A role for pabAB, a p-aminobenzoate synthase gene of Streptomyces venezuelae ISP5230, in chloramphenicol biosynthesis

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INTRODUCTION

p-Aminobenzoic acid (PABA) participates in primary metabolism as an intermediate in the formation of tetrahydrofolate. Its biosynthesis diverges from the pathway to aromatic protein amino acids through a branch-point reaction (Fig. 1) in which chorismic acid is channelled towards PABA formation by the successive actions of PABA synthase and 4-amino-4-deoxychorismic acid lyase (Nichols et al., 1989; Green & Nichols, 1991). PABA synthase is a complex of two enzymes: an amidotransferase removes the amido group from glutamine and donates it to an aminase that substitutes it for the C-4 hydroxyl group of chorismic acid to form 4-amino-4-deoxychorismic acid. The two enzymes are encoded by pabA and pabB, respectively. The genes have been cloned and sequenced from a variety of organisms (Kaplan et al., 1985; Goncharoff & Nichols, 1988; Tran et al., 1990; Slock et al., 1990; Arhin & Vining, 1993). In enteric bacteria, they are at separate locations on the chromosome (Huang & Pittard, 1967), whereas in Bacillus subtilis and Streptomyces lividans they are adjacent and are co-transcribed. The two genes in B. subtilis are clustered in an operon with other genes for folic acid biosynthesis (Slock et al., 1990). The conversion of 4-amino-4-deoxychorismic acid to PABA is catalysed by a lyase, the gene for which (pabC) has now been identified and cloned (Green et al., 1992). In B. subtilis, a gene resembling pabC is present in the putative folic acid operon, but in Escherichia coli, pabC, like pabA and pabB, is individually located on the chromosome (Green & Nichols, 1991).

In addition to its role in primary metabolism, PABA serves as a secondary metabolic pathway intermediate in Streptomyces griseus, where it appears to form the starter unit for the biosynthesis of the polyketide-derived antibiotic candicidin (Gil et al., 1980). In Streptomyces venezuelae, the secondary metabolic pathway to the antibiotic

Mutagenesis of Streptomyces venezuelae ISP5230 and selection for p-aminobenzoic acid-dependent growth in the presence of sulfanilamide yielded pab mutants VS519 and VS620 that continued to produce chloramphenicol (Cm), although with increased medium dependence. Transforming the mutants with pDQ102 or pDQ103, which carried a pab-complementing fragment from S. venezuelae ISP5230 in alternative orientations, restored uniformly high Cm production in VS620, but did not alter the medium dependence of Cm production in VS519. The cloned S. venezuelae DNA fragment was subcloned and trimmed to the minimum size conferring pab complementation. The resulting 2.8 kb BamHI-Sac1 fragment was sequenced. Codon preference analysis showed one complete ORF encoding a polypeptide of 670 amino acids. Comparison of the deduced amino acid sequence with database proteins indicated that the N- and C-terminal regions resembled PabA and PabB, respectively, of numerous bacteria. The gene product showed overall sequence similarity to the product of a fused pabAB gene associated with secondary metabolism in Streptomyces griseus. Insertion of an apramycin resistance gene into pabAB cloned in a segregationally unstable vector and replacement of the S. venezuelae chromosomal pabAB with the disrupted copy lowered sulfanilamide resistance from 25 to 5 µg ml⁻¹ and blocked Cm production.

Keywords: Streptomyces venezuelae, p-aminobenzoate synthase gene, chloramphenicol

Abbreviations: Am, apramycin; Cm, chloramphenicol; PABA, p-aminobenzoic acid; Ts, thiostrepton
The GenBank accession number for the pabAB nucleotide sequence reported in this paper is U21728.
M. P. BROWN, K. A. AIDOO and L. C. VINING

Fig. 1. Reactions following the chorismic acid branch point in aromatic metabolite biosynthesis.

