose/asparagine/salts medium of Hopwood *et al.* (1985). The tryptophan-free medium used for liquid cultures of *S. venezuelae* was prepared as described by Hopwood *et al.* (1985) but contained (per litre): glucose (10 g), maltose (5 g), asparagine (0.5 g), vitamin-free Casamino acids (5.0 g; Difco), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.6 g), 0.1 M $\text{NaH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer, pH 6.8 (150 ml), and the minor elements solution (1 ml) of Hopwood *et al.* (1985). For *E. coli*, LB medium or M9 (glucose/ammonium chloride/salts) minimal medium was used (Sambrook *et al.*, 1989). For strains derived from *E. coli* JA221 and JA200, M9 medium was supplemented as needed with amino acids at 10 mg ml⁻¹, and thiamin at 1 mg ml⁻¹. Cultures of *E. coli* W3110 *trpCER* (pDQ187) used to determine the effect of tryptophan on expression of *trpE(G)* were grown in M9 medium supplemented with vitamin-free Casamino acids at 0.5 mg ml⁻¹ and ampicillin at 0.1 mg ml⁻¹.

DNA manipulations. The general procedures described by Sambrook *et al.* (1989) were followed. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method (Kieser, 1984), except during the screening of colonies, when the boiling method of Holmes & Quigley (1981) was adopted. Genomic DNA was obtained as described by Hopwood *et al.* (1985).

Transformation procedures. Competent cells of *E. coli* *trp* auxotrophs were prepared and transformed as described by

Table 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype/phenotype	Source/reference
<i>Streptomyces venezuelae</i>		
ISP5230	Wild-type	Doull <i>et al.</i> (1985)
VS153	<i>trpC cml-5</i>	Stuttard (1983)
VS194	<i>hisA6 adeA10 SVP1⁻ SVP2⁻</i>	Doull <i>et al.</i> (1986)
AP55	VS194 with a mutation in <i>trpE(G)</i>	This work
<i>Escherichia coli</i>		
TG1	<i>supE hsd Δ(lac-proAB) F'(traD36 proAB⁺ lacI^q lacZ ΔM5)</i>	Carter <i>et al.</i> (1985)
JA221	<i>r⁻m⁺ leu thi ΔtrpE5</i>	C. Yanofsky, Stanford University
JA200	<i>thr-1 leuB6 ΔtrpE63 recA56 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 λ⁻ supE44</i>	CGSC 6059/Clarke & Carbon (1976)
W3110 <i>trpC</i>	<i>trpC</i>	C. Yanofsky, Stanford University
W3110 <i>trpCER</i>	<i>trpC10243 trpE9829 trpR</i>	C. Yanofsky, Stanford University
Plasmids		
pBluescriptII SK(+)	Phagemid Ap ^R	Stratagene
pHJL400	Bifunctional Par ⁻ Ap ^R Ts ^R	Larson & Hersherberger (1986)
pDQ187	pTZ18R with a 2.4 kb <i>trpE(G)</i> insert	Paradkar <i>et al.</i> (1991a)
pDQ189	pHJL400 with a 2.4 kb <i>trpE(G)</i> insert	Paradkar <i>et al.</i> (1993)
pDQ190	pTZ18R with a modified <i>trpE(G)</i> insert	This work
pDQ191	pHJL400 with a modified <i>trpE(G)</i> insert	This work
pDQ192-193	pBluescriptII SK(+) with <i>trpE(G)</i> insert	This work
pDQ290	pBR322 with a 2.7 kb <i>pabB pabA</i> insert	Arhin & Vining (1993)

Kushner (1978). For other *E. coli* strains the procedure of Hopwood *et al.* (1985) was used. Transformants were plated on LB agar (Sambrook *et al.*, 1989) containing ampicillin (0.1 mg ml^{-1}). A genomic library was prepared by partially digesting DNA from *S. venezuelae* ISP5230 with *Mbo*I and size-fractionating it in a sucrose gradient to give 6–12 kb fragments. The fragments were ligated to pTZ18R that had been linearized with *Bam*HI and dephosphorylated (Sambrook *et al.*, 1989). The ligation mixture was used to transform *E. coli* JA221 ($\Delta trpE$) to prototrophy. For introducing plasmid DNA into *S. venezuelae* we used strain VS194, which is transformed more efficiently than the wild-type strain ISP5230.

