JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 463–471
0021-9193/91/020463-09$02.00/0
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Vol. 173, No. 2

Molecular Characterization and Expression Analysis of the Anthranilate Synthase Gene of *Pseudomonas syringae* subsp. *savastanoi*†

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Received 1 May 1990/Accepted 22 October 1990

The *trpE* gene, which encodes the large component of the enzyme anthranilate synthase, was isolated from a *Pseudomonas syringae* subsp. *savastanoi* (*P. savastanoi*) cosmid library. Cosmids that complemented an *Escherichia coli trpE* mutation contained a gene whose product is 86% homologous at the deduced amino acid level to TrpE of *Pseudomonas aeruginosa* and *Pseudomonas putida*. Amino acid sequence comparison with other TrpE sequences revealed the existence of conserved regions between the procaryotic and eucaryotic polypeptide sequences analyzed, regions that might be of functional importance. We also report on studies on the expression pattern of this gene. We analyzed the promoter activity of a *trpE::lacZ* transcriptional fusion, the relative amount of *trpE* steady-state mRNA, and the activity of anthranilate synthase from cells grown in minimal medium with or without exogenously added tryptophan and in complete medium. We concluded that under the conditions tested, expression of the *trpE* gene of *P. savastanoi* is independent of the concentration of tryptophan in the culture medium. Implications of such an expression pattern on the virulence of this bacterium are discussed.

The amino acid tryptophan is the precursor of indoleacetic acid (IAA), which is one of the virulence factors of the plant-pathogenic bacterium *Pseudomonas syringae* subsp. *savastanoi* (*P. savastanoi*) (22). Increases in the intracellular concentration of tryptophan resulted in the production of larger amounts of IAA and, consequently, a greater severity of symptoms caused by this bacterium on its hosts, as measured by the size of the galls formed (23, 33). It was recently demonstrated that expression of the IAA biosynthetic genes of *P. savastanoi*, *iaaM* and *iaaH*, is constitutive and independent of the concentration of tryptophan in the medium (17). The levels of IAA produced by *P. savastanoi* are dependent on the availability of tryptophan and on feedback inhibition of the enzyme tryptophan 2-monooxygenase (EC 1.13.12.3), the *iaaM* gene product, by IAA (20). Thus, regulation of the tryptophan biosynthetic pathway may play an important role in the virulence of this organism.

Tryptophan biosynthesis has been best studied in *Escherichia coli* and *Salmonella typhimurium* (for a recent review, see reference 37). A variety of other organisms have also been studied, among them some *Pseudomonas* species (6, 9, 19, 28). The number and organization of the tryptophan biosynthetic genes vary considerably among the organisms studied so far. Only the enteric bacteria have all *trp* genes organized in a single operon (37). In the *Pseudomonas* species studied so far, there are seven tryptophan biosynthetic genes found in four locations on the chromosome (6, 19).

Anthranilate synthase (EC 4.1.3.27) catalyzes the first step in the pathway specific for the synthesis of tryptophan, the chorismate-dependent synthesis of anthranilate with glutamine as the amino donor. In all procaryotic enzymes examined in detail, anthranilate synthase is a two-component enzyme system (9, 28). The large component carries the chorismate binding and the tryptophan feedback inhibition sites and is capable of converting chorismate to anthranilate with ammonia as the amino donor (9, 28). The small component functions in the glutamine binding (9, 28). The large and small subunits of anthranilate synthase in *Pseudomonas aeruginosa* and *Pseudomonas putida* are the gene products of the *trpE* and *trpG* loci, respectively (9).

In the enteric bacteria, the tryptophan operon is regulated by a dual transcriptional control: (i) tryptophan repression, mediated by the *trp* repressor, the *trpR* gene product (4), and (ii) attenuation, which provides an option of transcription termination, depending on the level of charged $tRNA^{Trp}$ (37). This sophisticated regulatory system permits variations in the level of expression of the tryptophan operon over a range of 600-, 80-, and 7-fold from repression and attenuation, respectively (38). Another factor that is important in determining the amount of tryptophan produced by the cell is feedback inhibition of anthranilate synthase by tryptophan.

Some information regarding regulation of the tryptophan biosynthetic genes of *Pseudomonas* spp. is available. Smidt (32a) has shown that the enzyme anthranilate synthase of *P. savastanoi* is under feedback inhibition by tryptophan. The inhibition seems to be competitive with respect to chorismate. Maurer and Crawford (26) and Calhoun et al. (6) reported the isolation of mutants of *P. putida* and *P. aeruginosa* resistant to indole and tryptophan analogs which overproduced the enzymes anthranilate synthase, phosphoribosyltransferase, and indoleglycerol phosphate synthetase in the presence or absence of tryptophan. They postulated that this mutation was located in a repressor gene of the *trp* genes, a *trpR* analog. In wild-type *P. aeruginosa* and *P. putida*, however, tryptophan did not alter the expression of the same genes, which indicates that they are always fully

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† Dedicated to the memory of Tsune Kosuge (deceased 13 March 1988). Without his support and encouragement, this work would have been impossible.