chloramphenicol (Cm) also branches from the route to aromatic protein amino acids at chorismic acid (see Fig. 1; Vining & Stuttard, 1994). Teng et al. (1985) established that p-aminophenylalanine, an intermediate in Cm biosynthesis, is derived from chorismic acid via 4-amino-4-deoxychorismic acid and thus shares this initial step with the biosynthesis of PABA. In the route to p-aminophenylalanine, the lyase reaction that would otherwise form PABA is replaced with a mutase that generates 4-amino-4-deoxyphenylpyruvic acid; this intermediate may be converted to p-aminophenylalanine via p-aminophenylpyruvic acid (Jones et al., 1978), although detection of both arogenate dehydrogenase and prephenate dehydratase in S. venezuelae (Lingens & Keller, 1983) suggests that an alternative reaction sequence via the arogenate intermediate should not be excluded. The dual role of 4-amino-4-deoxychorismic acid as an intermediate in both PABA and Cm biosynthesis suggests that it represents a branch point for primary and secondary metabolism and raises questions about the regulation of the branching reactions, as well as about the evolution of the secondary pathway.

Aidoo (1989) isolated a putative PABA synthase gene from a Cm-producing organism by cloning in the Streptomyces vector pIJ41 a fragment of S. venezuelae ISP5230 DNA complementing a pab mutation in S. lividans (Gil & Hopwood, 1983). This pab-containing DNA has now been subcloned in an E. coli vector as a 2.8 kb fragment and sequenced, revealing features of PABA synthase that bear on its relationship to primary and secondary metabolism in S. venezuelae. The chromosomal gene in S. venezuelae has been disrupted to determine whether it has a role in Cm production.

METHODS

Bacteria, plasmids and cultures. The micro-organisms and plasmids used are described in Table 1. Streptomyces venezuelae strains were maintained on MYM agar (Stuttard, 1982); S. lividans JG10 was maintained on K1 medium, which contained (1·5 g·l⁻¹): maltose (10 g), yeast extract (5 g, Difco), Casamino acids (0·2 g, Difco), KH₂PO₄ (0·5 g), MgSO₄⋅7H₂O (0·2 g), FeSO₄⋅7H₂O (0·1 g) and agar (15 g). Cultures of S. venezuelae and S. lividans were grown as described by Aidoo et al. (1990). Spores of S. venezuelae ISP5230 were mutagenized with NTG in Tris/maleate buffer, pH 8·0, as described by Delić et al. (1970). Mutants defective in PABA biosynthesis were selected by plating the spores on minimal medium (MM) supplemented with PABA (1·0 µg ml⁻¹) and then screened by replica plating on MM containing sulfanilamide (10 µg ml⁻¹). The sensitivity level of colonies that failed to grow in the presence of sulfanilamide was tested by incubating spores on MM agar supplemented with sulfanilamide at various concentrations; reversal of the inhibition by PABA was tested by incubating spores on MM agar containing sulfanilamide and PABA at 10 and 1·0 µg ml⁻¹, respectively. MM consisted of a glucose/asparagine/salt solution (Hopwood, 1967); unwashed agar (Difco) was used in...
Table 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><em>S. venezuelae</em></td>
<td></td>
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<tr>
<td>ISP5230</td>
<td>Wild-type</td>
<td>Sturtard (1982)</td>
</tr>
<tr>
<td>VSS19</td>
<td>Sulfanilamide-sensitive mutant (pab-2,3) of ISP5230</td>
<td>Atkinson (1987)</td>
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<tr>
<td>VSS620</td>
<td>Sulfanilamide-sensitive mutants (pab-10, pab-11) of ISP5230</td>
<td>This work</td>
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<tr>
<td>VSS629</td>
<td>Wild-type transformed with pDQ501</td>
<td>This work</td>
</tr>
<tr>
<td><em>S. lividans</em></td>
<td></td>
<td></td>
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<tr>
<td>JG10</td>
<td>pab-1 str-6 SLP2- SLP3-</td>
<td>Gil &amp; Hopwood (1983)</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG1</td>
<td>supE hsd Δ(lac-pro-AB) F'(traD36 pro.AB' lacI2 lacZ ΔM5)</td>
<td>Hanahan (1983)</td>
</tr>
<tr>
<td>DH5xF1Q</td>
<td>F' 600lacZ ΔM15 Δ(lacZYA-argF)W169 deoR recA1 hsdR17 (rKm·) supE44 thi-1 gyrA96 relA1 F' pro.AB' lacP2 ΔM15 (τ53::Tn5[Km·])</td>
<td>Carter et al. (1985)</td>
</tr>
<tr>
<td>ET12567</td>
<td>dam dcm hsdM</td>
<td>MacNeil et al. (1992)</td>
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<td><strong>Plasmids</strong></td>
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<td>pBluescript II</td>
<td>E. coli phagemid vector: ApB lacZ'</td>
<td>Stratagene</td>
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<tr>
<td>pT7188/19R</td>
<td>E. coli phagemid vector: ApB lacZ'</td>
<td>Pharmacia</td>
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<td>pJ141</td>
<td>Modified S. lividans plasmid SLP1.2: NeoR TsR</td>
<td>Chater et al. (1982)</td>
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<tr>
<td>pDQ101</td>
<td>Modified pJ941 (transforms <em>S. venezuelae</em>)</td>
<td>Aaido et al. (1990)</td>
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<td>pDQ101/103</td>
<td>pDQ101 with a 6.4 kb Parl insert from pDQ116 (two orientations)</td>
<td>Aaido et al. (1990)</td>
</tr>
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<td>pDQ116</td>
<td>pJ141 with a 5.6 kb <em>S. venezuelae</em> insert containing pabAB</td>
<td>This work</td>
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<tr>
<td>pDQ121</td>
<td>pJ141 with a 3.4 kb <em>S. venezuelae</em> insert containing pabAB</td>
<td>This work</td>
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<tr>
<td>pDQ372</td>
<td>pJHL400 with a 6.4 kb Parl insert from pDQ116</td>
<td>This work</td>
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<td>pDQ373</td>
<td>pDQ372 lacking the 2.7 kb BamHI–BamHI segment</td>
<td>This work</td>
</tr>
<tr>
<td>pDQ500</td>
<td>pDQ373 lacking 1.4 kb XhoI–XhoI and 0.21 kb Nol–NolI segments</td>
<td>This work</td>
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<tr>
<td>pDQ501</td>
<td>pDQ500 with an apr insert</td>
<td>This work</td>
</tr>
<tr>
<td>pKC462a</td>
<td>Cosmid vector containing apr</td>
<td>Stanzak et al. (1986)</td>
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Determining requirements for PABA and sensitivity to sulfanilamide.

