The gene cluster for chloramphenicol biosynthesis in *Streptomyces venezuelae* ISP5230 includes novel shikimate pathway homologues and a monomodular non-ribosomal peptide synthetase gene

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Regions of the *Streptomyces venezuelae* ISP5230 chromosome flanking *pabAB*, an amino-deoxychorismate synthase gene needed for chloramphenicol (Cm) production, were examined for involvement in biosynthesis of the antibiotic. Three of four ORFs in the sequence downstream of *pabAB* resembled genes involved in the shikimate pathway. BLASTX searches of GenBank showed that the deduced amino acid sequences of ORF3 and ORF4 were similar to proteins encoded by monofunctional genes for chorismate mutase and prephenate dehydrogenase, respectively, while the sequence of the ORF5 product resembled deoxy-*arabino*-heptulosonate-7-phosphate (DAHP) synthase, the enzyme that initiates the shikimate pathway. A relationship to Cm biosynthesis was indicated by sequence similarities between the ORF6 product and membrane proteins associated with Cm export. BLASTX searches of GenBank for matches with the translated sequence of ORF1 in chromosomal DNA immediately upstream of *pabAB* did not detect products relevant to Cm biosynthesis. However, the presence of Cm biosynthesis genes in a 7.5 kb segment of the chromosome beyond ORF1 was inferred when conjugal transfer of the DNA into a blocked *S. venezuelae* mutant restored Cm production. Deletions in the 7.5 kb segment of the wild-type chromosome eliminated Cm production, confirming the presence of Cm biosynthesis genes in this region. Sequencing and analysis located five ORFs, one of which (ORF8) was deduced from BLAST searches of GenBank, and from characteristic motifs detected in alignments of its deduced amino acid sequence, to be a monomodular nonribosomal peptide synthetase. GenBank searches did not identify ORF7, but matched the translated sequences of ORFs 9, 10 and 11 with short-chain ketoreductases, the ATP-binding cassettes of ABC transporters, and coenzyme A ligases, respectively. As has been shown for ORF2, disrupting ORF3, ORF7, ORF8 or ORF9 blocked Cm production.

Keywords: *p*-aminophenylalanine, gene disruption, mutant complementation, sequence analysis

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Abbreviations: ADC, 4-amino-4-deoxychorismate; Am, apramycin; Cm, chloramphenicol; DAHP, deoxy-*arabino*-heptulosonate-7-phosphate; Km, kanamycin; NRPS, non-ribosomal peptide synthetase; PABA, *p*-aminobenzoic acid; PAPA, *p*-aminophenylalanine; PAP5, *p*-aminophenylserine; Ts, thiostrepton.

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INTRODUCTION

Chloramphenicol (Cm; Fig. 1) is produced by the filamentous Gram-positive soil bacterium *Streptomyces venezuelae* and certain other actinomycetes. The phenylpropanoid moiety of Cm has its origins in the general shikimate pathway for assembling aromatic metabolites (Vining & Westlake, 1984). The route to Cm branches at chorismic acid to generate *p*-aminophenylalanine (PAPA), which serves as a precursor of the *p*-nitrophenylserinol component of the antibiotic (Teng *et al.*, 1985). Incorporation of isotopically labelled Cm precursors and characterization of intermediates accumulated by *S. venezuelae* mutants blocked in Cm biosynthesis have suggested a sequence of reactions involving *p*-aminophenylserine (PAPS) and *N*-dichloroacetyl *p*-aminophenylserinol (see Fig. 1) through which PAPA is converted to the *p*-nitrophenylserinol derivative (Vining & Stuttard, 1994). Genes associated with mutational loss of Cm biosynthesis have been mapped by analysing conjugational crosses and cotransduction frequencies in *S. venezuelae* ISP5230 and shown to constitute a Cm biosynthesis cluster (Doull *et al.*, 1986; Vats *et al.*, 1987). Mapping data for all blocked Cm producers in which the mutation has been located have placed the cluster at approximately 2 o'clock on a circular *S. venezuelae* ISP5230 chromosome. However, relatively little is known of the molecular genetics of Cm production and until now no gene in the mapped cluster has been isolated.