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TABLE 1. Bacterial strains, vectors, and plasmid constructs

Strain or plasmid	Description[a]	Reference or source
Strains		
P. savastanoi		
PB213	Oleander isolate, IAA$^+$, pIAA2	8
PB213-rif	Rf^r PB213	Laboratory strain
E. coli		
HB101	F$^-$ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ^- *thi leu*	5
DH5α	F′ *endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1* φ80*lacZ*ΔM15 Δ(*lacZYA-argF*)	Bethesda Research Laboratories
JM101	*supE thi* Δ(*lac-proAB*)(F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15)	36
W3310/Δ*trp*	*trp*Δ*E5 tna*	C. Yanofsky
Vectors		
pUC118	Ap^r; M13mp18 multicloning region	35
pTZ18R	Ap^r; M13mp18 multicloning region	U.S. Biochemicals
pSP72	Ap^r; T7 and SP6 promoters	Promega Biotec
pLAFR3	Tc^r; cosmid vector	34
pGD499	Ap^r Tc^r; vector for *lacZ* transcriptional fusions	12
pGD500	Tc^r; control plasmid, promoterless *lacZ* gene	12
pRK2013	Km^r; helper plasmid in triparental matings	13
Plasmid constructs		
pSAV301	0.6-kb *Pst*I-*Eco*RI fragment of pCJP10 cloned in pSP65 (*iaa* probe)	17
p1460	3.8-kb *Hin*dIII-*Bgl*II fragment of pUC19 (*trp*E gene of *P. aeruginosa* PAO1)	15
pSAV609	1-kb *Sal*I deletion of pSAV660 in pUC118	This study
pSAV610	0.6-kb *Eco*RI deletion of pSAV660 in pUC118	This study
pSAV613	1.2-kb *Sph*I deletion of pSAV660 in pUC118	This study
pSAV614	0.7-kb *Sph*I deletion of pSAV660 in pUC118	This study
pSAV615	0.7-kb *Bam*HI-*Hin*dIII fragment of pSAV660 in pGD499	This study
pSAV616	3.2-kb *Pst*I fragment of pSAV660 in pTZ18R	This study
pSAV616Δ15/3	1.6-kb exonuclease III derivative of pSAV616 in pTZ18R	This study
pSAV617	Opposite orientation of pSAV616 in pTZ18R	This study
pSAV633	29-kb partial *Sau*3AI insert in pLAFR3 (library clone)	This study
pSAV634	0.18-kb *Sac*I-*Cla*I fragment of pSAV616Δ15/3 in pSP72 (residues 1615 to 1793 in Fig. 2) (*trp*E probe)	This study
pSAV660	3.2-kb *Pst*I fragment of pSAV633 in pUC118	This study

[a] IAA$^+$, Strain carrying the *iaa* operon (IAA-producing strain); Ap^r, ampicillin resistant; Tc^r, tetracycline resistant; Km^r, kanamycin resistant; Rf^r, rifampin resistant; T7 and SP6, bacteriophage RNA polymerase promoters.

repressed by the intracellular concentration of tryptophan even when the cells are growing in minimal medium. These results seem to argue that feedback inhibition of anthranilate synthase by tryptophan plays a major role in the regulation of tryptophan biosynthesis in pseudomonads.

Because of the very likely close link between tryptophan biosynthesis and virulence of *P. savastanoi*, we are interested in studying this pathway. For this purpose, we have cloned, from a genomic cosmid library, the *trpE* gene of this bacterium. This clone restored anthranilate synthase activity to a *trpE*-deletion *E. coli* mutant. Extensive amino acid similarity was found between the deduced sequences of *P. savastanoi* and other *Pseudomonas* spp. TrpE proteins. Blocks of conserved amino acid residues were found in the C-terminal portions of procaryotic and eucaryotic TrpE proteins, which might indicate that these residues are functionally important. This gene is a chromosomal single-copy gene and seems to be transcribed as a monocistronic message. We also present results of studies on the regulation of expression of this gene. We analyzed the promoter strength of the gene, the steady-state concentrations of the *trpE* mRNA, and anthranilate synthase activity from cells growing in minimal medium, in minimal medium with excess tryptophan, and in complete medium. We present evidence that the *trpE* gene of *P. savastanoi* is always expressed at similar levels, under the experimental conditions tested, independently of the tryptophan concentration in the culture medium and speculate on the implications of such an expression pattern on the fitness and virulence of this bacterium on its hosts.

MATERIALS AND METHODS

Enzymes and chemicals. Enzymes were obtained from New England BioLabs, Inc., Boehringer Mannheim Biochemicals, IBI Inc., or Amersham Corp. The Sequenase sequencing kit was obtained from United States Biochemical Corp. Radiolabeled nucleotides and the Multiprime labeling kit were purchased from Amersham. Giga Pack Gold was purchased from Stratagene. The Riboprobe kit, containing bacteriophage T7 and SP6 RNA polymerases, was obtained from Promega Biochemical. All other chemicals were obtained from Sigma Chemical Co.

Bacterial strains and plasmids. The bacterial strains and plasmids used in these studies are described in Table 1.

Culture of bacteria. *P. savastanoi* was routinely cultured in King B medium (peptone, 10 g/liter; glucose, 15 g/liter; $MgSO_4 \cdot 7H_2O$, 0.4 g/liter; pH 7.0) (21) or minimal A medium (27) at 28°C. The antibiotics for these cultures were tetracycline at 15 or 12 μg/ml and rifampin at 100 μg/ml. *Escherichia coli* was grown in LB (27) or minimal A medium at 37°C. The antibiotics used were ampicillin at 50 or 20 μg/ml and tetracycline at 25 or 15 μg/ml. L-Tryptophan was supplemented when needed at 50 μg/ml.

Bacterial transformations and conjugations. *E. coli* transformations were performed as described in Alexander et al. (2). Triparental matings were routinely done by using pRK2013 as a helper plasmid as described elsewhere (30). Incubations were done on King B medium plates at room temperature for 12 to 16 h. Filter matings of the amplified *P. savastanoi* PB213 library into *E. coli* W3310/Δ*trp* were done

as described by Miller (27) with an approximate cell concentration ratio (recipient/donor/helper) of 1:1:1.