**DNA manipulations.** The general procedures described by Sambrook et al. (1989) were followed. Plasmid DNA was isolated from streptomycetes by the alkaline lysis method of Kieser (1984). Genomic DNA was obtained as described by Hopwood et al. (1985). For Southern hybridizations (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 x SSPE (SSPE is 0.18 M NaCl, 10 mM Na2HPO4 and 1 mM EDTA, pH 7.7), 5 x Denhardt's solution (Denhardt, 1966), 0.1% SDS and denatured salmon sperm DNA (100 μg ml−1). Membranes were washed at 65 °C with SSPE solutions (twice with 2 x, then with 1 x and 0.1 x) containing 0.1% SDS.

**Cloning and transformation.** Competent cells of *E. coli* strains were prepared and transformed as described by Sambrook et al. (1989). To prepare genomic libraries of *S. venezuelae* DNA, wild-type strain ISP5230 DNA was digested with restriction enzymes. Each digested DNA was used to transform the sulfanilamide-sensitive *S. lividans* mutant JG10, using the general procedures of Hopwood et al. (1985). Incubating excess *S. lividans* JG10 protoplasts with the ligation mixture containing *BclI* fragments yielded 1.2 x 10⁶ transformants, 20% of which the vector contained an insert; the ligation mixture containing *BamHI* fragments yielded 5 x 10⁶ transformants. Thiostrepton (Ts)-resistant colonies were replica-plated on MM agar supplemented with 0.2 μg sulfanilamide ml−1. At this concentration, the drug selected for transformants carrying a *pab* gene that complemented the host mutation and allowed growth similar to that of the wild-type. For transforming *S. venezuelae*, the procedures described by Aaido et al. (1990) were used.

**DNA sequencing and sequence analysis.** DNA fragments were subcloned in both orientations in pBluescript II SK(+) and overlapping deletions were generated with exonuclease III (Henikoff, 1984). The ligated DNA was used to transform *E. coli* DH5αF1Q (BRL) and plasmid DNA, extracted from the transformants by the rapid boiling method (Holmes et al., 1990), was isolated. Phagemid DNA, extracted from the transformants by the rapid boiling method (Holmes & Quigley, 1981), was screened for size by agarose gel electrophoresis. Single-strand DNA templates were generated from phagemid inserts by incubating cultures with helper phage VCSM13 as described by Karger & Jessee (1990), except that the culture medium contained yeast extract (10%), Bacto-Tryptone (1.6%), NaCl (0.8%). The single strands were sequenced by the dideoxy chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 (Amersham) and [α-35S]dATP. The sequence was analysed with version 7.0 software developed by the Genetics Computer Group (GCG), University of Wisconsin, Madison, WI, USA.

