***p*-Aminobenzoic acid and chloramphenicol biosynthesis in *Streptomyces venezuelae*: gene sets for a key enzyme, 4-amino-4-deoxychorismate synthase**

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Amplification of sequences from *Streptomyces venezuelae* ISP5230 genomic DNA using PCR with primers based on conserved prokaryotic *pabB* sequences gave two main products. One matched *pabAB*, a locus previously identified in *S. venezuelae*. The second closely resembled the conserved *pabB* sequence consensus and hybridized with a 3.8 kb *NcoI* fragment of *S. venezuelae* ISP5230 genomic DNA. Cloning and sequence analysis of the 3.8 kb fragment detected three ORFs, and their deduced amino acid sequences were used in BLAST searches of the GenBank database. The ORF1 product was similar to PabB in other bacteria and to the PabB domain encoded by *S. venezuelae* *pabAB*. The ORF2 product resembled PabA of other bacteria. ORF3 was incomplete; its deduced partial amino acid sequence placed it in the MocR group of GntR-type transcriptional regulators. Introducing vectors containing the 3.8 kb *NcoI* fragment of *S. venezuelae* DNA into *pabA* and *pabB* mutants of *Escherichia coli*, or into the *Streptomyces lividans* *pab* mutant JG10, enhanced sulfanilamide resistance in the host strains. The increased resistance was attributed to expression of the pair of discrete translationally coupled *p*-aminobenzoic acid biosynthesis genes (designated *pabB/pabA*) cloned in the 3.8 kb fragment. These represent a second set of genes encoding 4-amino-4-deoxychorismate synthase in *S. venezuelae* ISP5230. In contrast to the fused *pabAB* set previously isolated from this species, they do not participate in chloramphenicol biosynthesis, but like *pabAB* they can be disrupted without affecting growth on minimal medium. The gene disruption results suggest that *S. venezuelae* may have a third set of genes encoding PABA synthase.

Keywords: *p*-aminobenzoate synthase genes, primary and secondary metabolism

INTRODUCTION

In their biosynthetic origins, *p*-aminobenzoic acid (PABA) and chloramphenicol (Cm) share the early steps

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Abbreviations: ADC, 4-amino-4-deoxychorismic acid; PABA, *p*-aminobenzoic acid; PAPA, *p*-aminophenylalanine; Am, apramycin; Cm, chloramphenicol; Ts, thiostrepton; Vio, viomycin.

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of the shikimate pathway leading to chorismic acid. At the chorismate branch point, separate routes have evolved to supply the proteinogenic aromatic amino acids (phenylalanine, tyrosine, tryptophan) and the folic acid cofactor component PABA. Although the *p*-nitrophenylserinol moiety of Cm bears obvious similarity to phenylalanine and tyrosine, it is not derived metabolically from them, but is synthesized *de novo* in the branch of the shikimate pathway that leads to PABA. The initial reaction, catalysed by an enzyme complex formed from the association of *pabA* and *pabB* gene products, converts chorismic acid to 4-amino-4-deoxychorismic acid (ADC). In turn, ADC is a metabolic branch point, leading either to PABA via a lyase reaction catalysed by the *pabC* product (Green *et al.*, 1992), or to

Fig. 1. Biosynthesis of primary and secondary aromatic metabolites in the shikimic acid pathway.

4-amino-4-deoxy-prephenic acid by a mutase-catalysed reaction (Teng *et al.*, 1985; Fig. 1). Conversion of 4-amino-4-deoxy-prephenic acid to *p*-aminophenylpyruvic acid gives Cm via the known precursor *p*-aminophenylalanine (PAPA) (Siddiquellah *et al.*, 1967; Jones & Vining, 1976). An alternative series of reactions from ADC via 4-amino-4-deoxyarogenic acid to PAPA and Cm is also feasible. The dichloroacetyl moiety of Cm is contributed by a still undetermined pathway that does not involve shikimic acid, but creates intermediates derived ultimately from glucose (Munro *et al.*, 1975).

Because genes associated with the biosynthesis of antibiotics in streptomycetes are commonly clustered on the chromosome of the producing organism, participation of ADC in Cm biosynthesis suggested that a cluster of Cm biosynthetic genes might be located in the vicinity of ADC synthase genes involved in PABA formation. Cloning PABA synthase genes could then

offer a potential route to cloning a Cm biosynthesis gene cluster. Consistent with these expectations, Brown *et al.* (1996) demonstrated that Cm production in *Streptomyces venezuelae* ISP5230 was severely impaired when an indigenous gene that complemented a *Streptomyces lividans* *pab* mutant was replaced with a disrupted allele. The locus has not yet been genetically linked to the mapped Cm biosynthesis gene cluster in *S. venezuelae* ISP5230 (Doull *et al.*, 1986; Vats *et al.*, 1987), but the DNA that complemented the *S. lividans* *pab* lesion has been shown by cloning and sequence analysis to be a gene consisting of fused *pabA* and *pabB* domains. In their deduced amino acid sequences, these domains closely resemble the products of discrete *pabA* and *pabB* genes in a variety of bacteria (Brown *et al.*, 1996). A strain of *S. venezuelae* in which the fused *pabAB* had been disrupted showed increased sensitivity to sulfanilamide, a metabolic inhibitor that competes with PAPA for the active site of a key enzyme required in the

synthesis of folate cofactors (Swerdberg & Skold, 1980). Sulfanilamide is normally included in culture media to counteract contaminants that obscure PABA assays based on a growth response. Because the interaction is competitive, the increased sensitivity to sulfanilamide observed after *pabAB* disruption implied reduced PABA synthesis. However, loss of *pabAB* did not completely prevent growth of the *S. venezuelae* strain in the absence of a PABA supplement. The results suggested that the ADC synthase encoded by *pabAB* acts predominantly in the secondary metabolic pathway and that *S. venezuelae* ISP5230 might contain another set of genes expressing ADC synthase to form PABA for primary metabolism.