Recombinant DNA techniques and plasmid constructs. Restriction enzyme digests, ligations with T4 DNA ligase, and plasmid isolations were performed as described by Maniatis et al. (25). Calf intestine alkaline phosphatase and DNA polymerase I Klenow fragment were used according to the manufacturer's protocols. Total DNA was isolated from *P. savastanoi* as described in reference 3. Partial digestion of total DNA with *Sau*3AI and fractionation of the resulting restriction fragments in sucrose density gradients for construction of the genomic library were done as described by Maniatis et al. (25). Restriction fragments to be subcloned into various plasmid vectors were isolated from TAE (40 mM Tris-acetate, 1 mM EDTA) (25) agarose gels with DEAE NA-45 membranes (Schleicher & Schuell, Inc.) as instructed by the manufacturer. The plasmid constructs used in these studies are shown in Table 1.

Construction of a *P. savastanoi* PB213 genomic library. A genomic library of *P. savastanoi* PB213 was constructed with partially *Sau*3AI-digested total DNA ligated into the *Bam*HI site of the broad-host-range cosmid vector pLAFR3 as described by Staskawicz et al. (34) except that Giga Pack Gold packaging extracts were used. For long-term storage, the library was amplified in *E. coli* HB101 by harvesting library-infected cells grown on selective plates with sterile 0.9% NaCl. Sterile glycerol was then added to a final concentration of 15%, and the cells were stored in aliquots at −70°C.

RNA preparations. RNA was extracted from *P. savastanoi* cells by the hot phenol method (1).

Nucleic acid gel electrophoresis. Agarose slab gels (15 wide by 10 cm long) were normally used with TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) run at 100 V (25). Uncut *P. savastanoi* plasmids were analyzed in 0.4% agarose gels; restriction enzyme analyses of plasmid constructs were done in 0.7 or 1.0% agarose gels. RNA samples were run in 1% agarose–6% formaldehyde gels (12 by 14 cm) in 1× MOPS buffer, pH 7.0 (20 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 5 mM sodium acetate, 1 mM EDTA) at 60 V (25). The RNA preparations were denatured at 60°C for 15 min in loading dye (50% formamide, 6% formaldehyde, 1× MOPS [pH 7.0], 0.25% bromophenol blue) and loaded on the gel. Gels used for DNA sequencing were wedge 0.2 to 0.6-mm gels (34 by 45 cm), 8% polyacrylamide (19:1, acrylamide/bisacrylamide)–7 M urea in TBE buffer (25). The gels were run at constant 75 W.

DNA hybridizations. Blotting of DNA from 0.7% agarose gels to nylon membranes (Nytran; Schleicher & Schuell) was done as recommended by the manufacturer. Prehybridizations and hybridizations were performed in 6× SSC (1× SSC is 0.15 M NaCl plus 15 mM sodium citrate, pH 7.0)–1% sodium dodecyl sulfate (SDS)–1× Denhardt solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin)–250 μg of denatured salmon sperm DNA per ml at 55°C for low-stringency blots or at 65°C for high-stringency blots. Blots were washed first at room temperature for 20 min in 6× SSC–0.5% SDS and then at 42°C for 20 min in 1× SSC–1% SDS; the last wash was done at 55°C for 45 min in 2× SSC–1% SDS for low-stringency blots and at 65°C for 45 min in 0.1× SSC–1% SDS for high-stringency blots. Labeling of DNA probes was done with [α-^{32}P]dCTP (800 Ci/mmol), using the Multiprime labeling kit (Amersham) as instructed by the manufacturer.

RNA hybridizations. Blotting of RNA from 1% agarose–6% formaldehyde gels to nylon membranes (Nytran) was done as recommended by the manufacturer. RNA slot blotting on nylon membranes (Nytran) were performed by using a Minifold-II slot blotter apparatus (Schleicher & Schuell) as instructed by the manufacturer. The RNA samples were denatured in 6% formaldehyde–10× SSC at 60°C for 15 min prior to loading on the blotting apparatus. Prehybridizations and hybridizations were carried out at 60°C in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], and 1 mM EDTA)–0.5% SDS–5× Denhardt solution–250 μg of denatured salmon sperm DNA per ml. Blots were washed twice for 20 min each in 1× SSPE–0.5% SDS at 65°C and once for 15 min in 0.1× SSPE–0.5% SDS at 60°C. RNA probes, labeled with [α-^{32}P]UTP (400 Ci/mmol), generated from a plasmid containing the bacteriophage T7 and SP6 RNA polymerase promoters flanking the multicloning site were used in these experiments and were made according to the manufacturer's protocols.

DNA sequencing. The dideoxy-chain termination method (32) was routinely used for sequencing inserts in the vector pTZ18R, using the M13 reverse sequencing primer (IBI). Nested exonuclease III deletions of the inserts to be sequenced were made as described by Henikoff (18). Single-stranded copies of the various constructs were obtained from *E. coli* JM101 cells infected with the mutant phage M13KO7 by a modification of the basic protocol of Dente et al. (7, 11). The Sequenase sequencing kit was used as instructed by the manufacturer with [α-^{35}S]dATP (600 Ci/mmol). Sequence data and homology analyses were performed by using the MicroGenie sequence analysis program (Beckman Instruments, Inc.).

Anthranilate synthase activity assay. *P. savastanoi* and *E. coli* cells were assayed for anthranilate synthase (EC 4.1.3.27) activity as described by Last and Fink (24). The crude cell extract was prepared by a modification of the protocols of Last and Fink (24): cells (1 to 1.5 g) grown in appropriate medium (1 liter) were harvested at an optical density at 600 nm of 0.5 to 0.8 and washed twice with 0.9% sterile saline. The cells were resuspended in 5 ml of buffer A (50 mM Tris hydrochloride [pH 7.5], 0.2 mM EDTA, 0.2 mM dithiothreitol, 8 mM $MgCl_2$, 5% [vol/vol] glycerol) and broken with two passages through a French press at 750 to 1,000 lb/in^2. The bacterial suspension was incubated on ice for 20 min, and the extract was cleared by centrifugation for 10 min at 10,000 rpm in an SS34 rotor at 4°C. A 2.5-ml sample of the supernatant was subjected to chromatography on a NAP-25 (Sephadex G-25; Pharmacia, Inc.) column that had been equilibrated with 5 column volumes of buffer B (50 mM Tris hydrochloride [pH 7.5], 0.05 mM EDTA, 0.05 mM dithiothreitol, 2 mM $MgCl_2$, 5% [vol/vol] glycerol). Fractions of 0.5 ml were collected and assayed for protein concentration (Bio-Rad protein assay; catalog no. 500-0006). The peak fractions were pooled, glycerol was added to a final concentration of 25%, and the samples were stored at −20°C.