**Construction and use of a *pabAB* replacement vector.** pDQ372 (see Table 1) was digested with XhoI to remove a 1.4 kb segment of DNA from the vector fragment, which was

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**References:**

- Sambrook et al. (1989).
- Carter et al. (1985).
- Aaido et al. (1990).
- Aaido et al. (1990).
- Chater et al. (1982).
- Aaido et al. (1990).
- Holmes et al. (1990).
- Sanger et al., 1977.
- Sambrook et al. (1989).
- Karger (1990), except that the culture medium contained yeast extract (10%), Bacto-Tryptone (1.6%), NaCl (0.8%).
then circularized with T4 DNA ligase. The resulting plasmid was digested with NolI to remove a 0.21 kb segment of DNA and again circularized by incubation with T4 DNA ligase. This plasmid was linearized with NolI and ligated with an NolI cassette carrying the apramycin (Am) resistance gene (apr) subcloned from pKC462a (Stanzak et al., 1986; Paradkar & Jensen, 1995). The ligation mixture was used to transform E. coli TG1; from a transformant exhibiting the Am<sup>+</sup> phenotype, plasmid pDQ51 was isolated. This was used to transform E. coli ET12567 (MacNeil et al., 1992); pDQ51 was reisolated from this host was used as the replacement vector. Protoplasts of S. venezuelae pDQ373 was isolated. This was used to transform S. venezuelae ET12567 (MacNeil et al., 1992) isolated after treatment of the wild-type strain with NTG. Several prototrophic transformants were screened on MM agar containing Ts<sup>+</sup>. Thepab mutation in S. venezuelae was identified within four of the Ts<sup>+</sup> transformants. Genomic DNA was isolated from these strains, digested with SaeI and examined by Southern hybridization using the 1.8 kb fragment from pDQ373 and the 1.45 kb cassette carrying the apramycin (Am) resistance gene as probes.

**Measurement of Cm production.** Cm production by S. venezuelae strains grown as patches on MYM agar was bioassayed against Micrococcus luteus as described by Aidoo et al. (1990). To measure Cm production in liquid medium, 25 ml cultures were incubated at 27 °C in a rotary shaker (220 r.p.m.) for 10 ml GNY medium (Malik & Vining, 1970). Cm in liquid culture is expressed in the form of sodium octadecyl sulfate (SDS)-PAGE and analysed by HPLC using a C<sub>18</sub> reverse phase column (80 x 4.6 mm) and stepped linear gradients from 0 to 25% (1 min), 25 to 50% (5 min) and 50 to 100% (1 min) in a 100 µl/min gradient returning to 100% water (1 min). Cm was eluted at 6-05 min; its concentration was calculated using a standard curve. The results were confirmed by analysis using specific reference samples.

**RESULTS**

**Sensitivity of S. lividans and S. venezuelae strains to sulfanilamide.** Strains of S. lividans that had no growth requirement for PABA were sensitive to sulfanilamide at concentrations above 2 µg ml<sup>-1</sup>. The pab mutation in S. lividans JG10 (Gil & Hopwood, 1983) markedly increased the sensitivity of this strain; when this strain was patched on a defined agar medium containing Ts<sup>+</sup>, the titres were lower than for the wild-type. In shaken cultures grown in the glucose/isoleucine medium optimising production of the antibiotic, the antibiotic was produced at wild-type levels (as determined by bioassay). When these were used to transform E. coli, none of the survivors were able to grow. Two of those inhibited (designated VS620) were examined; each showed marginal growth with 5 µg sulfanilamide ml<sup>-1</sup> and grew normally when additionally supplemented with PABA.