Gene disruption. pDQ187 was linearized by digestion with *Mlu*I, incubated with mung bean nuclease and treated with the Klenow fragment of DNA polymerase I. The blunt ends were ligated and the recircularized plasmid was used to transform *E. coli* JA200. Plasmid DNA isolated from *Trp*[−] transformants was screened for loss of the *Mlu*I site by gel electrophoresis of *Eco*RI and *Mlu*I digests.

DNA sequencing and sequence analysis. Both orientations of the DNA fragment cloned in pDQ187 were subcloned in the phagemid vector pBluescriptII SK(+), and overlapping deletion clones were generated. Single-stranded DNA templates were isolated as described by Karger & Jessee (1990) from cultures of *E. coli* TG1 transformed with the phagemid and infected with helper phage VCSM13 (Stratagene). The DNA strands were sequenced by the dideoxy chain-termination procedure (Sanger *et al.*, 1977) using Sequenase version 2.0 (Amersham) and [³⁵S]dATP α -S. The sequence was analysed with GCG software (Genetics Computer Group). The region containing the *Mlu*I site in pDQ191 was sequenced using the double-stranded cloned DNA insert as the template and a synthetic oligonucleotide primer.

Hybridization. Plasmids and genomic DNA digested with restriction enzymes were fractionated by electrophoresis and the DNA was transferred to Hybond-N nylon membranes (Amersham) as described by Southern (1975). DNA probes were labelled with [α -³²P]dCTP by the random priming procedure. Hybridization was carried out at 65 °C in a solution containing 5 $\times$ SSPE (SSPE is 0.18 M NaCl, 10 mM Na₂HPO₄ and 1 mM EDTA, pH 7.7), 5 $\times$ Denhardt's solution (Denhardt, 1966), 0.5% SDS and denatured salmon sperm DNA (200 $\mu\text{g ml}^{-1}$). Membranes were routinely washed at 65 °C with SSPE solutions (twice with 2 $\times$, then with 1 $\times$ and 0.1 $\times$), all containing 0.1% SDS. The stringency of the wash should have removed DNA of lower than 70% sequence identity with the probe. [³²P]DNA was detected by autoradiography.

Enzyme assays. Cells from cultures of *E. coli* or *S. venezuelae* were harvested by centrifugation and washed with extraction buffer. Extraction buffer for *E. coli* contained 0.1 M potassium phosphate, pH 7.8, 1 mM EDTA and 1 mM DTT (Smith & Yanofsky, 1963); for *S. venezuelae* it contained 0.1 M potassium phosphate, pH 7.8, 0.5 M potassium chloride, 0.1 mM EDTA, 0.8 M sucrose and 6 mM 2-mercaptoethanol (Smithers & Engel, 1974). Washed cells were resuspended in the buffer, disrupted by sonication and centrifuged. The supernatant solution was used as the cell extract. All procedures during and after harvest were at 4 °C. AS was assayed by incubating cell extracts at 37 °C for 30 min with chorismic acid (1 μmol), glutamine (20 μmol), magnesium chloride (10 μmol) and Tris/HCl (50 μmol), pH 7.5 (final volume 1 ml). Anthranilic acid was measured in the deproteinized solution by diazotization and coupling with *N*-naphthylethylenediamine (Jones