METHODS

Bacterial strains, plasmids and vectors. These are listed in Table 1.

Culture conditions. *Escherichia coli* was grown as described by Sambrook *et al.* (1989). For *Micrococcus luteus* the medium used was GNY (Malik & Vining, 1970). *Streptomyces venezuelae* was maintained on MYM agar (Stuttard, 1982) supplemented with antibiotics as needed to select strains with plasmids; *Streptomyces lividans* JG10 was maintained on K1 agar (Aidoo, 1989), except when TOY agar (20 g each of Heinz tomato paste, Pablum and agar, and 2.5 g yeast extract in 1 litre water, pH 6.8), was required to enhance sporulation. For long-term maintenance of streptomycetes, spores in 20% (v/v) glycerol were stored at -70°C . Growth and sensitivity to sulfanilamide of streptomycete strains was assessed on minimal medium (MM; Hopwood, 1967). Sensitivity of *E. coli* strains to sulfanilamide was determined on minimal agar (MA; Clowes & Hayes, 1968) supplemented with vitamin-free Casamino acids (0.2 mg ml^{-1}) and thiamine (1.0 mg ml^{-1}). Sulfanilamide inhibition of *E. coli* and streptomycetes was measured by plating cells or spores at densities that gave well-separated colonies with no cross-feeding.

Transformation. Competent *E. coli* cells were prepared and transformed as described by Sambrook *et al.* (1989). Protoplasts of *S. lividans* were prepared, transformed and regenerated by standard methods for streptomycetes (Hopwood *et al.*, 1985); for *S. venezuelae* these methods were modified (Aidoo *et al.*, 1990). Procedures for conjugal transfer of plasmids from *E. coli* to streptomycetes were essentially those of Flett *et al.* (1997).

DNA manipulations. λ phage DNA, *E. coli* plasmid DNA and single-stranded DNA templates for sequencing were prepared as described by Sambrook *et al.* (1989). Genomic and plasmid DNA were isolated from streptomycetes by the methods of Hopwood *et al.* (1985). For rapid screening of plasmids in *E. coli* the Sekar (1987) procedure was adopted. DNA fragments excised in agarose gel slices were extracted with the QIAEX II kit (Qiagen).

Amplification of *S. venezuelae* chromosomal DNA by PCR. The primers used were 5'-ATGATCGTGGACCTSGACCG-SAACGAC-3' (sun-3) and 5'-CTGCGGCTCSAGCTCGTC-GATGATCTCCAT-3' (sun-4). The reaction mixture (total volume 100 μl) consisted of: dNTPs, 10 μl ($44 \times 1.25\text{ mM}$); primer sun-3, 1 μl (268 pmol); primer sun-4, 1 μl (182 pmol); chromosomal DNA, 100 ng; $10 \times$ Promega PCR buffer, 10 μl ; and water to 100 μl . It was aliquotted (20 μl) in thin-walled 0.5 ml microcentrifuge tubes and layered with mineral oil (ca 20 μl). Each aliquot was mixed with 1 U *Taq* DNA polymerase and heated to 80°C , then thermal cycling was started (model PTC-100; MJ Research). The programme for the first 7 cycles

was: 96°C for 1 min (denaturing), 67°C for 1 min (annealing) and 72°C for 1 min (extension). For the next 30 cycles denaturing and annealing times were each reduced to 45 s. Amplified *S. venezuelae* DNA was inserted into the *Sma*I site of pUC18 (SureClone Kit; Pharmacia Biotech) and sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977).

Construction of an *S. venezuelae* genomic library in λ GEM-11. Genomic DNA (50–100 μg) from *S. venezuelae* was partially digested at 37°C in a 100 μl reaction mixture containing 3 U *Sau*3AI. The incubation time was adjusted to optimize the yield of 9–23 kb fragments; digestion was terminated with EDTA (20 mM). The DNA recovered was fractionated by sucrose-gradient centrifugation and 3'-CTAG-5' overhangs of the 9–23 kb fragments were partially filled-in by incubation with the Klenow fragment of DNA polymerase I in the presence of dGTP and dATP. λ GEM-11 arms digested with *Xho*I (giving 3'-AGCT-5' overhangs) were partially filled in with dTTP and dCTP; the modified fragments and arms, after incubation with T4 DNA ligase and a λ phage packaging system (Promega), were used to infect *E. coli* LE392. The phage library was amplified as described by Sambrook *et al.* (1989) and stored at -70°C in SM buffer containing 7% (v/v) DMSO.