**Cm production by S. venezuelae pab mutants.** Atkinson (1987) was unable to detect antibiotic production by the pab mutant strain VS519 in bioassays on MYM agar, although positive bioassays were obtained when this strain was patched on a defined agar medium favoring production of the antibiotic. On MYM agar both VS620 strains gave positive bioassays for Cm, but the titres were lower than for the wild-type. In shaken cultures grown in the glucose/isoleucine medium optimized for Cm production (Chatterjee et al., 1983; Doull et al., 1985), HPLC analyses showed wild-type titres of the antibiotic in all of the mutant strains. The anomalous results from bioassays of cultures grown on agar media were attributed to growth rate effects (Liao et al., 1995) and were not investigated further.

**Cloning of the p-aminobenzoate synthase gene.** By restriction enzyme mapping of plasmid DNA extracted from sulfanilamide-resistant S. lividans transformants, the same 5.65 kb segment (e.g. the insert in pDQ116; Fig. 2a) was identified within four of the BsrEI inserts of S. venezuelae DNA. Since the same insert was present in two of the recombinant pJ41 vectors in alternative orientations, complementation of the host phenotype by both plasmids suggested that the fragment included a promoter for the pab gene. A restriction map of the 3.4 kb insert in pDQ121, the plasmid extracted from the single sulfanilamide-resistant transformant derived from the BamHI fragments of S. venezuelae genomic DNA, indicated that this fragment formed part of the 5.65 kb region common to the BsrEI-derived inserts (see Fig. 2a).

To determine whether the gene was expressed in E. coli, a 6.4 kb fragment containing most of the 5.65 kb insert and an additional 0.75 kb of adjacent vector DNA (see Fig. 2a) was excised with PstI from pDQ116 and ligated into the PstI site of the E. coli vector pTZ18R (Mead & Kemper, 1988). Incubating E. coli TG1 with the pTZ18R ligation mixture gave transformants from which plasmids containing the insert in alternative orientations were isolated. When these were used to transform E. coli mutants AB3292 and AB3295, defective in pabA and pabB, respectively, none of the 500 transformants tested was prototrophic, implying that the promoter region of the cloned S. venezuelae gene(s) did not function in E. coli.

**Transformation of S. venezuelae pab mutants.** Aidoo et al. (1990) recloned the 6.4 kb PstI–PstI insert from pDQ116 in the S. venezuelae-compatible vector pDQ101 to give pDQ102 and pDQ103 (alternative orientations). Both plasmids complemented the pab mutation when introduced into S. lividans JG10, but did not restore Cm production in an S. venezuelae mutant blocked at an early step in biosynthesis of the antibiotic (Aidoo et al., 1990). Transformation of the S. venezuelae pab mutants VS519 and VS620 with pDQ102 yielded Ts-resistant colonies at the same frequency as transformants of the wild-type. All of the eight colonies examined from the VS620 transformation showed resistance to sulfanilamide and produced Cm at wild-type levels (as determined by HPLC analyses).
bioassay against *M. luteus*). Seven of nine putative transformants of VS519 examined were sulfanilamide-resistant; the other two retained the sensitivity (inhibition at 2 μg ml⁻¹) of the host. All of the putative VS519 transformants showed by bioassay the pleiotropic, medium-dependent Cm production exhibited by the host. Since no plasmid DNA could be extracted from either VS519 or VS620 transformants, but a signal was detected in genomic DNA by Southern hybridization using the *Ts* resistance gene excised from the vector as a probe, integration was presumed to have occurred.

**Sequencing the pab gene in pDQ116**

To facilitate subcloning and disruption of the *pab*-complementing DNA, the 6·4 kb *PstI*-*PstI* fragment from pDQ116 was recloned in the *Streptomyces-E. coli* shuttle vector pHJL400 (Larson & Hershberger, 1986), yielding pDQ371 and pDQ372 with the insert in each orientation. Both plasmids transformed *S. lividans* JG10 to sulfanilamide resistance. Digestion of pDQ372 (Fig. 2b) with *BamHI* and ligation to recircularize the plasmid yielded pDQ373, in which the 3·0 kb segment between the *BamHI* sites at 1·5 kb (in the insert) and 6·4 kb (in the multiple cloning region of the vector) had been deleted. pDQ373 failed to confer sulfanilamide resistance, as did pDQ374, in which digestion of pDQ372 with *XhoI* followed by ligation had deleted the 1·24 kb DNA segment between the two *XhoI* sites. Therefore, the gene conferring sulfanilamide resistance was presumed to lie in the region between the *XhoI* site at 1·3 kb and the end of the insert at 5·65 kb (see Fig. 2a). To locate the gene more precisely, the region between the *SacI* site at 0·6 kb and the *BamHI* site at 3·4 kb was subcloned as smaller fragments in both orientations in the phagemid pBluescript II SK(+) and sequenced (Fig. 3).
Sequence analysis