& Westlake, 1974). A unit of AS is defined as the amount of enzyme catalysing the formation of 1 nmol anthranilic acid min^{-1} . Protein was assayed with bicinchoninic acid (BCA protein assay reagent, Pierce & Smith, 1985) using bovine serum albumin as the standard. To avoid interference from tryptophan in assays for feedback effects on enzyme activity, anthranilic acid was extracted from acidified reaction mixtures with ethyl acetate. After back-extraction with aqueous buffer at pH 4.5, the solvent was evaporated and the residue, redissolved in water, was assayed colorimetrically as above. To confirm the identity of the enzyme reaction product, the residue from representative assays was examined by TLC on silica gel plates using toluene/acetic acid (19:1, v/v) as the mobile phase. Anthranilic acid was distinguished as a blue-fluorescent zone at R_F 0.28, giving a purple colour after spraying with 5% sodium nitrite in 0.1 M hydrochloric acid and overspraying with 0.2% *N*-naphthylethylenediamine in 2 M hydrochloric acid. Neither *p*-aminobenzoic acid (purple zone at R_F 0.18) nor indole (R_F 0.58) was formed in the enzyme reaction mixtures.

RESULTS

Nucleotide sequence of the DNA fragment encoding anthranilate synthase

The 2.4 kb fragment of *S. venezuelae* DNA cloned in the recombinant *E. coli* plasmid pDQ187 (Paradkar *et al.*, 1991a) was excised as a *Bam*HI–*Sal*I fragment. It was blunt-ended by treatment with the Klenow fragment of DNA polymerase I, and subcloned (both orientations) in the *Eco*RV site of pBluescriptII SK(+). Single-stranded DNA from each of the phagemids (Vieira & Messing, 1987) was sequenced. Analysis of the nucleotide sequence using the CODONPREFERENCE program (Devereux *et al.*, 1984) with the codon usage table for streptomycetes compiled by Wright & Bibb (1992) indicated that the 2.4 kb fragment contains a small ORF (ORF1) followed by a much larger one (ORF2; see Fig. 1). ORF1 begins with an ATG codon at nt 93–95 and ends with the TGA codon at nt 147–149. The region upstream of the translational start codon does not show an obvious RBS, but at nt 81–86 the sequence TACAGT fits the consensus (TAgPuPuT) for the –10 hexamer of

Fig. 1. The 2.43 kb *Bam*HI–*Sal*I fragment of *S. venezuelae* DNA cloned in pDQ187 (Paradkar *et al.*, 1991a). The nucleotide sequence was determined after recloning the DNA insert in pBluescriptII SK(+), and ORFs were detected in the nucleotide sequence by codon usage analysis with the CODONPREFERENCE program of the GSG software package (Devereux *et al.*, 1984). Regions in the deduced amino acid sequence showing similarity to ASI and ASII were identified by comparison with protein databases using BLASTP (Altschul *et al.*, 1990).

(a)
S. venezuelae *E. coli* (b)
S. venezuelae *E. coli*

Fig. 2. Comparison of secondary structures postulated for *trpL* mRNA in *S. venezuelae* with those described for *E. coli* (Landick *et al.*, 1996). (a) The 1:2 (pause) and 3:4 (terminator) stem-loop structures that result in transcription terminating when tryptophan is adequate. (b) The 2:3 (antiterminator) structures that allow tryptophan synthesis when the amino acid is depleted. Codons for tryptophan are in bold face; the translational stop codon is underlined. In the *E. coli* gene, nucleotides are numbered relative to the 5' end of the transcript; in the *S. venezuelae* gene, numbering is from the 5' end of ORF2.

streptomycete promoter sequences recognized by σ^{70} of *E. coli* RNA polymerase (Strohl, 1992); the corresponding -35 hexamer is not apparent, but a tandem six-base (GTTCCC) direct repeat is present at nt 61-66 and 67-72. No indication of a palindromic operator sequence was found. ORF2 begins with ATG at nt 251-253 and terminates with TGA at nt 2120-2122. A putative RBS (GGA; $\Delta G -9.2$ kcal mol⁻¹; -38.5 kJ mol⁻¹) at nt 244-246 is present five to seven bases upstream of the translational start codon; the upstream region lacks recognizable promoter sequences.