Construction of disruption vectors. The vector used to disrupt *pabAB* has been described by Brown *et al.* (1996). To disrupt the putative *pabB* in pJV305, a 1.5 kb DNA fragment containing an apramycin resistance (Am^{R}) gene (Paradkar & Jensen, 1995) was inserted into the *Eco*72I site of ORF1 to give pJV307. The disrupted ORF1 in pJV307 was then recloned as pJV323 in pHJL400. To construct a disruption vector containing the viomycin resistance (Vio^{R}) gene, the 2.0 kb *Streptomyces vinaceus* DNA fragment containing the gene (Thompson *et al.*, 1982) was excised from pJV230 (Chang, 1999) and introduced into pJV305 at the *Eco*72I site in ORF1, giving pJV308. The 5.8 kb *Bam*HI fragment containing ORF1 disrupted with the Vio^{R} gene was recloned from pJV308 into pHJL400, giving pJV324. Since protoplast procedures failed to give Vio^{R} transformants of VS629 with pJV324, the 5.8 kb *Bam*HI insert of pJV324 was subcloned in the conjugal vector pJV326 to give pJV325. Conjugal transfer of pJV325 from *E. coli* into *S. venezuelae* VS629 using methodology developed by Mazodier *et al.* (1989) and Flett *et al.* (1997) yielded single- and double-crossover mutants VS1003 and VS1004.

DNA sequencing and analysis. The 3.8 kb *Nco*I fragment of *S. venezuelae* ISP5230 DNA from recombinant phage YSB1 was subcloned (both orientations) into pBluescript II SK(+), giving pJV305 and pJV306. Nested overlapping deletions were introduced into the inserts by the Henikoff (1984) procedure and the DNA was used to transform *E. coli* DH5 α . Inserts in plasmid DNA extracted from the transformants were sequenced (ABI Prism model 373 DNA Sequencer). To detect ORFs by codon third position mol% G + C bias and codon usage, the CODONPREFERENCE programme (GCG Wisconsin Package, version 9.0; Devereux *et al.*, 1984) was used. Restriction enzyme sites were located with GeneRunner (Hastings Software) and similarities between derived amino acid sequences and proteins in GenBank were assessed from BLAST searches (Altschul *et al.*, 1997). Sequences were aligned using CLUSTAL W (Higgins *et al.*, 1996); the MacVector software of the Oxford Molecular Group was used for phylogenetic analysis.

Hybridization. For phage library screening (Hopwood *et al.*, 1985) and Southern analyses (Southern, 1975), DNA samples bound to nylon membranes were incubated with ^{32}P -labelled probes at 65°C overnight in hybridization solution (Sambrook *et al.*, 1989). The membranes were washed at room tem-

Table 1. Strains and vectors

Strain	Genotype/phenotype	Reference/source
<i>Streptomyces venezuelae</i>		
ISP5230	Wild-type	Stuttard (1982)
VS692	ISP5230 with <i>pabAB</i> disrupted	Brown <i>et al.</i> (1996)
VS1001	ISP5230 transformed with pJV323	This study
VS1002	ISP5230 with ORF1 disrupted	This study
VS1003	VS692 transformed with pJV325	This study
VS1004	VS692 with ORF1 disrupted	This study
<i>Streptomyces lividans</i>		
JG10	SLP2 ⁻ SLP3 ⁻ <i>pab-1 str-6</i>	Gil & Hopwood (1983)
<i>Escherichia coli</i>		
DH5 α	F'/ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^R) <i>relA1</i> Δ (<i>lacZYA-argF</i>) U169 <i>deoR</i> ϕ 80dlac <i>lacZ</i> Δ M15	BRL
ET12567	<i>dam</i> ⁻ <i>dcm</i> ⁻ <i>hsdM</i> ⁻	MacNeil <i>et al.</i> (1992)
ET12567(pUZ8002)		M. Paget, John Innes Institute, Norwich
LE392	F ⁻ <i>hsdR514 supE44 supF58 lacY1</i> or Δ (<i>lacIZY</i>)6 <i>galK2 galT22 metB1 trpR55</i>	Maniatis <i>et al.</i> (1982)
Plasmids		
pBluescript II SK(+)	Phagemid, <i>amp lacZ</i>	Stratagene
pHJL400	<i>tsr amp lacZ</i>	Larson & Hersherberger (1986)
pJV225	Am ^R gene flanked by MCSs	Chang (1999)
pJV230	pUC18::Tn4560	Chang (1999)
pJV291	pUC18 with YH1 cloned in <i>Sma</i> I site	This study
pJV292	pUC18 with YH2 cloned in <i>Sma</i> I site	This study
pJV293	pUC18 with YH3 cloned in <i>Sma</i> I site	This study
pJV294	pUC18 with YH4 cloned in <i>Sma</i> I site	This study
pJV295	pUC18 with YH5 cloned in <i>Sma</i> I site	This study
pJV305/306	pBluescript II SK(+) with 3.8 kb <i>Nco</i> I cloned (both orientations) in the <i>Eco</i> RV site	This study
pJV307	pJV305 containing 1.45 kb Am ^R gene fragment from pJV225 inserted at <i>Eco</i> 72I site	This study
pJV308	pJV305 containing 2.0 kb Vio ^R gene fragment from pJV230 inserted at <i>Eco</i> 72I site	This study
pJV323	pHJL400 containing 5.3 kb <i>Bam</i> HI fragment from pJV307 inserted at <i>Bam</i> HI site	This study
pJV324	pHJL400 containing 5.8 kb Vio ^R gene fragment from pJV308 inserted at <i>Bam</i> HI site	This study
pJV325	pJV326 containing 5.8 kb Vio ^R gene fragment from pJV308 inserted at <i>Bam</i> HI site	This study
pJV326	pHJL400 containing 0.76 kb <i>Pst</i> I fragment with <i>oriT</i> from pOJ446	This study
pJV327/328	pJV326 containing 3.8 kb <i>Bam</i> HI fragment of pJV305 cloned in <i>Bam</i> HI site (both orientations)	This study
pUC18	<i>E. coli</i> vector, <i>amp lacZ</i>	Pharmacia
Phage		
VCSM13	M13K07 derivative, Km ^R	Stratagene
GEM-11	λ vector	Promega
YSB1	λ phage from <i>S. venezuelae</i> DNA library in λ GEM-11; plaque hybridizing with YH3	This study

perature with 2 $\times$ SSC, and then twice at 65 °C with 0.1 $\times$ SSC (each solution containing 0.1 % SDS).