The overall G+C content (72.4%) of the sequenced region is within the range reported (61.0–79.7%) for streptomycete genes (Wright & Bibb, 1992). Analysis of codon usage with the CODONPREFERENCE program (GCG) confirms the observation of Bibb (1992) for streptomycetes that the BamHI–SacI region of the S. venezuelae ISP5230 chromosome containing the pabAB operon contains a high proportion of ATG initiation codons (indicated by asterisks) in ORF1.

Fig. 3. Nucleotide and deduced amino acid sequence of the BamHI–SacI region of the S. venezuelae ISP5230 chromosome containing pabAB. A ribosome binding site (RBS, underlined) and the initiation codon (ATG, bold type) for ORF2 are indicated. Asterisks indicate termination codons (TGA).
Fig. 4. Alignment by the PILEUP program (GCG) of the amino acid sequences deduced from ORF2 of S. venezuelae 15P230 and PABA synthase genes from other bacteria. (a) The pabA regions of ORF2 (E. coli) and pabAB of S. griseus (Sg) are compared with pabA from Klebsiella aerogenes (Ka), Salmonella typhimurium (St), E. coli (Ec), B. subtilis (Bc), and Azospirillum brasilense (Ab). (b) The pabB regions of ORF2 (E. coli) and pabAB of S. griseus (Sg) compared with pabB of K. aerogenes (Ka), S. typhimurium (St), E. coli (Ec), Pseudomonas putida (Pp) and S. lividans (Sl). Asterisks indicate amino acids conserved in all sequences compared.
Fig. 5. (a) Southern hybridization of *S. venezuelae* genomic DNA from mutant (VS629; lanes 1 and 3) and wild-type (ISP5230; lanes 2 and 4) strains. The mutant contained the disruption vector pDQ501 integrated in its chromosome. Lane λ, λ phage DNA digested with PstI as size markers. Genomic DNA was digested with Sacl and probed with either a 1.45 kb Ncol-Ncol cassette containing *apr* (lanes 1 and 2) or a 1.8 kb Sacl-Sacl fragment containing a segment of *pabAB* from pDQ373. (b) Restriction map showing the predicted result of a double crossover between homologous regions of DNA in pDQ501 and the *S. venezuelae* ISP5230 chromosome. The tips and shaded segment of the lower broad arrow represent regions in VS629 that hybridize with the *pabAB* probe. For abbreviations, see Fig. 2 legend.

The amino acid sequences for ORFs 1 and 2 were compared with those in the GenBank database using the BLASTP program (Altschul et al., 1990). No meaningful similarities were found for the partial sequence of ORF1. However, the sequence of ORF2 showed marked similarity (47.5% identical amino acids) to that deduced for the product of *pabAB* from *S. griseus* (Criado et al., 1993). The sequence of about 200 amino acids nearest the N terminus of ORF2 from *S. venezuelae* also resembled that of the *pabA* products from many bacteria, while the sequence of the approximately 450 amino acids remaining was similar to *pabB* product sequences. These relationships were confirmed by alignment (Fig. 4) of the two regions of the *pabAB* product with the corresponding *pab* gene products using the PILEUP program (GCG) based on the algorithm of Feng & Doolittle (1987). The marked similarity between the *pabAB* products of *S. griseus* and *S. venezuelae* was supported by a comparison of the sequences using the PLOTSIMILARITY program (GCG). In general, the areas of high similarity correspond to conserved regions in PabA and PabB (Crawford, 1989).