Anthranilate synthase activity was measured as the chorismate-dependent production of anthranilate in a 2-ml reaction mixture containing 1.0 ml of buffer B, 0.1 μmol of chorismic acid, 20 μmol of glutamine, 2 μmol of $MgCl_2$, and 25 μmol Tris hydrochloride (pH 8.0). The reaction was started by adding crude extract, incubated at 37°C, and stopped with 0.2 ml of 1 M HCl. The assay yielded linear results when 5 to 20 μl of crude extract was used and the reaction mixture was incubated for 10 to 30 min. The anthranilate produced was then extracted with 2 ml of ethyl acetate, and the extraction mixture was clarified by low-speed centrifugation at room temperature. The anthranilate

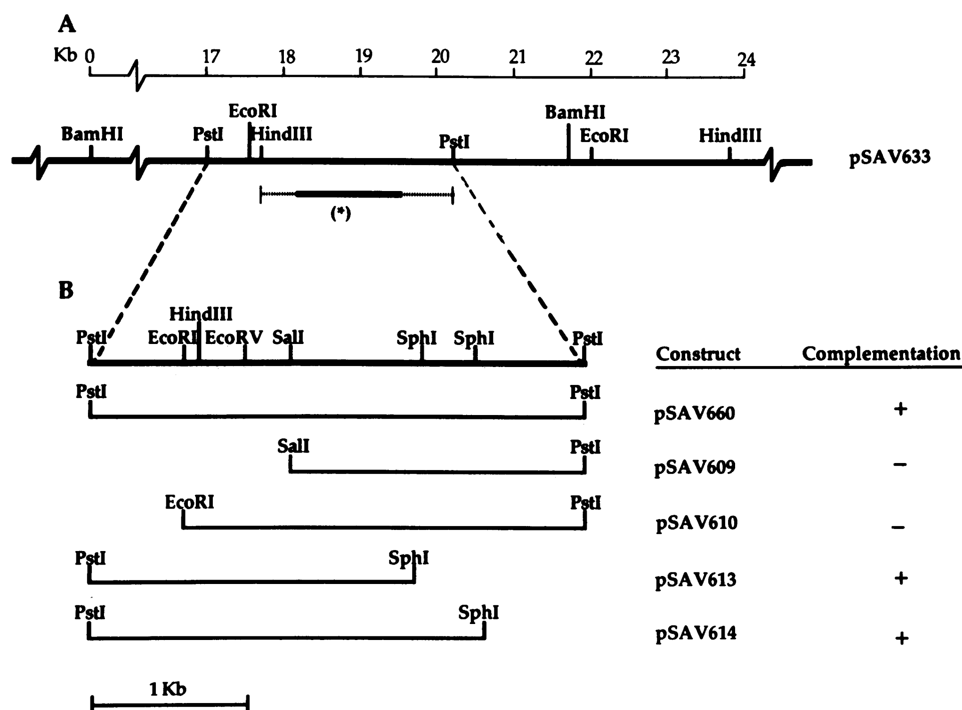

FIG. 1. Partial restriction enzyme map of the cosmid clone pSAV633 (A) and restriction enzyme map of pSAV660 (B). Deletions derivatives of pSAV660 are also shown, together with their ability to complement the *trpΔE5* mutation of *E. coli* W3310 *Δtrp*. (A) The *Xho*I-*Sal*I fragment of p1460 used as probe in the Southern blot analysis done to construct this map is shown as a black bar with an asterisk. The exact location of the probe on the 2.5-kb *Hin*dIII-*Pst*I fragment is unknown (hatched lines). (B) Complementation of the *trpΔE5* mutation of *E. coli* W3310/*Δtrp* by each deletion derivative was determined by inoculating this strain harboring the different constructs in minimal A medium (+, growth; −, no growth).

produced was quantitated in Perkin-Elmer MPF-44A fluorescence spectrophotometer (excitation 340 nm, slit width 10; emission 400 nm, slit width 4) with anthranilate solutions as standards.

β-Gal activity assay. The assay of β-galactosidase (β-gal) activity of *P. savastanoi* cells harboring a *trpE*::*lacZ* transcriptional fusion and control plasmid was done as described by Miller (27). Cultures were used for the assay at an optical density at 600 nm of 0.4 to 0.6, and the cells were permeabilized with SDS and chloroform.

Nucleotide sequence accession number. The nucleotide sequence of the *P. savastanoi trpE* gene has been assigned GenBank accession number M55911.

RESULTS

Molecular characterization. (i) Genomic library of *P. savastanoi* PB213. The genomic library of *P. savastanoi* constructed with size-fractionated partially *Sau*3AI-digested *P. savastanoi* total DNA in the vector pLAFR3 contained more than 2×10^5 transductants per μg of insert DNA used in the ligation. Analysis of random clones revealed that the average insert size was 25 to 30 kb (data not shown).