Cm production. Strains of *S. venezuelae* were grown and analysed for Cm production by methods described by Brown *et al.* (1996). The Cm content of filtered broths was determined by HPLC.

RESULTS

Cloning a second ADC synthase gene set from *S. venezuelae*

To isolate *S. venezuelae* ADC synthase homologues different from the *pabAB* cloned by Brown *et al.* (1996),

Fig. 2. (a) Alignment of deduced amino acid sequences for *pabB* from the GenBank database. The amino acids boxed and underlined with arrows are the conserved sequences used to design oligonucleotide primers sun-3 and sun-4. Identical and similar amino acids are indicated below the alignment by asterisks and periods, respectively. (b) Nucleotide sequences of fragments amplified by PCR from an *S. venezuelae* ISP5230 genomic DNA template using sun-3 and sun-4 as primers. Primer sequences recognized in the amplicons are boxed. Asterisks and periods below the alignment signify nucleotides present in all or most sequences, respectively.

candidate genomic sequences were amplified by PCR using primers chosen from consensus regions in an alignment of GenBank PABA synthase genes. The pair of oligonucleotides, sun-3 and sun-4 (Fig. 2a), was

designed from a streptomycete codon usage table (Wright & Bibb, 1992) to prime the amplification of 250 bp of *S. venezuelae* ISP5230 genomic DNA lying between two such consensus regions. Conditions for the

Fig. 3. Top: restriction map of *S. venezuelae* ISP5230 DNA cloned in λ phage YSB1. # indicates that not all *SacI* sites are shown. The expanded region below shows the 3.82 kb *NcoI*-*NcoI* fragment blunt-ended with *S1* nuclease and subcloned (alternative orientations) in the *EcoRV* site of pBluescript II SK(+) as pJV305 and pJV306 (the orientation shown); it also locates ORF1, ORF2 and the incomplete ORF3 in the cloned 3.82 kb *NcoI* fragment.

PCR were optimized to obtain a dominant 250 bp product. Amplified genomic DNA was cloned in pUC18 and used to transform *E. coli* DH5 α . Plasmid DNA was extracted from 21 individual transformants purified by plating on LB agar and the cloned inserts were sequenced. CLUSTAL W alignments showed four distinguishable patterns (YH1, YH2, YH3 and YH5; Fig. 2b) among the nucleotide sequences of the inserts. A BLASTX search of GenBank indicated that the deduced amino acid sequence of the type-YH1 amplicon matched the ADC synthase encoded by *pabAB* from *S. venezuelae*; a CLUSTAL W alignment confirmed the identity of the two sequences. With YH5 a BLASTX search detected no significant similarities between deduced amino acid sequences and GenBank proteins, but YH2 showed 30–45% similarity to bacterial anthranilate synthases, while the deduced amino acid sequence of the YH3 amplicon gave much closer matches (66–78% similarity) to PABA synthases. By using [32 P]dCTP-labelled YH3 to probe an *S. venezuelae* ISP5230 genomic library constructed in λ GEM-11, a hybridizing recombinant phage (YSB1) was recovered. Restriction mapping and Southern hybridization of the DNA insert in YSB1 (Fig. 3) located a 3.82 kb *NcoI* fragment of DNA that hybridized with the YH3 probe. The fragment was excised, blunt-ended and subcloned (both orientations) in the *EcoRV* site of pBluescript II SK(+) to give pJV305 and pJV306 (Fig. 4). Both strands of the YSB1 insert were sequenced and the sequence was deposited in GenBank (accession no. AF189258). The YH3 probe sequence was present at nt 1285–1496 in the cloned fragment.

Sequence analysis of the cloned 3.82 kb *NcoI* fragment

Analysis of the pJV306 insert sequence located ORF1 between nt 668 and 1843 in frame +2; a second ORF (ORF2) was present between nt 1840 and 2472 in frame +1. (see Fig. 3). Both ORF1 and ORF2 showed high G + C content (74 and 73.9 mol%, respectively) and a strong G + C bias (>94%) in the third position of codons, typical of streptomycete genes. The two ORFs, which are transcribed in the same direction but in different reading frames, overlap by 3 nt. An incomplete ORF (ORF3) was located from nt 2289 to the end of the sequenced region in frame -3. The ORF3 sequence overlapped one end of ORF2 (in frame +1) by 183 nt, but was transcribed in the opposite direction. Using BLASTX to search GenBank for proteins with sequences similar to the deduced amino acid sequences of ORF1 and ORF2 showed 30–40% identity between the ORF1 product and PabB or TrpE from a variety of organisms; similar close matches were found between the ORF2 product and PabA or TrpG proteins. The ORF1 product resembled (40% identity, 53% similarity) PabB of *Bacillus subtilis* and the PabB domain in products of the *pabAB*-type genes in *Streptomyces griseus* and *Streptomyces pristinae-spiralis* (35% identity, 51–52% similarity). CLUSTAL W alignments confirmed that the ORF1 and ORF2 products corresponded to the C- and N-terminal regions, respectively, in *pabAB* products (Fig. 5).