### Disruption of *pabAB*

The potential involvement of *pabAB* in the biosynthesis of PABA and Cm was examined by disrupting the gene in *S. venezuelae* ISP5230. A disruption vector was prepared from pDQ373, which contained *pabAB* within a 3-4 kb fragment cloned between BamHI and PstI in the multiple cloning region of the *Streptomyces*—*E. coli* bifunctional vector pHJL400; this vector is segregationally unstable in streptomycetes (Larson & Hershberger, 1986). pDQ373 was modified by removing 1.4 kb *XhoI—XhoI* and 0.21 kb *NcoI—NcoI* segments of DNA from within *pabAB* to give pDQ500. Introducing an *NcoI* cassette carrying *apr* into
the unique NcoI site of the resulting plasmid furnished the disruption vector pDQ501 (see Fig. 2b). To avoid restriction in *S. venezuelae* ISP5230 apparently due to enzymes recognizing methylated DNA (Han *et al.*, 1994), the plasmid was isolated from *E. coli* ET12567, which lacks DNA methylating systems (MacNeil *et al.*, 1992). Transformation of *S. venezuelae* ISP5230 with pDQ501 initially yielded Am<sup>R</sup> Ts<sup>R</sup> colonies; when these were propagated without antibiotic selection, Am<sup>R</sup> Ts<sup>S</sup> strains (e.g. VS629) were obtained.

Hybridization of SacI-digested genomic DNA using *apr* as probe gave signals for 0·23, 0·70 and 0·75 kb fragments only in the DNA from VS629, confirming the presence of *apr* in this strain (Fig. 5a). Probing of similar digests with a 1·8 kb *SacI* fragment containing most of *pabAB* showed hybridizing fragments of 1·8 and 2·8 kb in the wild-type DNA; in VS629 DNA, a 2·9 kb fragment was present, but the 1·8 kb fragment was replaced by one at 0·70 kb that coincided with the 0·70 kb fragment hybridizing to the *apr* probe. The results are consistent with interruption of the 1·8 kb chromosomal segment by insertion of *apr* and with the presence in the VS629 chromosome of the expected segments of *pabAB* DNA (see Fig. 5b). From restriction enzyme and Southern analyses of the replacement vector, it was determined that *apr* was oriented for transcription in the same direction as *pabAB*. When cultures of these strains and the wild-type were grown in media favouring Cm production (Dowill *et al.*, 1985), the antibiotic titres measured by HPLC in the Am<sup>R</sup> Ts<sup>R</sup> transformants were similar to wild-type levels (60–70 μg ml<sup>−1</sup> at 6 d), whereas those in the Am<sup>R</sup> Ts<sup>S</sup> replacement strains were below 2 μg ml<sup>−1</sup>. Assays of the sensitivity of the replacement strain to sulfanilamide showed that growth was inhibited at concentrations above 5 μg ml<sup>−1</sup>.

**DISCUSSION**

Since the inhibition of some streptomycetes by sulfonamides can be reversed by adding PABA to the culture medium (Gil & Hopwood, 1983), the approximately 10-fold greater resistance of *S. venezuelae* than *S. lividans* to sulfanilamide could be explained by a higher endogenous level of PABA synthesis in *S. venezuelae*. This might be associated with the formation of 4-amino-4-deoxychorismic acid as a common intermediate in the biosynthesis of PABA and Cm. Considerably more 4-amino-4-deoxychorismic acid must be generated to meet the needs of the secondary metabolic process than is required for the synthesis of folic acid cofactors in primary metabolism. An increased supply of the intermediate could be achieved by regulating the activity of a single PABA synthase, or by using a second enzyme supplying 4-amino-4-deoxychorismic acid in the amounts needed for Cm biosynthesis. The results obtained in the present investigation indicate that two types of PABA synthase exist in *S. venezuelae* and thus support the second possibility.

Because the mutations causing sensitivity to sulfanilamide in VS519 and VS620 did not prevent Cm production, whereas replacing the chromosomal copy of the cloned *pabAB* with a disrupted copy virtually eliminated synthesis of the antibiotic without causing auxotrophy, the genes associated with sulfanilamide sensitivity are likely to be involved in folic acid biosynthesis. PABA synthesis can potentially be affected by mutations in *pabA*, *pabB* or *pabC* of this pathway; phenotypic differences between such mutants might account for the apparent differences in Cm production detected in bioassays, but these might also be due to additional mutations introduced by mutagenesis with NTG (Baltz, 1986). The restoration of sulfanilamide resistance as well as normal bioassay titres for Cm production when VS620 was transformed with pDQ102, which contains *pabAB*, supports the assumption that 4-amino-4-deoxychorismic acid generated in the Cm biosynthetic pathway can supply a similar need in the folic acid pathway. The failure of VS519 to respond similarly to transformation with pDQ102 may again be due to additional mutations introduced by mutagenesis with NTG.