Identity of ORF2

The polypeptide encoded by ORF2 contains 624 aa. Comparison of the amino acid sequence in the N-terminal region of the deduced ORF2 product with protein databases using BLASTP (Altschul *et al.*, 1990) showed 29-34% identity to ASI of enteric bacteria in the overlap regions; the sequence of the C-terminal region showed 30-33% identity to ASII. Alignment of the ASI-like domain in the ORF2 product with the deduced aminase products of *trpE* and *pabB*, which have

Fig. 3. Specific activity of AS in cell extracts from cultures supplemented with increasing concentrations of tryptophan: (a) *S. venezuelae* VS153, (b) *E. coli* W3110 *trpC* and (c) *E. coli* W3110 *trpCER*(pDQ187).

common ancestry (Goncharoff & Nichols, 1984), and also with aminase products of related secondary metabolic pathway genes (see below) from a variety of bacteria, showed well-conserved sequence in this region. A similar alignment of the ASII-like domain with products from *trpG*, *pabA* and related secondary metabolic pathway genes showed comparable sequence conservation in the C-terminal region of the ORF2 polypeptide. Relationships between the deduced protein sequences determined in alignments by the PILEUP program (Higgins & Sharp, 1989) showed clustering of the products from both domains of *S. venezuelae* ORF2 with the corresponding products of *phzB* from *Pseudomonas aureofaciens* (Pierson *et al.*, 1995), and of *phzE* from *Pseudomonas fluorescens* (Mavrodi *et al.*, 1995). In a BLASTP search of the GenBank database the *phzB* and *phzE* gene products ranked highest in sequence similarity (56% identical and 71% similar amino acids) to the product of ORF2 from *S. venezuelae*. As noted above, the percentage of identical amino acids in sequence comparisons between products from the ORF2 domains and those from *trp* and *pab* genes were much lower, ranging from 29 to 34%. The *phz* gene products exhibit AS activity, and participate in the biosynthesis of phenazine metabolites (Pierson *et al.*, 1995). In the two *Pseudomonas* species, as in *S. venezuelae*, both ASI and ASII are encoded by domains within a single ORF.

Identity of ORF1

The small ORF at nt 93–149, upstream of ORF2, contains codons for 18 amino acids. The size of the translated product is similar to that of leader peptides functioning as attenuators to regulate gene expression (Yanofsky, 1981; Crawford, 1989). A role in attenuation of a *trp* gene is supported by the presence in ORF1 of three Trp codons in tandem at nt 117–125. The location of the tandem codons matches that in the *trpL* genes of other bacteria; in other respects the deduced peptide sequences of these genes are not strongly conserved. Like known *trp* attenuators, the *S. venezuelae* sequence contains inverted repeats capable of forming alternative

stem-loop mRNA structures (Yanofsky, 1984). In the region beginning within the tandem Trp codons, the pairing sequences would allow an initial 1:2 stem-loop ending with a translational stop codon (Fig. 2a). Formation of this stem-loop would allow folding of the subsequent RNA sequence to give the G + C-rich 3:4 stem-loop, which is followed by a U-rich sequence typical of rho-independent transcriptional terminators (Chater & Hopwood, 1989). The 3:4 stem contains well-conserved nucleotide sequences matching those in the *trp* attenuators of *E. coli* and *Rhizobium* (*Sinorhizobium*) *meliloti* aligned by Bae *et al.* (1989). Recognizable also in the ORF1 sequence is an alternative 2:3 pattern (Fig. 2b) that could form a stem-loop if translation of the tandem Trp codons is prevented by tryptophan deficiency; in preempting the sequence giving the 1:2 and 3:4 structures this alternative stem-loop would act as an antiterminator.