(ii) Cloning of the *P. savastanoi trpE* gene. The approach used to isolate cosmid clones containing the *P. savastanoi trpE* gene was to complement the *trpΔE5* mutation of *E. coli* W3310/*Δtrp*, a tryptophan auxotroph. This was attempted by mobilizing, via triparental matings, the *P. savastanoi* genomic library into this *E. coli* strain and selecting for growth on minimal medium. Restriction enzyme analysis of plasmid DNA extracted from colonies isolated by this scheme revealed the presence of four different classes of complementing overlapping cosmid clones with common restriction fragments shared by the four classes (data not shown).

(iii) Mapping and subcloning of the *P. savastanoi trpE* gene. The cosmid clone pSAV633 was used for further mapping of the *trpΔE5* mutation-complementing region by Southern blot analysis using a *P. aeruginosa* PAO1 *trpE* clone (p1460) as a probe. Various restriction enzyme digests of pSAV633 were submitted to electrophoresis in agarose gels, blotted onto nylon membranes, and hybridized to the labeled 1.3-kb *Xho*I-*Sal*I fragment of p1460 under low-stringency conditions (data not shown). These experiments allowed us to construct the partial restriction map of pSAV633 shown in Fig. 1A. From this map, the 3.2-kb *Pst*I fragment was chosen as a candidate for containing the entire *P. savastanoi trpE* gene. This fragment was subcloned in the vector pUC118, and it indeed complemented the *trpΔE5* mutation; this construct was designated pSAV660.

Deletion derivatives of pSAV660 were constructed, and their ability to complement the *trpΔE5* mutation was determined (Fig. 1B). We concluded that the *P. savastanoi trpE* gene was located in the 2.2-kb *Pst*I-*Sph*I fragment of pSAV660, construct pSAV613.

(iv) Sequencing of the *P. savastanoi trpE* gene. The 3.2-kb *Pst*I fragment of pSAV660 was subcloned in the vector pTZ18R in the two orientations relative to the vector polylinker, designated pSAV616 and pSAV617. Exonuclease III nested deletions of these two constructs were made, and appropriate clones were used for sequencing by the dideoxy-chain termination method (32). The nucleotide sequence of pSAV613 (the clone containing the *P. savastanoi trpE* gene)

and the deduced amino acid sequence of the anthranilate synthase large component are presented in Fig. 2. The anthranilate synthase large component of *P. savastanoi* is 505 amino acids long and has a calculated molecular size of 56 kDa.

Figure 2 also shows the results of alignment of the deduced amino acid sequences of *P. savastanoi* PB213, *P. aeruginosa* PAO1, and *P. putida trpE* genes. The Dayhoff algorithm used in this analysis takes also into consideration conserved changes, i.e., replacement of an amino acid by another of similar function and structure (10). The similarity level with the *P. aeruginosa* sequence is 85% (22 of the 70 changes are conserved) and 87% with the *P. putida* sequence (there are 15 conserved changes among 62 mismatches). Homology between the three sequences ended 25, 12, and 13 amino acids before the stop codons in the *P. savastanoi*, *P. aeruginosa*, and *P. putida* sequences, respectively.

The deduced sequence of the *P. savastanoi* TrpE protein was also compared with the sequences of the TrpE proteins from other, more distant organisms (a gram-negative [*E. coli*] and a gram-positive [*Bacillus subtilis*] bacterium and a eucaryote [*Saccharomyces cerevisiae*]) to identify putative important domains of these proteins that have been conserved through the evolution of these organisms. These comparisons revealed the existence of blocks of conserved residues in the C-terminal portions of the proteins (Fig. 3).

(v) Organization of the *P. savastanoi trpE* locus. The location of the cloned *trpE* gene in the genome of *P. savastanoi* was established by Southern blot analysis performed with different restriction enzyme digests of total and plasmid DNA extracted from *P. savastanoi* hybridized to labeled pSAV660 under high-stringency conditions. Since the pattern of cross-hybridizing bands obtained by using total DNA (data not shown) corresponded to the restriction maps of pSAV633 and pSAV660 (Fig. 1) and no bands were detected in the plasmid DNA (data not shown), we concluded that the cloned *trpE* gene of *P. savastanoi* is a unique copy in the *P. savastanoi* genome located on the chromosome and that no fragment rearrangements occurred during the cloning of pSAV660.

Northern hybridization experiments using a *trpE*-specific probe (pSAV634) revealed cross-hybridization to an mRNA population in the 1.5- to 1.7-kb range (data not shown). These results imply that the transcript of the *trpE* gene is a monocistronic message, since the coding region of the gene is 1,518 bases long, which would encompass the whole mRNA. At this point, however, the possibility of message degradation or processing cannot be ruled out. No known transcription terminators were found in the sequenced 3′ untranslated region, which would indicate that the mRNA extends past the *Sph*I site. Sequence comparison of the sequenced 5′ and 3′ untranslated regions failed to reveal similarities to other known *trp* genes. In other fluorescent *Pseudomonas* spp., the *trpE* gene has been reported as being in a single chromosomal location (14, 15).

Expression analyses. (i) β-Gal activity assay of *trpE::lacZ* transcriptional fusions. We studied the effect of exogenous tryptophan on the transcription level of the *P. savastanoi trpE* gene promoter, as measured by the β-gal activity of a *trpE::lacZ* transcriptional fusion, pSAV615, in which detection of β-gal activity relied on transcription directed by the insert sequences. The direction of transcription, determined by sequencing and Northern (RNA) hybridization experiments and deletion analysis of pSAV660, suggested that the 0.7-kb *Pst*I-*Hin*dIII fragment of pSAV660 used in the construction of this fusion contains the promoter of the *trpE* gene. pSAV615 was mobilized into *P. savastanoi* PB213-rif, and the transconjugants were grown in either King B medium, minimal A medium, or minimal A medium with tryptophan (50 μg/ml) and used to determine levels of β-gal activity. Plasmid pGD500 (promoterless *lacZ* plasmid) was also mobilized into PB213-rif and used as a control in these experiments (Table 2). Levels of expression of the promoter of the *P. savastanoi trpE* gene, as measured by the β-gal activity levels of pSAV615, were approximately the same in the three growth media used and independent of the concentration of exogenous tryptophan. The β-gal activity levels obtained with pSAV615 were reproducibly ~1.4-fold higher than background levels obtained with pGD500.

