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

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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 *Bam*HI–*Sac*I 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

INTRODUCTION

p-Aminobenzoic acid (PABA) participates in primary metabolism as an intermediate in the formation of tetrahydrofolic acid. 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

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.

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-deoxyprephenic 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 arogenic acid 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 (l⁻¹): maltose (10 g), yeast extract (5 g, Difco), Casamino acids (0.2 g, Difco), K₂HPO₄ (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/salts solution (Hopwood, 1967); unwashed agar (Difco) was used in

Table 1. Bacterial strains and plasmids used

Strain	Genotype/phenotype	Source/reference
<i>S. venezuelae</i>		
ISP5230	Wild-type	Stuttard (1982)
VS519	Sulfanilamide-sensitive mutant (<i>pab-2,3</i>) of ISP5230	Atkinson (1987)
VS620	Sulfanilamide-sensitive mutants (<i>pab-10 pab-11</i>) of ISP5230	This work
VS629	Wild-type transformed with pDQ501	This work
<i>S. lividans</i>		
JG10	<i>pab-1 str-6 SLP2⁻ SLP3⁻</i>	Gil & Hopwood (1983)
<i>E. coli</i>		
TG1	<i>supE hsd Δ(lac-proAB) F'(traD36 proAB⁺ lacI^q lacZ ΔM5)</i>	Carter <i>et al.</i> (1985)
DH5αF'IQ	<i>F' φ80dlacZ ΔM15 Δ(lacZYA-argF)W169 deoR recA1 hsdR17 (τ_km_k⁺) supE44 thi-1 gyrA96 relA1 F' proAB⁺ lacI^q ΔM15 xzf::Tn5[Km^r]</i>	Hanahan (1983)
ET12567	<i>dam dcm hsdM</i>	MacNeil <i>et al.</i> (1992)
Plasmids		
pBluescript II	<i>E. coli</i> phagemid vector: Ap ^R lacZ'	Stratagene
pTZ18R/19R	<i>E. coli</i> phagemid vector: Ap ^R lacZ'	Pharmacia
pIJ41	Modified <i>S. lividans</i> plasmid SLP1.2: Neo ^R Ts ^R	Chater <i>et al.</i> (1982)
pHJL400	<i>E. coli-Streptomyces</i> bifunctional vector: Par ⁻ Ap ^R Ts ^R	Larson & Hershsberger (1986)
pDQ101	Modified pIJ941 (transforms <i>S. venezuelae</i>)	Aidoo <i>et al.</i> (1990)
pDQ102/103	pDQ101 with a 6.4 kb <i>Pst</i> I insert from pDQ116 (two orientations)	Aidoo <i>et al.</i> (1990)
pDQ116	pIJ41 with a 5.65 kb <i>S. venezuelae</i> insert containing <i>pabAB</i>	This work
pDQ121	pIJ41 with a 3.4 kb <i>S. venezuelae</i> insert containing <i>pabAB</i>	This work
pDQ372	pHJL400 with a 6.4 kb <i>Pst</i> I insert from pDQ116	This work
pDQ373	pDQ372 lacking the 2.7 kb <i>Bam</i> HI– <i>Bam</i> HI segment	This work
pDQ500	pDQ373 lacking 1.4 kb <i>Xho</i> I– <i>Xho</i> I and 0.21 kb <i>Nco</i> I– <i>Nco</i> I segments	This work
pDQ501	pDQ500 with an <i>apr</i> insert	This work
pKC462a	Cosmid vector containing <i>apr</i>	Stanzak <i>et al.</i> (1986)

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 × SSPE (SSPE is 0.18 M NaCl, 10 mM Na₂HPO₄ and 1 mM EDTA, pH 7.7), 5 × Denhardt's solution (Denhardt, 1966), 0.5% SDS and denatured salmon sperm DNA (100 µg ml⁻¹). Membranes were washed at 65 °C with SSPE solutions (twice with 2 ×, then with 1 × and 0.1 ×) 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 to completion with either *Bcl*I or *Bam*HI and a 5 µg sample was ligated to 1 µg of pIJ41 (Chater *et al.*, 1982) linearized with *Bam*HI. Each of the ligation mixtures 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 *Bcl*I fragments yielded 1.2 × 10⁴ transformants, in 20% of which the vector contained an insert; the ligation mixture containing *Bam*HI