Regulation of anthranilate synthase

When grown in minimal medium, *S. venezuelae* ISP5230 mutants blocked at various steps in tryptophan biosynthesis contained cellular AS activity two orders of magnitude above the wild-type (Paradkar *et al.*, 1991b). On the assumption that the difference in AS activity was due to differences in size of the cytoplasmic tryptophan/tryptophanyl-tRNA^{Trp} pools that regulate AS synthesis by feedback effects, we examined the response to tryptophan supplements of an *S. venezuelae* *trpC* mutant, where such pools should be depleted (Fig. 3a). As the exogenous tryptophan concentration was increased stepwise from 25 to 100 μM, the AS activity in rapidly growing mycelium was reduced 9.5-fold. For comparison, adding tryptophan to a *trpC* mutant of *E. coli* W3110 caused a similar decrease in AS activity, but in a lower response range (4–25 μM; Fig. 3b). The results were consistent with the presence of feedback repression in both organisms. The specific activity of AS extracted from the mycelium of VS153 and assayed *in vitro* with 0, 0.06, 0.25 and 2 mM tryptophan in the reaction mixture showed 6, 28 and 44% inhibition,

respectively, by the supplement. The results indicated that, although a relatively high concentration of tryptophan is required, feedback inhibition of enzyme activity could also be a factor in regulating tryptophan biosynthesis in *S. venezuelae*.

When the *E. coli trpE* mutant JA200 was transformed to prototrophy by introducing the cloned AS gene of *S. venezuelae* in pDQ187, the specific activity of AS from the mycelium of transformants grown in M9 minimal medium was below that in wild-type *S. venezuelae* [0.01 versus 0.06 nmol anthranilic acid min⁻¹ (mg protein)⁻¹]; moreover, the AS specific activity was not reduced further by supplementing cultures with tryptophan. To avoid the presumed feedback effect of endogenous tryptophan synthesis, we used an *E. coli* W3110 *trpE* strain with additional mutations (*trpC trpR*) that maintained tryptophan auxotrophy in cultures transformed with pDQ187. As anticipated, transformants in minimal medium responded to tryptophan, but grew more slowly than the wild-type. The highest specific activity of AS in the *E. coli trpCER* mutant carrying pDQ187 was 0.063 nmol anthranilic acid min⁻¹ (mg protein)⁻¹ (Fig. 3c); this was much lower than that in *E. coli* W3110 *trpC* [0.85 nmol anthranilic acid min⁻¹ (mg protein)⁻¹], where AS activity was expressed from the native *E. coli trpE* and *trpG* genes (Fig. 3b). The differences were reflected in the sensitivity of the two strains to exogenous tryptophan; in *E. coli trpCER* (pDQ187), repression of AS activity was virtually complete at 10 µM tryptophan, whereas *E. coli* W3110 *trpC* required 50 µM for a similar response. The results indicated that the cloned *S. venezuelae* DNA fragment mediated a weak feedback response to tryptophan when transferred to an *E. coli* host. The low level of enzyme activity and interference from tryptophan in the assay prevented accurate measurement of feedback inhibition in extracts of *E. coli trpCER* transformed with pDQ187.

Gene disruption and replacement

The ORF2 sequence in pDQ187 was disrupted by digesting the plasmid at its unique *MluI* site (see Fig. 1), and recircularizing the linear DNA after blunt-ending by the procedure of Anzai *et al.* (1988). Transformation of *E. coli* JA200 (*trpE*) with the ligation mixture gave prototrophic and tryptophan-requiring colonies in about equal proportions. Plasmid pDQ190 isolated from one of the Trp⁻ auxotrophs resembled pDQ187 in giving a single fragment of 5.3 kb by digestion with *EcoRI*, but was not digested by *MluI*. The insert was excised from pDQ190 as a 2.4 kb *EcoRI*–*HindIII* fragment, and ligated to the *EcoRI* and *HindIII* sites in pHJL400. Transformation of *E. coli* TG1 with the ligation mixture yielded pDQ191, which was then introduced into *S. venezuelae* VS194. Transformants selected for thiostrepton resistance were maintained without selection through three rounds of sporulation to allow loss of free plasmids. Reimposing selection for the antibiotic yielded resistant strains in which pDQ191 had integrated into the chromosome by a single crossover.