(ii) Steady-state concentrations of *trpE* message. We determined, in Northern hybridization experiments, the amount of the *trpE* message present in *P. savastanoi* PB213 cells grown in minimal medium containing tryptophan (50 μg/ml) and in King medium B relative to the amount present in cells grown in minimal medium. Different amounts of total RNA extracted from cells growing in the appropriate medium were spotted, in duplicate, onto nylon membranes by using a slot blotter apparatus and hybridized to labeled *trpE* probe or *iaa* probe (data not shown). The amount of message of the IAA biosynthetic genes in each RNA sample was used to normalize the results obtained with the *trpE* probe, since the IAA biosynthetic genes were shown to be equally expressed in minimal medium either containing or lacking tryptophan and in King B medium (17). In a control experiment, hybridization of the RNA samples to a labeled *trpE* sense probe, we determined that these samples did not contain any detectable amounts of DNA (data not shown). The relative intensities of hybridized probes were quantitated from Kodak X-Omat film, using a laser densitometer (Zeineh SL-504-XL). Levels of expression of the *trpE* gene of *P. savastanoi*, as measured by the steady-state levels of message present in the cell, were approximately the same in the three treatments under our level of detection (Table 2).

(iii) Anthranilate synthase activity assays. Crude protein extracts were prepared from *P. savastanoi* cells grown in minimal medium, minimal medium with supplemented tryptophan (50 μg/ml), and complete medium (King B) and used to determine anthranilate synthase activity (Table 2). The levels of enzyme activity in minimal medium with tryptophan and King B medium were very similar. An approximately threefold increase in anthranilate synthase activity was observed in cells grown in minimal medium. We attributed this difference to feedback inhibition of the enzyme activity by residual tryptophan in the two previous samples (33) (a similar increase was observed when anthranilate synthase was assayed from *E. coli* W3310/Δ*trp* harboring pSAV660 grown in minimal medium and minimal medium with tryptophan; data not shown). Unfortunately, the enzyme activity proved to be very unstable and was lost after dialysis of the crude extracts, possibly because of denaturation or dissociation of the two components of the enzyme. We concluded that the activity of the enzyme anthranilate synthase was independent of the concentration of tryptophan in the medium.

DISCUSSION

We have isolated the *trpE* gene, which encodes the large component of the enzyme anthranilate synthase, from *P. savastanoi*. The cloned *trpE* gene was able to restore growth in minimal medium lacking tryptophan, as well as anthranilate synthase activity, to *E. coli* W3310/Δ*trp*, a *trpE*