fragments yielded 5 × 10³ 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 Aidoo *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* DH5αF'IQ (BRL) and plasmid 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 (1.0%), Bacto-Tryptone (1.6%, w/v) and 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. pDQ373 (see Table 1) was digested with *Xho*I to remove a 1.4 kb segment of DNA from the vector fragment, which was

then circularized with T4 DNA ligase. The resulting plasmid was digested with *Nco*I to remove a 0.21 kb segment of DNA and again circularized by incubation with T4 DNA ligase. This plasmid was linearized with *Nco*I and ligated with an *Nco*I 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*^R phenotype, plasmid pDQ501 was isolated. This was used to transform *E. coli* ET12567 (MacNeil *et al.*, 1992); pDQ501 reisolated from this host was used as the replacement vector. Protoplasts of *S. venezuelae* ISP5230 were transformed with pDQ501 by the procedure of Aidoo *et al.* (1990). One colony from about 80 that grew on the protoplast regeneration medium containing Ts (20 µg ml⁻¹) was confirmed to be resistant to both *Am* (50 µg ml⁻¹) and Ts. It was then transferred to MYM agar and allowed to sporulate. Replica plating on MYM agar containing *Am* or Ts identified colonies with an *Am*^R Ts^S phenotype. Genomic DNA was isolated from these strains, digested with *Sac*I and examined by Southern hybridization using the 1.8 kb *Sac*I–*Sac*I fragment from pDQ373 and the 1.45 kb *apr* cassette 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 in 250 ml Erlenmeyer flasks on a rotary shaker (220 r.p.m.) at 27 °C. The glucose/isoleucine medium (Doull *et al.*, 1985) was inoculated (2% v/v) from a vegetative culture prepared by incubating *S. venezuelae* spores for 24 h in 10 ml GNY medium (Malik & Vining, 1970). Cm in production cultures grown for 2, 4 and 6 d was extracted from 3 ml samples of the clarified broth with an equal volume of ethyl acetate. The extract was evaporated until dry, taken up in 0.1 ml methanol/water (3:1, v/v) and analysed (20 µl injection) by HPLC using a C₁₈ reverse phase column (80 × 4.0 mm) and stepped linear gradients from 0 to 25% (1 min), 25 to 50% (5 min) and 50 to 100% (1 min) methanol in water (v/v), followed by 100% methanol for 1 min and a gradient returning to 100% water (1 min). Cm was eluted at 6.05 min; its concentration was estimated by comparing the peak area with peak areas given by 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⁻¹. The *pab* mutation in *S. lividans* JG10 (Gil & Hopwood, 1983) markedly increased the sensitivity; growth of this strain was completely inhibited at 0.2 µg ml⁻¹. Wild-type *S. venezuelae* ISP5230 was appreciably less sensitive to sulfanilamide and could grow at concentrations up to 25 µg ml⁻¹. A *pab* mutant (VS519; Atkinson, 1987) isolated after treatment of the wild-type with NTG was inhibited by 2 µg sulfanilamide ml⁻¹. To isolate additional *pab* mutants, mutagenized spores of *S. venezuelae* ISP5230 were screened on MM agar containing 10 µg sulfanilamide ml⁻¹. At this inhibitor concentration, 0.2% of the survivors were unable to grow. Two of those inhibited (designated VS620) were examined; each showed marginal growth with 5 µg sulfanilamide ml⁻¹ 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 favouring 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 *Bcl*I inserts of *S. venezuelae* DNA. Since the same insert was present in two of the recombinant pIJ41 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 *Bam*HI fragments of *S. venezuelae* genomic DNA, indicated that this fragment formed part of the 5.65 kb region common to the *Bcl*I-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 *Pst*I from pDQ116 and ligated into the *Pst*I 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 *Pst*I–*Pst*I 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 transformations 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

Fig. 2. (a) Restriction maps for pDQ116 and pDQ121, and locations of ORF1 and *pabAB*. (b) Plasmids constructed during the cloning and disruption of *S. venezuelae pabAB*. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Nco*I; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sac*I; Sm, *Sma*I; X, *Xho*I.