Fig. 4. (a) Southern hybridization of DNA digests probed with pDQ189. Lanes: H and E, *HindIII* and *EcoRI* digests, respectively, of λ DNA; VS194, *MluI* digest of *S. venezuelae* VS194 genomic DNA; Thio^S transformants 1–7, *MluI* digests of genomic DNA from *S. venezuelae* VS194(pDQ191) transformants that were Thio^S and failed to hybridize with pHJL400. (b) Model accounting for integration of pDQ191 into the *S. venezuelae* chromosome, plasmid excision, and retention of alternative alleles by homologous recombination.

Plating spores from these strains to give 100–150 colonies per plate, and replica-plating to screen for antibiotic sensitivity and auxotrophy yielded about 120 (10%) thiostrepton-sensitive (Thio^S) colonies, none of which required tryptophan for growth.

Southern analysis of genomic DNA from seven of the Thio^s strains digested with *Mlu*I and probed with pHJL400 showed no hybridization, as predicted if the plasmid had been excised by a double crossover. Probing the DNA with pDQ189 (carrying the 2.4 kb *Eco*RI–*Hind*III *S. venezuelae* fragment from pDQ187 recloned in pHJL400) gave two hybridization patterns (Fig. 4a). In two of the seven strains (designated AP54), the size of the fragments (16.5 and 12 kb) matched those given by the VS194 parent, and were thus consistent with an *Mlu*I site within the hybridizing region. The other five strains (AP55) showed only the single hybridizing fragment of approximately 28.5 kb predicted if the region lacked an internal *Mlu*I site. The results imply that the second crossover occurred at two alternative locations, one restoring the wild-type chromosomal sequence, and the other replacing it with the disrupted sequence (Fig. 4b).

In the nucleotide sequence of the pDQ187 insert, the restriction site for *Mlu*I is at nt 1140–1145, near the middle of ORF2 (nt 251–2122), and in the domain encoding TrpE. Predictably, pDQ191, carrying the disrupted ORF2, failed to confer prototrophy when it was used to transform the *E. coli trpE* mutant JA200. However, *S. venezuelae* strain AP55, in which the disrupted ORF2 sequence had replaced the wild-type DNA, grew on minimal medium without a tryptophan supplement, implying endogenous synthesis. To confirm the hybridization evidence for mutation at the *Mlu*I site in pDQ191, the region was sequenced. This showed that ORF2 had been altered by the deletion of two bases (AA) at nt 1139–1140, shifting the reading frame to create a TGA stop codon at nt 1165–1167. The 304 aa polypeptide encoded by the mutated gene should constitute a severely truncated ASI; ASII should be absent. In an attempt to clone a second fragment of *S. venezuelae* DNA conferring anthranilic acid synthesis, a genomic library of *S. venezuelae* AP55 was prepared in pTZ18R and used to transform *E. coli* JA221 (Δ *trpE*). Approximately 17000 transformants containing recombinant vector DNA were screened for growth on minimal medium, but no prototrophic colonies were obtained.

DISCUSSION

In encoding both ASI and ASII from domains in a single ORF the AS gene cloned from *S. venezuelae* ISP5230 (Paradkar *et al.*, 1991a) resembles the *trpE*(G) gene used in tryptophan biosynthesis by *R. meliloti* (Bae *et al.*, 1989) and *Azospirillum brasilense* (De Troch *et al.*, 1997), and the *phzB* and *phzE* genes used in phenazine biosynthesis by *P. aureofaciens* and *P. fluorescens*, respectively (Pierson *et al.*, 1995). In all of these genes, the N-terminal two-thirds of the encoded polypeptide is similar in sequence to the discrete ASI subunit proteins that exhibit aminase activity in enteric and other bacteria; the sequence of the C-terminal regions resembles the ASII subunit responsible for glutamine amidotransferase activity. However, the 624–637 aa products of the streptomycete and pseudomonad genes are smaller than the TrpE(G) polypeptides of *R. meliloti* and *Azospirillum brasilense*, which match the combined