In a BLASTP search of proteins encoded by the *S.*

Fig. 4. Plasmids constructed for this study. Solid filled regions represent *S. venezuelae* DNA from YSB1. The two regions of ORF1 separated by disruption with *apr* or *vph* at *Eco72I* are distinguished in pJV323 and pJV325 as ORF1⁻ and -ORF1. *amp*, *apr*, *tsr* and *vph* designate genes for ampicillin, Am, Ts and Vio resistance.

coelicolor A3(2) genome (http://www.sanger.ac.uk/Projects/S_coelicolor) using translated *pabB* from *S. venezuelae* as the query sequence, the two closest matches were the anthranilate synthase component I products of *trpE2* in cosmid E8 (46% identical, 59% similar amino acids) and *trpE3* in cosmid 4G6 (39% identical, 54% similar amino acids). The ORF1 and ORF2 products were also related (41–43% identity, 59–60% similarity) to the *trpE* and *trpG* products, respectively, of several plants (e.g. *Arabidopsis thaliana*, *Ruta* sp.), as well as to *trp* products of the extreme bacterial thermophile *Aquifex aeolicus* (ca 37% identity, 51% similarity). A BLASTX search of the GenBank database with the partial ORF3 sequence showed no similarity to shikimate pathway gene products, but instead a strong resemblance to the sequence of MocR, a GntR-type transcriptional regulator in *Rhizopus* species.

Expression of genes for PABA biosynthesis

The 3.82 kb *NcoI* fragment of *S. venezuelae* ISP5230 DNA excised from λ YHB1 was blunt-ended and

subcloned in the *EcoRV* site of pBluescript II SK(+) as pJV305 and pJV306 (alternative orientations; see Fig. 3). To determine whether their inserts expressed enzymes for PABA biosynthesis, each of these plasmids was used to transform the *E. coli* PABA auxotrophs AB3292 and AB3295, which are defective in *pabA* and *pabB* functions, respectively. Plasmid pJV305 contained ORFs 1 and 2 oppositely oriented from the vector *lacZ*, whereas in pJV306, ORFs 1 and 2 were in the same orientation as *lacZ* (see Fig. 4). To assess whether PABA synthase genes were expressed, the growth response of the *E. coli* *pab* mutants and transformants on medium supplemented with sulfanilamide was determined. Expression was inferred from enhanced sulfanilamide resistance. The higher sulfanilamide resistance in mutants transformed with pJV306 than in those transformed with pJV305 (Table 2) implied that the cloned DNA was transcribed more efficiently when ORF1/ORF2 and *lacZ* were oriented in the same direction. This would be consistent with expression of the cloned *E. coli* insert from the vector *lacZ* promoter and with little or no expression from a native promoter. Comparable results have been reported for *S. griseus* *pabAB*,

Fig. 5. Alignment with CLUSTAL W of the deduced amino acid sequences of the pJV306 ORF1 (*pabB*) product (top line) and ORF2 (*pabA*) product (bottom line) with those of the *pabAB* product (centre line). Identical amino acids in comparisons of either the PabA and PabAB, or the PabB and PabAB sequences are highlighted by reverse printing; similar amino acids are outlined.

Table 2. Effect of sulfanilamide on growth of *E. coli* *pab* auxotrophs alone or transformed with pJV305 and pJV306

+, Normal growth; —, no growth.

Strain	Growth on sulfanilamide ($\mu\text{g ml}^{-1}$)			
	10	15	20	25
AB3292*	+	+	—	—
AB3292(pJV306)†	+	+	+	—
AB3292(pJV305)†	+	+	—	—
AB3295‡	+	+	—	—
AB3295(pJV306)†	+	+	+	+
AB3295(pJV305)†	+	+	+	—

* PabA auxotroph.

† The orientation of the 3.8 kb insert in pBluescript II SK(+) was opposite to *lacZ* in pJV305 and same as *lacZ* in pJV306.

‡ PabB auxotroph.

which was shown by Gil & Hopwood (1983) to be expressed in *E. coli* only from the vector promoter.

When the *S. venezuelae* DNA in pJV305 was subcloned in both orientations as a *Bam*HI fragment in the bifunctional *E. coli*/*Streptomyces* vector pJV326, the

resulting plasmids (pJV327 and pJV328; see Fig. 4) differed in their ability to confer sulfanilamide resistance on *S. lividans* PABA auxotrophic strain JG10. Cultures transformed with pJV328, in which the cloned fragment and vector promoter were oriented in the same direction, grew in a medium containing $10 \mu\text{g}$ sulfanilamide ml^{-1} , whereas cultures transformed with pJV327 (where the insert was oppositely oriented) were, like the auxotrophic parent, completely inhibited by $2 \mu\text{g}$ sulfanilamide ml^{-1} (Table 3). Again the higher resistance to sulfanilamide of the pJV328 transformant is attributed to expression from the vector promoter of the PABA synthase genes cloned from *S. venezuelae*. Because the results in both *E. coli* and *S. lividans* indicate that the 3.82 kb ADC synthase gene set cloned from *S. venezuelae* ISP5230 can express enzymes supporting PABA biosynthesis, we propose to designate the relevant gene pair *pabB/pabA*.