bioassay against *M. luteus*). Seven of nine putative transformants of VS519 examined were sulfanilamide-resistant; the other two retained the sensitivity (inhibition at $2 \mu\text{g ml}^{-1}$) 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 *Pst*I–*Pst*I 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 *Bam*HI and ligation to recircularize the plasmid yielded pDQ373, in which the 3.0 kb segment between the *Bam*HI sites at 3.4 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 *Xho*I followed by ligation had deleted the 1.24 kb DNA segment between the two *Xho*I sites. Therefore, the gene conferring sulfanilamide resistance was presumed to lie in the region between the *Xho*I 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 *Sac*I site at 0.6 kb and the *Bam*HI site at 3.4 kb was subcloned as smaller fragments in both orientations in the phagemid pBluescript II SK(+) and sequenced (Fig. 3).

<i>Bam</i> HI -----ORF1----->			
1	GGATCCGCCG	GAGTCTGTCG	COCTGCCCTT
1	P P A E V V A	L P F L G E	H G D L
51	TGCGCATCCG	CAGCAAGACC	GGATGGCTGA
18	R I R S K T	G W L I R F G	E R S
101	GTGCTGTTTC	CCGCGGACTC	CACCAACATC
33	V L F A A D S	T N I S P T I	Y T K
151	GGTGGCCGAG	GTGATCGGCC	CGTGGGACAC
51	V A E V I G F	S D T V F I	G M E S
201	GCATCGGC	GGCGGCCAGC	TGATCTACG
68	I G A A A S	W I Y G P L Y	G E P
251	CTCGACCGCA	GGACCGACCA	GAGCGGACGG
84	L D R R T D Q	S R R L N G S	N F P
301	CCAGCGGAGG	GAGATCGTCG	ACGCCCTGGA
101	Q A R E I V D	A L E P D G	V Y V Y
351	ACGCGATGCG	CTCGAACCTG	GATGCGCTCG
118	A M A S N L D	G V V M A V D	Y D
401	GAGAGCCACC	CGGCCATCGT	CGACAGCGAC
134	E S H P A I V	D S D L L V R	H V Q
451	GGACAAGGGC	GGGACCGCCG	AACGGCTGCA
151	D K G G T A E	R L H L R R	T L R L
501	TCTGAGCGAC	CGGACGCCCG	CGGCCGGGGC
168	*		
551	CCCGGCCCT	CCGSCACGGC	COCTCCGGCC
			CGTCCCGCTG
			GCCCGCCGGC
601	GCCCCCGCGC	CCTCCGGAGA	GCCCTTCGAG
1			ACAAGGTGCC
651	CACGCTTCG	ATCGACAAC	ACGACTCGTT
3	T L L I D N Y	D S F T Q N	L F Q Y
701	ACATCGGCGA	GGCCACCGGG	CAGCCCCCGG
20	I G E A T G	Q P P V V P N	D A D
751	TGGTCCGGCG	TGCCCTTCGA	GGACTTCGAC
36	W S R L P L E	D F D A I V V	S P G
801	CCCCGGCAGC	CCGACCGGGG	AACGGGACTT
53	P G S P D R E	R D F G I S	K R A I
851	TCACCGACAG	CGGCTGCCC	GTCCCGCGC
70	T D S G L P	V L G V C L G	H Q G
901	ATCGCCGAGC	TCTCGGCGGA	ACCCATCGAC
86	I A Q L S A E	P M H G R V S	E V R
951	GCACACCGGC	GAGGACGTCT	TCCGGGGCCT
103	H T G E D V F	R G L P S P	F T A V
1001	TGCGTACCA	CTCCCTGGCC	GCCACCGACC
120	R Y H S L A	A T D L P D E	L E P
1051	CTCGCTGGA	CGGACGACGG	CGTCTCATG
136	L A W S D D G	V V M G L R H	R E K
1101	GCCGCTGATG	GGCGTCCAGT	TOCCACCGGA
153	P L M G V Q F	P P E S I G	S D F G
1151	GCCGGGAGAT	CATGGCCAAC	TTCGGCGACC