size (700–900 aa) of most pairs of discrete AS subunits. Sequence alignments indicate that size reduction in the *S. venezuelae*, *P. aureofaciens* and *P. fluorescens* enzymes has occurred in the distal portion of the ASI domain; the region between 150 and 400 aa from the N termini closely matches the region 250–500 aa from the N terminus of ASI in other organisms. Retention of C-terminal sequence would be consistent with the presumption from sequence conservation (Crawford, 1989) that this region is associated with important functional features in TrpE polypeptides.

The two-base deletion introduced by gene replacement into the ORF2 of *S. venezuelae* AP55 would be expected to change the translational reading frame 296 codons from the start site. Consequently, strain AP55 should lack both ASI and ASII activity. Because tryptophan is synthesized in *S. venezuelae* by the pathway common to most organisms (Paradkar *et al.*, 1991a), the ability of AP55 to grow without a tryptophan supplement implies that it has an alternative means of synthesizing anthranilic acid. The evidence is supported by our inability to create auxotrophic mutants by integration within the gene of random, small fragments of ORF2 DNA cloned in pHJL400 (data not shown). The possible presence of a second enzyme with AS activity is raised by evidence obtained earlier (Francis *et al.*, 1978) that partial purification of AS activity from *S. venezuelae* gave an enzyme with two discrete subunits different in size from the single bifunctional protein deduced from nucleotide sequence data in the present work. Although efforts to obtain a second gene by complementing *E. coli* JA221 (*trpE*) with cloned genomic DNA from the *S. venezuelae* AP55 were unsuccessful, this may well have been due to lack of expression in *E. coli*.

Southern hybridizations at relatively high stringency using as a probe the 2.4 kb *Bam*HI–*Sac*I *S. venezuelae* DNA fragment containing ORF1 and ORF2 detected only one hybridizing region in *S. venezuelae* genomic DNA (Paradkar *et al.*, 1991a; see also Fig. 4a). Unique homologues were also present in other streptomycetes, though lower stringencies were sometimes needed (Paradkar *et al.*, 1991a). At lower stringencies, additional hybridizing fragments might be detected in *S. venezuelae*; these could include related genes conferring AS activity, since genes propagated within an organism after duplication of a common ancestral form would be expected to undergo paralogous divergence (Crawford & Milkman, 1991). *p*-Aminobenzoate synthase is related in structure and function to AS (Goncharoff & Nichols, 1984), and could potentially substitute for this enzyme in streptomycetes. A potential candidate for residual AS activity would be the secondary metabolic *pabAB* gene identified in *S. venezuelae* (Aidoo *et al.*, 1990; Brown *et al.*, 1996). Whether this gene can substitute for *trpE*(G) has not been tested. However, a cloned *S. lividans* DNA fragment complementing *pab* mutations in both *S. lividans* and *E. coli* (Arhin & Vining, 1993) did not restore prototrophy in *E. coli* JA221 (Δ *trpE*) (data not included). The existence of more than one gene encoding AS activity in streptomycetes might explain why there

are no previous reports of *trpE* or *trpG* mutants from these organisms.