Disruption of the second ADC gene set in *S. venezuelae*

Transformation of wild-type *S. venezuelae* protoplasts with the ORF1 disruption vector pJV323 (see Fig. 4) yielded the Am^R and thiostrepton-resistant (Ts^R) strain VS1001 containing both the free and integrated plasmid. Plasmid derivatives of pHJL400 are segregationally unstable in streptomycetes (Larson & Hersherberger, 1996) and are lost during propagation without selection for Ts^R ; thus cultivation of VS1001 on unsupplemented MYM agar yielded a double-crossover transformant

Table 3. Effect of sulfanilamide on growth of *Streptomyces lividans* JG10 auxotroph transformed with pJV327 or pJV328

Colonies per plate: + + + + +, $>10^6$; + + +, $>10^3$; + +, 10–100; –, 0.

Strain	Growth on sulfanilamide ($\mu\text{g ml}^{-1}$)				
	0.25	0.5	1.0	2.0	5.0
JG10	+ + + + +	+ + +	+ +	–	–
JG10(pJV328)	+ + + + +	+ + + + +	+ + + + +	+ + + + +	–
JG10(pJV327)	+ + + + +	+ + + +	+ +	–	–

Fig. 6. (a) Southern hybridization of *S. venezuelae* genomic DNA from strains ISP5230 (lane 1) and VS1002 (lane 2) digested with *Nco*I and probed with the 3.8 kb *Bam*HI fragment of pJV305. The increased size of the hybridizing fragment in VS1002 is accounted for by integration of the 1.5 kb *apr* (see Fig. 4). (b) Southern hybridization of *S. venezuelae* genomic DNA from strains ISP5230 (lane 1) and VS1004 (lane 2) digested with *Mlu*I and probed with the 5.8 kb *Bam*HI fragment of pJV325. The increased size of the 1.6 kb hybridizing fragment in VS1004 is accounted for by integration of the 2.0 kb *vph* (see Fig. 4).

Table 4. Effect of sulfanilamide on growth of *Streptomyces venezuelae* ISP5230 and strains disrupted in ADC synthase genes

Colonies per plate: + +, $>10^3$; +, 10–100; $\pm$, 0–10; –, 0.

Strain	Gene disrupted	Growth on sulfanilamide ($\mu\text{g ml}^{-1}$)				
		0.5	1.0	1.5	2.0	10
ISP5230	None	+ +	+ +	+ +	+ +	+
VS629	<i>pabAB</i>	+ +	+	$\pm$	–	–
VS1002	ORF1	+ +	+ +	+ +	+ +	$\pm$
VS1004	<i>pabAB</i> and ORF1	+ +	+	$\pm$	–	–

Fig. 7. Sequence relationships between 35 gene products in the PabB/TrpE superfamily. Sequences were aligned with CLUSTAL W in the MacVector software of the Oxford Molecular Group. Numbers in the dendrogram are distance measurements given as changes per residue. The sequences used in the alignment and their protein IDs are: *Aquifex aeolicus* PabB (AAC07173); *Bacillus subtilis* PabB (AAA22694), TrpE (NNB51); *Bacillus halodurans* PabB (BAB03809); *Campylobacter jejuni* PabB (CAB73127); *Clostridium thermocellum* TrpE (P14953); *Corynebacterium glutamicum* TrpE (B24723); *Escherichia coli* PabB (BAB35944), TrpE (AAC74346); *Haemophilus influenzae* PabB (AAC22834), TrpE (AAC22033); *Klebsiella aerogenes* PabB (AAA88207); *Lactococcus lactis* PabB (AAK05412); *Mycobacterium tuberculosis*

(VS1002), the identity of which was confirmed by Southern hybridization (Fig. 6a). Strain VS1002, in which ORF1 had been insertionally inactivated, was slightly more sensitive to sulfanilamide than *S. venezuelae* ISP5230 (Table 4), but produced a wild-type titre of Cm.

To inactivate both *pabAB* and the putative *pabB* (ORF1) in the same wild-type strain by the methods used to disrupt the genes individually (see above), it was necessary to construct a conjugal disruption vector carrying a different resistance gene. Transformation of the *pabAB*-disrupted *S. venezuelae* strain VS629 (Brown *et al.*, 1996) with the new vector (pJV325; see Fig. 4) gave single-crossover transformants (VS1003) from which a double-crossover strain (VS1004) resistant to both Am and Vio, but sensitive to Ts, was isolated. Disruption of ORF1 in VS629 by insertion of the *Vio*^R gene, giving strain VS1004, was confirmed by Southern hybridization (Fig. 6b). When the growth of *S. venezuelae* strains ISP5230, VS629, VS1002 and VS1004 was compared in minimal medium supplemented with increasing concentrations of sulfanilamide (see Table 4), the difference in sensitivity to sulfanilamide between ORF1-disrupted strains and their parents was marginal (compare strains VS1004 and VS629). It was also noted that the Cm titre in strain VS1004 was not significantly different from that in strain VS629.