170	R E I M A N	F R D L A L A	H H R
1201	GCACGTCCG	ACGCGGCCGA	CTGGGGCTAC
186	A R R D A A D	W G Y E L H V	R R V
1251	CGACGTGCTG	CCGACGCGCG	AAGAGGTACG
203	D V L P D A E	E V R R A A	C F A E
1301	AGGGCCGCAC	GTCTGGCTG	GACAGCACT
220	G A T F W L	D S S S V L E	G A S
1351	CCGTTCTCT	TCCTCGGCGA	CGACCGCGGC
236	P F S F L G D	D R G P L A E	Y L T
1401	CTACCGCGTC	GCCGACGGCG	TGCTCTCCGT
253	Y R V A D G V	V S V R G S	D G T T
1451	CGACCGGGA	CGCGCGGACC	CTCTTCAGCT
270	T R D A A T	L F S Y L E E	Q L E
1501	CCGCGGGCGG	GTCCCGTCCG	CCCCGACCTG
286	P P A G P V A	P D L P F E F	N L G
1551	CTACGTCGCG	TACCTCGGCT	ACGAGCTGAA
303	Y V G Y L G Y	E L K A E T	T G D P
1601	CCGCACTACC	GGCCCCGAC	CCCCGACCGG
320	A V P A P H	P D A A F L F	A D R
1651	GCCATCGGCC	TCGACACCA	GGAAGCTGC
336	A I A L D H Q	E G C C Y L L	A L D
1701	CCGCGGGGCG	CACGACGACG	CGCCCCGCGC
353	R R G H D D G	A R A W L R	E T A E
1751	AGACCTTCAC	CGCCCTGGCC	GTCCCGCTCC
370	T L T G L A	V R V R P R P	T P A
1801	ATGGTCTTCG	GGGTCCCCGA	GGCGGGCGCC
386	M V F G V P E	A A A G F G P	L A R
1851	CGCAGCGCAC	GACAAGGACG	CCTCGGCGCT
403	A R H D K D A	S A L R N G	E S Y E
	<i>Bgl</i> III		
1901	AGATCTGCTT	GACCAACATG	GTACCGCGCG
420	I C L T N M	V T A P T E A	T A L
1951	CCGCTCTACT	CGCGCTGCG	CGGCATCAGC
436	P L Y S A L R	R I S P V P S	G A L
	<i>Xho</i> I		
2001	GCTCGAGTTC	CCCGAGCTGT	CGGTGCTCAG
453	L E F P E L S	V L S A S P	S R L
2051	TCACGATCGG	CGCCGACGGC	GGCGTCCAGT
470	T I G A D G	G V E S K P I	K G T
2101	CGCCCCCGGG	CGCACCGGCG	GGAGGAGGAC
485	R P R G A P A	E E D E R L R	A D L
2151	GGCGGGCGGG	GAGAAGGACC	GGGCCGAGAA
503	A G R E K D R	A E N L M I	V P L V
2201	TCCGCAACGA	CCTCAACAGC	GTCTGCGCGA
520	R N D L N S	V C A I G S V	H V P
2251	CGGCTCTTCG	AGGTGGGAGA	CCTCGCGCCC
536	R L F E V G D	L A P V H Q	L V S T
	<i>Pvu</i> II		
2301	CATCGGGGGA	CGGCTGCGGC	CGGCACCCAG
553	I R G R L R P	G T S T A A	C V R A
2351	CGGCTCTCCC	CGGCGGCTCC	ATGACCGCGG
570	A C F P G G S	M T G A P K K	R P M
2401	GAGATCATCG	ACCGCTGGA	GGAAGCCCCC
586	E I I D R L E	E G F R G V L	P G A
2451	GCTCGGATGG	TTGCGCTCA	GCGGCGCGCG
603	L G W F A L S	G A A D L S	I V I R
2501	GCACCATCGT	GCTGGCGGAC	GGCGGCGCGG
620	T I V L A D	G R A E F G V	G G A
2551	ATCGTGTCCC	TCTCGACCA	GGAGGAGGAG
636	I V S L S D Q	E E E F R Q T	V V K
2601	GGCCCCGCGC	ATGGTCACCG	CCCTCGACCG
653	A R A M V T A	L D G S A V	A G A R
2651	GATGAGCGGC	TTCCCCCGGA	GCCTGCTCGT
670	*		
2701	GCGCGGCATG	TTGCGCGGGC	TGCTGCGGGA
			GGCGGGCAGC
2751	TGCTCGACCT	CGTACCGCGG	CCGGGACGGC
			CGACGCCTCG
2801	ACGTACACCG	GCCGGGGGCC	<i>Sac</i> I
			GAGCTC

Fig. 3. Nucleotide and deduced amino acid sequence of the *Bam*HI–*Sac*I 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).