Two genes for AS activity have been demonstrated in *P. aeruginosa* (Essar *et al.*, 1990); one of the gene products functions in tryptophan biosynthesis and the other in production of the phenazine antibiotic pyocyanine. Under some conditions the functions are interchangeable, and either set of AS genes can complement *trpE* or *trpG* mutations in *E. coli* (Essar *et al.*, 1990). Phenazines have been isolated from several actinomycetes (Gerber, 1984), but as yet *S. venezuelae* has not been reported to produce any secondary metabolites derived from anthranilic acid. The marked similarities between the AS from *S. venezuelae* and those reported from phenazine-producing pseudomonads point to a possible role in phenazine biosynthesis, and raise a question about the function of ORF2 in *S. venezuelae*. A persuasive argument in favour of a role in tryptophan biosynthesis is the presence immediately upstream of a small ORF with the characteristics of a *trpL* attenuator gene. Its predicted gene product is a polypeptide in the size range (14–19 amino acids) of *trpL* leader peptides from other bacteria (Bae *et al.*, 1989). It resembles the *trpL* region associated with regulation of the *trp* operons in *Brevibacterium lactofermentum* and *R. meliloti* in containing three Trp codons in tandem; the leader peptides of enteric bacteria have two tandem Trp codons. Strong sequence similarity between the region predicted to generate the 3:4 stem structures in *S. venezuelae*, *R. meliloti* and *E. coli* suggests that the rho-independent transcriptional terminator known to form in the latter two bacteria probably forms also in *S. venezuelae*. The presence of a putative –10 hexamer upstream of the leader region, the absence of a recognizable –35 consensus sequence, and the existence nearby of a small direct repeat is consistent with a promoter containing auxiliary regulatory sequences.

An earlier suggestion (Paradkar *et al.*, 1991b) that the concentration of tryptophan/tryptophanyl-tRNA in wild-type *S. venezuelae* mycelium is sufficient to cause feedback repression of AS synthesis was supported by the marked increase in AS activity that occurred when endogenous pool concentrations were lowered by introducing a *trpC* mutation. Supplementation of the mutant cultures with tryptophan provided further evidence of endproduct repression. However, the apparent ability of *S. venezuelae* to obtain anthranilic acid by more than one route raised a question about whether *trpE(G)* or the alternative process was the feedback target. This issue was addressed by introducing the ORF1 and ORF2 genes from *S. venezuelae* into *E. coli trp* mutants. As anticipated from earlier results (Paradkar *et al.*, 1991b), *E. coli* JA200 ($\Delta trpE$) transformed to tryptophan independence by pDQ187 showed low AS activity, which did not change appreciably when cultures in minimal medium were supplemented with increasing concentrations of tryptophan. That the low AS activity was due to feedback from endogenous tryptophan was indicated by the response to tryptophan of *E. coli* W3110 *trpCER* transformed to prototrophy with pDQ187. Although

the level of AS activity in transformants was low, and feedback repression saturated at a relatively low supplement concentration, the graded response to tryptophan suggested that the enzyme was involved in tryptophan biosynthesis. The low intensity of the effect might be attributed to imperfect regulation and/or weak expression of the ORF2 gene in the heterologous system.

Sensitivity to endproduct inhibition is reported to be a feature of AS involved in tryptophan biosynthesis (Zalkin, 1980; Ross & Winkler, 1988), but not of the AS involved in biosynthesis of the secondary metabolite pyocyanine (Essar *et al.*, 1990). Thus the concentration-dependent inhibition by tryptophan of AS activity in extracts from *S. venezuelae* VS153 implicates this activity in tryptophan biosynthesis. However, the response was weak: 44% inhibition with 2 mM tryptophan compared with 75% inhibition of *R. meliloti* TrpE(G) obtained with only 5 μ M tryptophan by Bae & Crawford (1990). Moreover, the response in *S. venezuelae* cannot be unequivocally attributed to the ORF2 product because more than one AS activity may have been present in the cell extracts. The evidence currently available does not allow a firm decision on the physiological function of the AS gene cloned from *S. venezuelae*. Its association with systems for feedback regulation by tryptophan suggests that it is *trpE(G)*, a key gene in the pathway for biosynthesis of this amino acid. On the other hand, the results from sequence alignments show a close evolutionary relationship with *phz* genes and implicate it in the production of a phenazine secondary metabolite.

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