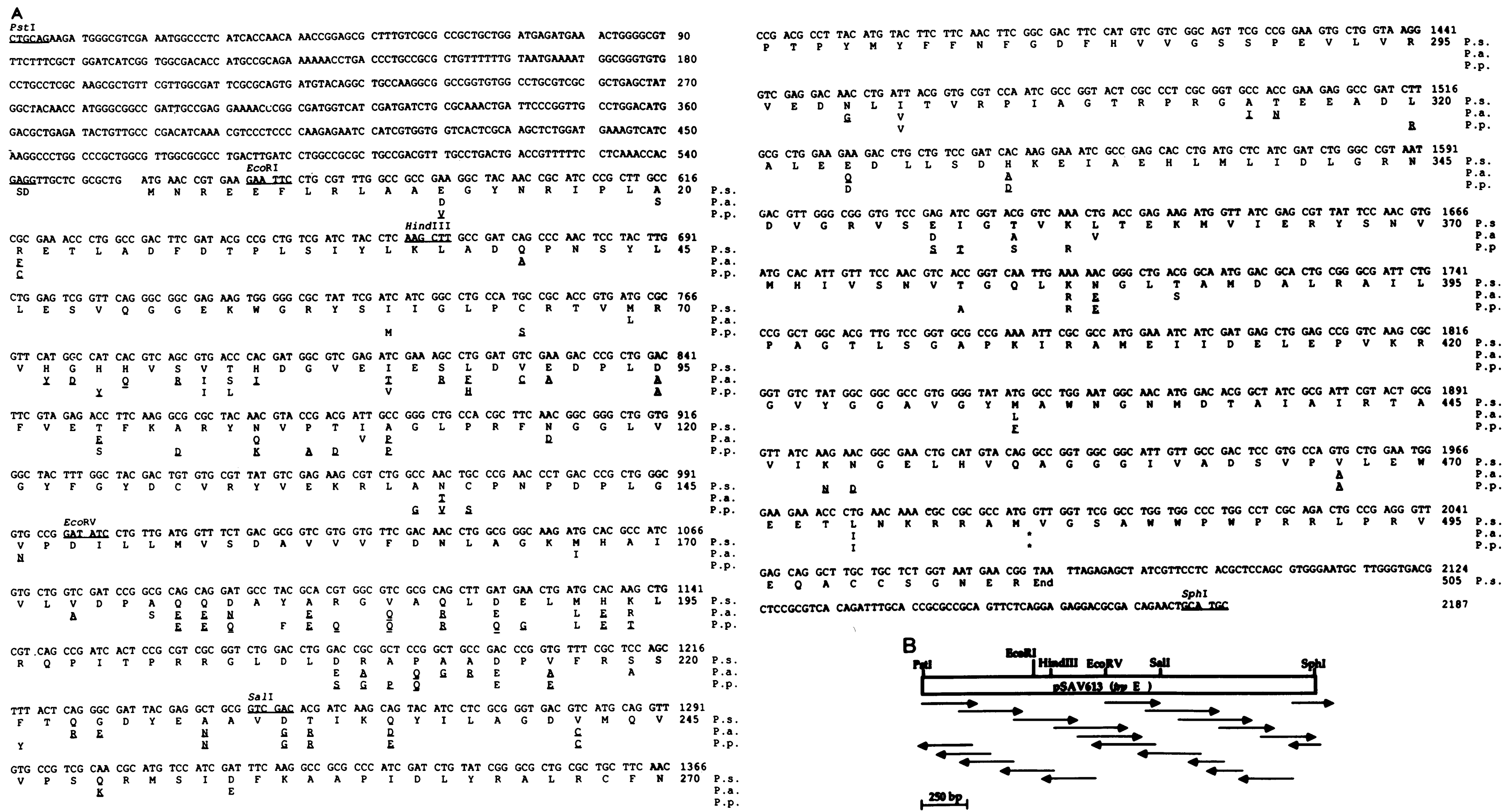

FIG. 2. (A) Nucleotide sequence of pSAV613, containing the *P. savastanoi trpE* gene, and alignment of the deduced amino acid sequences of *P. savastanoi* PB213 (P.s.), *P. aeruginosa* PAO1 (P.a.) (15), and *P. putida* (P.p.) (14) *trpE* genes, performed with the MicroGenie sequence analysis program using the Dayhoff algorithm (10). Only the differences with respect to the *P. savastanoi trpE* amino acid sequence are shown for the *P. aeruginosa* and *P. putida* sequences. Underlined letters correspond to nonconserved changes in a particular amino acid residue as defined by the algorithm used. *, Endpoint of homology with the *P. savastanoi* sequence. No gaps were introduced in the sequences. The one-letter amino acid abbreviations are used. The presumptive Shine-Dalgarno sequence (SD), translation stop (End), and relevant restriction enzyme sites are indicated. Numbers on the right refer to nucleotides (top) and amino acid residues of the *P. savastanoi trpE* amino acid sequence (bottom). Numbering for the *P. aeruginosa* and *P. putida* sequences is the same as for the *P. savastanoi* sequences. (B) Sequencing strategy used for pSAV613. Arrows indicate the different exonuclease III deletion derivatives of pSAV616 (rightward arrows) and pSAV617 (leftward arrows) used for sequencing of both DNA strands.

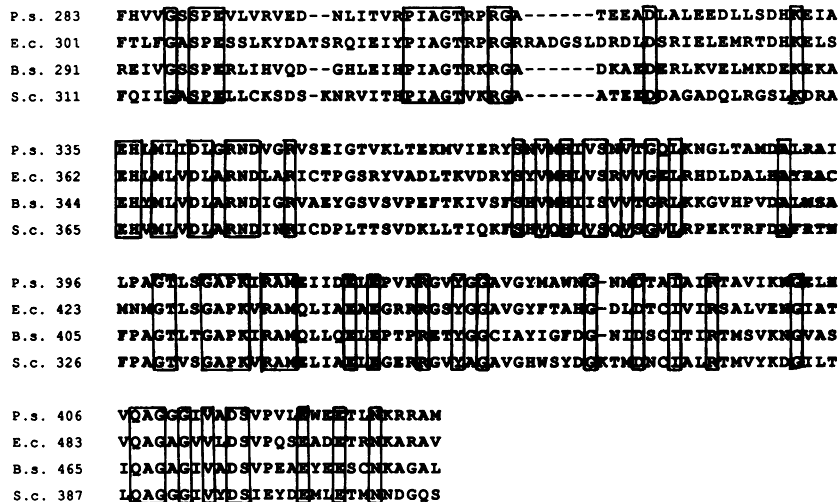

FIG. 3. Alignment of the deduced amino acid sequences of *P. savastanoi* PB213 (P.s.), *E. coli* (E.c.), *B. subtilis* (B.s.) (15), and *S. cerevisiae* (S.c.) (15) *trpE* genes. Gaps were introduced in the sequences to maximize homology. Identical residues among all sequences are boxed. The one-letter amino acid abbreviations are used. Numbers on the left indicate the amino acid residues of each sequence.

deletion mutant (data not shown). Interestingly, the *P. savastanoi* TrpE polypeptide was probably able to form an active complex with the *E. coli* anthranilate synthase small subunit, since glutamine-dependent enzyme activity was measured.

There is remarkable amino acid sequence similarity between the TrpE sequences of *P. savastanoi*, *P. aeruginosa*, and *P. putida*; these sequences are on average 86% similar. The *P. savastanoi* TrpE contains a slightly larger C terminus, 12 amino acid residues, which is unrelated to the other two sequences (Fig. 2).

Amino acid sequence comparisons between evolutionarily distant organisms can prove to be extremely helpful in the identification of functionally relevant regions on proteins whose functional domains are unknown, as is the case for the enzyme anthranilate synthase. These functionally important domains should have been conserved with little change throughout evolution. Such analysis (Fig. 3) revealed the presence of conserved stretches in the C-terminal portions of three procaryotic and one eucaryotic TrpE proteins. These results would imply that the conserved amino acids are relevant for the correct catalytic activity of this enzyme.

TABLE 2. *P. savastanoi trpE* gene expression analysis

Medium	Promoter activity[a] (avg ± SE)		Steady-state mRNA[b] (avg ± SE)	Anthranilate synthase activity[c] (U; avg ± SE)
	pSAV615	pGD500		
Minimal A	678 ± 22	484 ± 3	1.0 ± 0.2	1.0 ± 0.27
Minimal A + Trp (50 μg/ml)	620 ± 5	482 ± 14	1.2 ± 0.1	0.3 ± 0.03
King B	676 ± 3	508 ± 7	1.2 ± 0.3	0.3 ± 0.11

[a] β-Gal activity (Miller units). pSAV615, *trpE::lacZ* fusion plasmid; pGD500, control plasmid. Results are averages of two experiments.