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) using the data of Wright & Bibb (1992) for streptomycetes indicated two potential ORFs. One of these (ORF1) is incomplete and begins upstream of the *Bam*HI end of the cloned fragment. ORF2 begins 140 bp downstream of the

Fig. 4. Alignment by the PILEUP program (GCG) of the amino acid sequences deduced from ORF2 of *S. venezuelae* ISP5230 and *pabA* and *pabB* genes from other bacteria. (a) The *pabA* regions of ORF2 (Sv) and *pabAB* of *S. griseus* (Sg) are compared with *pabA* from *Klebsiella aerogenes* (Ka), *Salmonella typhimurium* (St), *E. coli* (Ec), *B. subtilis* (Bs) and *Azospirillum brasilense* (Ab). (b) The *pabB* regions of ORF2 (Sv) and *pabAB* of *S. griseus* (Sg) compared with *pabB* of *K. aerogenes* (Ka), *Sal. 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 *Pst*I as size markers. Genomic DNA was digested with *Sac*I and probed with either a 1.45 kb *Nco*I–*Nco*I cassette containing *apr* (lanes λ , 1 and 2) or a 1.8 kb *Sac*I–*Sac*I 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.

TGA stop codon (nt 503–505) of ORF1 and contains 2010 bp. Its ATG start codon (nt 646–648) is preceded, with a 7 bp separation, by a putative ribosome binding site (GGTGG; $\Delta G = -9.4 \text{ kcal mM}^{-1}$; Tinoco *et al.*, 1973). The stop codon for ORF2 is at nt 2654–2656.

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 *Bam*HI and *Pst*I 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 *Xho*I–*Xho*I and 0.21 kb *Nco*I–*Nco*I segments of DNA from within *pabAB* to give pDQ500. Introducing an *Nco*I cassette carrying *apr* into

the unique *Nco*I 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^R Ts^R colonies; when these were propagated without antibiotic selection, Am^R Ts^S strains (e.g. VS629) were obtained.

Hybridization of *Sac*I-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 *Sac*I 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 (Doull *et al.*, 1985), the antibiotic titres measured by HPLC in the Am^R Ts^R transformants were similar to wild-type levels (60–70 µg ml⁻¹ at 6 d), whereas those in the Am^R Ts^S replacement strains were below 2 µg ml⁻¹. Assays of the sensitivity of the replacement strain to sulfanilamide showed that growth was inhibited at concentrations above 5 µg ml⁻¹.

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 aminase (*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 *pabAB* of *S. venezuelae*, it was unable to confer sulfanilamide resistance in transformants of *S. lividans* JG10. This and the absence of recognizable promoter sequences for ORF2 suggest that transcription does not begin immediately upstream of *pabAB*. Instead, the promoter site may be associated with an upstream region cotranscribed with *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 *S. griseus* the native *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 candidin biosynthesis gene cluster (Criado *et al.*, 1993). It is noteworthy that pDQ121, with the same *pabAB*-containing *S. venezuelae* DNA insert as pDQ373, conferred sulfanilamide resistance in *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 *S. griseus* and *S. venezuelae*, *pabAB* encodes an enzyme that acts near the beginning of unique biosynthetic sequences. If the pathways to Cm and candidin have arisen by forward rather than retrograde metabolic evolution, the distinctiveness and complexity of these two specialized biosynthetic systems suggest that *pabAB* has existed for a relatively long time. This being so, secondary metabolism itself is likely to have been an ancient process.

ACKNOWLEDGEMENTS

We are grateful to Dr J. Gil, Oviedo University, for the culture of *S. lividans* JG10, to Dr A. S. Paradkar, University of Alberta, for the *Nco*I cassette containing the apramycin resistance gene and to Dr C. L. Hersherberger, Eli Lilly & Company, for the vector pHJL400. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

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Received 2 October 1995; revised 18 January 1996; accepted 22 January 1996.