DISCUSSION

Functions of ADC gene sets in *S. venezuelae*

The loss of over 97% in Cm production when the native *pabAB* was replaced with a disrupted allele, and restoration of production when disrupted cultures were supplemented with PABA have provided strong evidence that this ADC synthase gene set encodes an enzyme converting chorismate to ADC as a precursor of PABA, and ultimately of Cm (Brown *et al.*, 1996; Halpern, 1997). Since *pabAB*-disrupted strains retained their prototrophic phenotype and were only slightly more sensitive than the wild-type to inhibition by sulfanilamide, specific conversion of ADC formed by the *pabAB* product was inferred and *S. venezuelae* ISP5230 was postulated to contain a second set of ADC synthase genes needed to synthesize PABA for folate cofactors used in primary metabolism (Brown *et al.*, 1996). In exploring *S. venezuelae* ISP5230 for the presence of genes encoding PABA synthase, we have discovered a new set of ADC synthase genes, cloned it in pJV305 and investigated its properties. This set differs in organization from *pabAB* and in this respect is more like the PABA synthase genes in the *fol* operon of *B. subtilis*

(Slock *et al.*, 1990), which not only contains discrete genes, but also contains its *pabB* component upstream of its *pabA* equivalent (which in *B. subtilis* is the amphibolic *trpG*). A noteworthy difference from *B. subtilis* is the absence of *pabC* immediately downstream of the amphibolic *trpG*. The organization of both the *pabAB* and *pabB/pabA* sets of ADC synthase genes in *S. venezuelae* contrasts with that in *Mycobacterium tuberculosis*, *E. coli* and other bacteria where *pabA* and *pabB* are separated on the chromosome by up to 1500 kb.

The probable identity of ORF1 and ORF2 with *pabB* and *pabA*, respectively, is indicated by comparisons of their deduced amino acid sequences with proteins in the GenBank database and by the ability of the ORFs to complement *pab* mutants in *E. coli* and *S. lividans*. However, the absence of a decisive loss of sulfanilamide resistance in *S. venezuelae* ISP5230 when *pabB* is disrupted implies that this gene set is not, as anticipated, primarily associated with PABA biosynthesis. The conclusion is supported by the negligible change in sulfanilamide resistance following disruption of *pabB* in the *pabAB* mutant VS629 to yield a strain inactivated in both of its known ADC synthase genes. It is possible that the weak PABA synthase activity in VS629 is due, at least in part, to incomplete inactivation of *pabAB*, so the effect of disrupting *pabB* is masked by the host's residual PabAB activity. Contributing to this might be the high normal level of *pabAB* expression implied by the exceptionally high resistance of *S. venezuelae* to sulfanilamide. Aidoo *et al.* (1990) postulated that this high sulfanilamide resistance reflects a large ADC pool associated with the level of *pabAB* expression needed to support Cm biosynthesis. Spillover from the pool in the wild-type, or residual activity in the *pabAB* mutant might outweigh a relatively weak contribution to PABA synthesis from *pabB* and obscure the response to *pabB* disruption. However, the presence in *S. venezuelae* ISP5230 of a third gene set involved in supplying most of the PABA needed to support growth must also be considered.

Anthranilate and ADC synthase component I genes of *S. venezuelae* ISP5230 and *S. coelicolor* A3(2)

Information currently available from the *S. coelicolor* A3(2) genome sequencing project (http://www.sanger.ac.uk/Projects/S_coelicolor) has located four genes encoding putative anthranilate synthase component I proteins on cosmids cloned from the chromosome: cosmid 6E10 contains *trpE1*, cosmid E8 contains *trpE2*, cosmid 4G6 contains *trpE3* and cosmid L11 contains an undesignated putative *trpE*. No PABA

EntC (CAB08301), PabB (CAB08140), TrpE (CAB08903), TrpE2 (CAB03739); *Pseudomonas aeruginosa* PchA (S58229), TrpE (20580); *Pseudomonas fluorescens* PhzE (Q5 1791); *Sinorhizobium meliloti* TrpEG (AAA26370); *Saccharomyces cerevisiae* PabAB (P37254); *Salmonella typhimurium* PabB (AAA88618); *Streptomyces coelicolor* cosmid E8, TrpE2 (CAB385), cosmid L11, TrpE (CAB76066), cosmid 4C2, TrpE (CAC17508), cosmid 4G6 TrpE3 (CAB51435), cosmid 6E10, TrpE1 (CAB51965); *Streptomyces griseus* PabAB (P32438); *Streptomyces pristinae-spiralis* PabAB (AAC44866); *Streptomyces venezuelae* PabAB (AAB30312), PabB (AAF01062), TrpEG (AAC35404); *Sulfolobus solfataricus* TrpE (AAA7379); *Thermus thermophilus* TrpE (CAA30566).

synthase component I genes have yet been detected. In a BLASTP search of GenBank for sequence similarity to the translated TrpE1, the highest score was obtained with the anthranilate synthase of *S. venezuelae* ISP5230 associated with the primary metabolic pathway for tryptophan biosynthesis (Lin *et al.*, 1998). This result is consistent with similarities between genes of the primary metabolic tryptophan biosynthetic pathways in *S. venezuelae* and *S. coelicolor*, and with the presence of another primary metabolic *trp* gene (*trpD*) on the overlapping cosmid 6G1 (Redenbach *et al.*, 1996). Similar BLASTP searches of GenBank with the translated TrpE2 and TrpE3 sequences ranked only anthranilate synthases in the 30 highest scoring protein sequences. Evidence that *trpE2* and *trpE3* are tryptophan biosynthesis genes associated with secondary metabolism supports these search results: *trpE2* is involved in biosynthesis of tryptophan residues for the lipopeptide calcium-dependent antibiotic of *S. coelicolor* A3(2) (Chong *et al.*, 1998), while the presence of three other *trp* genes (*trpA*, *trpB* and *trpC*) distinct from the primary metabolic *trp* genes on the same cosmid (4G6) as *trpE3* implies an involvement with secondary metabolic tryptophan biosynthesis activity. Therefore, three of the four genes predicted to encode anthranilate synthases appear to be unambiguously assigned, but assignment of the cosmid L11 gene as *trpE* is less certain. A BLASTP search of GenBank with the translated sequence of this gene ranked the PabB sequences of *Mycobacterium tuberculosis*, *Xanthomonas albilineans* and *Pseudomonas aeruginosa* ahead of TrpE sequences in order of similarity and also gave high scores to the PabBs of enteric bacteria and *Vibrio cholerae*. The gene on *S. coelicolor* cosmid L11 may, therefore, encode an ADC synthase rather than an anthranilate synthase.