The rapid integration of plasmids noted earlier (Aidoo *et al.*, 1990) in *S. venezuelae* transformed with recombinant vectors containing inserts of homologous DNA facilitated replacement of the chromosomal *pabAB* with a disrupted copy containing a gene for Am resistance. The increased sensitivity of the replacement mutant to sulfanilamide, in addition to its almost complete loss of Cm production, are consistent with the postulated linkage between sulfanilamide resistance and Cm production in *S. venezuelae*. The small amount of the antibiotic still made in the replacement strain can be attributed to cross-feeding of the Cm biosynthesis pathway by 4-amino-4-deoxychorismic acid made by the intact primary PABA synthase.

The striking similarity between the *pabAB* genes from *S. griseus* and *S. venezuelae* lies not only in the deduced amino acid sequences but also in features of gene organization. In most bacteria, the glutamine amidotransferase component (*pabA* product) of PABA synthase is about 190 amino acids in length and the average aminate (*pabB* product) contains about 450 amino acids. Thus the combined length of PABA synthase (about 640 amino acids) is somewhat smaller than the 670 amino acids estimated for the *pabAB* product of *S. venezuelae*. In *S. griseus*, *pabAB* is even longer and encodes 723 amino acids. Sequence alignment of *pabB* proteins from bacteria indicates that much of the additional length of this component in *pabABs* (about 10 unpaired amino acids in the *S. venezuelae* product and 30 in the *S. griseus* product) is at the C-terminal end. In both *pabABs*, the component genes are fused in the same order, with the *pabA* component transcribed first. In *S. lividans* and *B. subtilis*, where *pabA* and *pabB* are separate but translationally coupled (Slock *et al.*, 1990; Arhin & Vining, 1993), *pabB* precedes *pabA*, and is itself preceded by a promoter sequence that is recognized in *E. coli*. In contrast, the indigenous promoter for *pabAB* in *S. venezuelae*, although expressed in streptomycetes (e.g. in pDQ116 transformants of *S. lividans* JG10), is not recognized in *E. coli*. A similar situation was reported for *pabAB* of *S. griseus*; here the gene was expressed in *E. coli* only after a deletion
allowed read-through from a vector promoter (Gil & Hopwood, 1983; Criado et al., 1993).

Although pDQ373 contains an intact ORF2 for \textit{pabAB} of \textit{S. venezuelae}, it was unable to confer sulfanilamide resistance in transformants of \textit{S. lividans} JG10. This and the absence of recognizable promoter sequences for ORF2 suggest that transcription does not begin immediately upstream of \textit{pabAB}. Instead, the promoter site may be associated with an upstream region cotranscribed with \textit{pabAB}. This appears to include ORF1, the identity of which could not be established by comparing the deduced partial sequence with proteins in the GenBank database. In \textit{S. griseus} the native \textit{pabAB} promoter was placed between 0-2 and 1-2 kb upstream of the gene (Gil & Hopwood, 1983) in a region containing a putative thioesterase gene that may form part of a candididin biosynthesis gene cluster (Criado et al., 1993). It is noteworthy that pDQ121, with the same \textit{pabAB}-containing \textit{S. venezuelae} DNA insert as pDQ373, conferred sulfanilamide resistance in \textit{S. lividans} JG10 transformants. Presumably the gene is expressed here by read-through from a streptomycete promoter in the vector (pIJ41).

The presence in two different streptomycetes of secondary metabolic pathway genes very similar in organization and function and also similar enough to related primary metabolic genes to indicate a common ancestry raises intriguing questions about the origin of secondary metabolic pathway genes. In both \textit{S. griseus} and \textit{S. venezuelae}, \textit{pabAB} encodes an enzyme that acts near the beginning of unique biosynthetic sequences. If the pathways to Cm and candididin have arisen by forward rather than retrograde metabolic evolution, the distinctiveness and complexity of these two specialized biosynthetic systems suggest that \textit{pabAB} has existed for a relatively long time. This being so, secondary metabolism itself is likely to have been an ancient process.

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