[b] Relative amounts. Values are normalized to those for mRNA from the IAA biosynthetic genes and relative to the value from minimal A medium. Results are averages of three experiments.

[c] Relative to the value from minimal A medium; 1 U = 1 nmol of anthranilate produced per min per mg of total protein. Results are averages of three experiments.

Final proof awaits the analysis of the effect on enzyme activity of point mutations in these conserved regions.

The *trp* genes have been shown to be transcriptionally regulated in *E. coli* depending on the intracellular concentration of the amino acid tryptophan (4). This allows the *E. coli* cell to adapt to tryptophan-rich and -poor environments. Two reports proposed that in the pseudomonads, this gene was always fully repressed by the intracellular concentration of tryptophan even when the cells were growing in minimal medium (6, 26).

We have investigated the expression pattern of the *P. savastanoi trpE* gene and determined that it is independent of the concentration of tryptophan in the culture medium, in agreement with two previous reports (6, 26). We arrived at this conclusion after measuring the promoter activity of a *trpE::lacZ* transcriptional fusion, the steady-state levels of *trpE* mRNA, and the activity of anthranilate synthase from *P. savastanoi* cells (Table 2). The large and small components of anthranilate synthase vary proportionally in concentration in the cell (26); because of this, the level of activity of this enzyme, under normal assay conditions, reflects the cellular concentration of each component.

The *P. savastanoi trpE* gene has a rather weak promoter; it is ~1.4-fold higher than background and ~3-fold less active than the promoter of the IAA biosynthetic genes (17). Nonetheless, this promoter is sufficient to supply the cell with enough tryptophan for protein synthesis and the secondary metabolism and virulence, IAA biosynthesis. Less hybridization of the *trpE* probe to total *P. savastanoi* RNA was also observed compared with the hybridization obtained with the *iaa* probe (data not shown).

An increase in the activity of the enzyme anthranilate synthase was observed with no apparent increase in the steady-state concentration of the *trpE* mRNA and activity of the *trpE* promoter in cells growing in minimal medium compared with the levels seen in cells grown in either tryptophan-supplemented minimal medium or King B medium, (Table 2). We believe that this difference is due to small amounts of tryptophan present in the extracts of cells grown in tryptophan-containing media which would inhibit

the enzyme (33). For unknown reasons, the enzyme activity was very unstable during the course of these experiments and could not be detected after dialysis of the extracts.

Regulation of the tryptophan biosynthetic pathway as it influences the total amount of tryptophan produced by *P. savastanoi* may play an important role in the virulence of this organism in its hosts, since tryptophan is the precursor for the synthesis of the virulence factor IAA. Moreover, expression of the IAA biosynthetic genes is constitutive, making variations in the synthesis of IAA completely dependent on the availability of tryptophan (17, 20). As mentioned previously, the intracellular concentration of tryptophan was directly correlated with the severity of the symptoms caused by this bacterium (33).

Production of IAA seems to be widespread among plant-pathogenic *Pseudomonas* species. However, not all are gall formers (39). Although *P. savastanoi*, *Pseudomonas syringae* subsp. *syringae*, and *P. syringae* subsp. *pisi* have nearly identical IAA biosynthetic genes, *P. savastanoi* is the only one that induces the formation of sizable galls in the host tissue. This could reflect the amounts of IAA synthesized by the different species. At least for *P. savastanoi*, symptom severity has been directly correlated with the amount of IAA produced in culture (33). Other factors besides IAA, cytokinins for example, may also be involved in gall formation by these phytopathogens (16, 29). Fett et al. (16) reported that in some cases there was no correlation between the amount of IAA produced in vitro and the symptoms observed in the plant.

In light of previous reports on the regulation of the *trp* genes of the pseudomonads (6, 26), we can draw no conclusion as to whether the *trpE* gene of *P. savastanoi* is constitutively expressed or fully repressed under the conditions tested, since we did not isolate *trp* gene regulatory mutants. However, we have learned that expression of the *trpE* gene of *P. savastanoi* is independent of the tryptophan concentration in the culture medium. Similar results were obtained by Ross and Winkler (31) working with the bacterium *Caulobacter crescentus*. By performing experiments with indoleacrylic acid, a potent derepressor of the *E. coli trp* operon, they excluded the possibility of continuous repression, since the anthranilate synthase activity remained unchangeably low compared with the activity of controls. Similar experiments were not performed with *P. savastanoi*; however, it is conceivable that because of the similarities in the chemical structures of IAA and indoleacrylic acid and the high levels of IAA produced by *P. savastanoi* in culture (up to 7 mg/liter in minimal medium), the *trpE* gene promoter would be derepressed if a *trpR* analog sensitive to indoleacrylic acid were present in the cell. This possibility has to be investigated further. The above-mentioned expression pattern presumably maintains appropriate intracellular levels of tryptophan for both protein synthesis and IAA production which do not impair the organism's fitness and virulence. Future experiments with *trp* regulatory mutants should bring a better understanding of how *P. savastanoi* controls this important primary biosynthetic pathway which is closely connected with secondary metabolism and the virulence of this organism.

ACKNOWLEDGMENTS

We are thankful to G. Bruening and T. Gaffney for critical reading of earlier versions of the manuscript. We thank G. Bruening for several discussions during the course of these studies, I. Crawford for the *P. aeruginosa trpE* clone (p1460), its nucleotide sequence, and the sequence of *P. putida trpE*, C. Yanofsky for the *E. coli* W3310/Δ*trp* strain, and P. Castlefranco for use of a fluorescence spectrophotometer. We also thank J. Hall for the photographic work.

This work was supported by NSF grant DCB-83 18782 to T.K. O.C.S. was supported by a predoctoral fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil.

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