In support of this conclusion, BLASTP searches of the *S. coelicolor* A3(2) genome with deduced amino acid sequences of the YH1, YH2 and YH3 PCR products amplified from the *S. venezuelae* chromosome showed that the cosmid L11 gene product resembled the YH1 and YH3 sequences more closely (63% similarity, 53% identity) than it resembled the YH2 sequence (53% similarity, 35% identity). Since the deduced YH1 and YH3 amino acid sequences matched PabAB and PabB, respectively, whereas the deduced YH2 sequence matched TrpE, the results indicate that the *S. coelicolor* gene in cosmid L11 is more likely to be *pabB* than *trpE*. In agreement with the assignments of *trpE* genes in the other *S. coelicolor* cosmids, the product of *trpE3* in cosmid 4G6 closely resembled (75% similarity, 70% identity) the deduced amino acid sequence of the YH2 PCR product, indicating that YH2 is amplified from a *trpE3* counterpart in *S. venezuelae*. The product of *trpE2* in cosmid E8 was less similar (65% similar, 55% identical amino acids) to the YH2 PCR product, confirming that YH2 is unlikely to have been amplified from a *trpE2* counterpart. Although these sequence comparisons suggested that the *pabB* cloned from *S. venezuelae* corresponds to the *S. coelicolor* A3(2) gene in cosmid L11, a preferential relationship could not be

demonstrated through BLASTP matching of *S. coelicolor* A3(2) genomic proteins with the translated sequence of *S. venezuelae* *pabB*. The highest scoring sequence was anthranilate synthase component I encoded by *trpE2* in cosmid E8 (59% similar, 46% identical amino acids), followed by the anthranilate synthase of *trpE3* in cosmid 4G6 (54% similar, 39% identical amino acids), and then the product of the putative *trpE* in cosmid L11 (48% similar, 37% identical amino acids). However, similar rankings were obtained when the products of *B. subtilis* *pabB* or *S. venezuelae* *pabAB* were used to query the *S. coelicolor* A3(2) genomic proteins, suggesting that such sequence comparisons are not reliable indicators of functional similarity. The indication that the putative *pabB/pabA* gene set cloned from *S. venezuelae* encodes proteins with weak PABA synthase activity, but appreciable anthranilate activity is, nevertheless, noteworthy in that it could account for the observation (Lin *et al.*, 1998) that disruption of the primary metabolic *trpE(G)* in *S. venezuelae* ISP5230 did not create an auxotrophic requirement for tryptophan. The analysis of *S. coelicolor* and *S. venezuelae* sequences presented above bears on the evidence from gene disruptions that implicates a third and so far undetected set of genes encoding PABA biosynthesis in *S. venezuelae* ISP5230 and suggests that this set may be related to the putative *trpE* cloned from *S. coelicolor* A3(2) in cosmid L11.

Phylogenetic analysis of genes in the *pabB/trpE* superfamily

The shared evolutionary origin of genes for PABA and anthranilate synthases, postulated from sequence data by Goncharoff & Nichols (1984), was demonstrated further in the results of a CLUSTAL W alignment of 35 PabB and TrpE sequences. The dendrogram showing sequence relationships (Fig. 7) indicated a superfamily derived from an ancestral sequence that diverged to form three initial groups. One (Group A) contains six TrpEs, the second (Group B) contains two TrpEs and the third (Group C) has both TrpEs and PabBs. The product of *pabB* cloned from *S. venezuelae* ISP5230 is present in Group C; its sequence shows early divergence from subgroups containing either TrpE or other PabB sequences and it has no closely related family member of recent origin.

In general, genes associated with secondary metabolic biosynthetic pathways diverge relatively early from homologues with roles in primary metabolism. The dendrogram implies the possible divergence of a cluster of streptomycete *pabABs* involved in antibiotic production from the *Saccharomyces cerevisiae* *pabAB*, which is dedicated to PABA biosynthesis (Edman *et al.*, 1993). The entire *pabAB* cluster shares ancestry with well separated clusters consisting of one or more discrete primary metabolic *pabBs* and with *trpE* from *S. coelicolor* cosmid L11. Only a small number of antibiotic-producing streptomycetes have PABA synthase genes that exhibit a correlation between fusion of *pabA/pabB* domains and a role in secondary metabolism. A second example where Pab/Trp superfamily

genes associated with secondary metabolism have diverged from primary metabolic homologues can be found in *Pseudomonas fluorescens*. Here the gene encoding anthranilate synthase (PhzE) for phenazine biosynthesis is related to anthranilate synthase genes for tryptophan biosynthesis in *S. coelicolor* and *S. venezuelae*. The dendrogram also supports the sequence similarity, noted in the preceding section, between *S. coelicolor* A3(2) TrpE in cosmid L11 and a PabB of *M. tuberculosis*. This protein appears to be the only gene product that might provide the PABA synthase activity required for primary metabolism in *S. coelicolor* A3(2), strengthening the possibility that the gene is distributed generally in streptomycetes and is indeed the third ADC synthase gene set in *S. venezuelae* ISP5230.

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