In an initial approach to cloning the genes, Aidoo *et al.*

(1990) transformed *S. venezuelae* *cml*-1, a mutant of *S. venezuelae* ISP5230 blocked in Cm biosynthesis at a step preceding PAPA, with a plasmid carrying wild-type genomic DNA fragments that had been shown to complement an auxotrophic *Streptomyces lividans* mutant with a requirement for *p*-aminobenzoic acid (PABA). The inability of transformants to produce Cm implied that the DNA fragment supporting PABA synthesis did not support PAPA synthesis and suggested a distinction between primary and secondary metabolic reactions generating the intermediate 4-amino-4-deoxychorismic acid (ADC; Fig. 2) common to the PABA and PAPA pathways. In subsequent work, Brown *et al.* (1996) cloned and sequenced an *S. venezuelae* DNA fragment that contained a distinctive, fused ADC synthase gene (*pabAB*), disruption of which created only a marginal growth dependence in *S. venezuelae* for exogenous PABA, but severely reduced Cm production. Thus *pabAB*, although not originally included in the genetically mapped cluster of *cml* genes, appeared to have a pivotal role in Cm biosynthesis. The role of the fused *pabAB* contrasts with that of the discrete *pabA* and *pabB* genes of *E. coli* and *Bacillus subtilis* (Slock *et al.*, 1990), where the products serve as components of an ADC synthase that, by associating with PabC (Nichols *et al.*, 1989), form the PABA synthase complex functioning in primary metabolism to generate PABA for incorporation into folic acid derivatives. The results obtained with the fused gene set in *S. venezuelae* implicate an alternative secondary metabolic role of ADC synthase. In association with ADC mutase, the

Fig. 1. Scheme showing potential roles for Cm biosynthesis genes referred to in this research.

Fig. 2. Shikimate pathway reactions associated with the biosynthesis of primary and secondary aromatic metabolites via chorismic acid in *S. venezuelae*.

enzyme is presumed to generate 4-amino-4-deoxy-prephenic acid (see Fig. 2). The ADC mutase activity parallels that of the chorismate mutase that converts chorismic acid to prephenic acid for phenylalanine and tyrosine synthesis in the primary shikimate pathway. The secondary metabolic pathway proposed here converts chorismic acid to ADC, 4-amino-4-deoxy-prephenic acid, PAPA and ultimately Cm. In accord with this hypothesis, further efforts to clone the Cm biosynthesis cluster were directed to regions of the *S. venezuelae* ISP5230 chromosome flanking *pabAB*.

METHODS

Micro-organisms and plasmids. The bacteria, phage and plasmids used are described in Table 1. *Streptomyces venezuelae* strains were maintained on MYM agar (Stuttard, 1982) supplemented when necessary with one or both of the antibiotics apramycin (Ap; 50 µg ml⁻¹) and thiostrepton (Ts; 25 µg ml⁻¹). Minimal (MM) agar used for *S. venezuelae* plate cultures contained the glucose/asparagine/salts solution of Hopwood (1967), but with maltose instead of glucose. *Streptomyces lividans* 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). For *Escherichia coli*, LB medium or 2× YT medium was used (Sambrook *et al.*, 1989).

DNA manipulations. The general procedures described by Sambrook *et al.* (1989) were used. Plasmid DNA was obtained

from *E. coli* by the alkaline lysis method. To isolate genomic DNA from *S. venezuelae*, the final aqueous solution obtained as described by Hopwood *et al.* (1985) was extracted with chloroform containing 1% (v/v) cetyl trimethylammonium bromide before the DNA was precipitated with an equal volume of 2-propanol. Competent *E. coli* cells were prepared and transformed as described by Sambrook *et al.* (1989). Procedures for transforming *S. venezuelae* ISP5230 were modified (Aidoo *et al.*, 1990) from those developed for *S. lividans* (Hopwood *et al.*, 1985). To avoid restriction by *S. venezuelae* enzymes recognizing methylated DNA (Brown *et al.*, 1996), plasmids were passaged before use through *E. coli* ET12567, which lacks DNA methylating systems (MacNeil *et al.*, 1992).

For Southern hybridization, restriction digests of genomic DNA electrophoresed in agarose gels were transferred to a positively charged nylon membrane (Qiagen) and probed with a DNA fragment labelled with [³²P]dCTP by the random primer method (Amersham Pharmacia Biotech). After hybridization at 65 °C in a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM Na₂HPO₄ and 1 mM EDTA, pH 7.7), 5× Denhardt's solution, 0.5% (w/v) SDS and denatured salmon sperm DNA (100 µg ml⁻¹), membranes were washed at 60 °C with SSPE solutions (twice with 2×, then with 1× and 0.1×) containing 0.1% SDS. Radioactive fragments were detected with a Bio-Rad CS phosphorimaging screen scanned in a Bio-Rad model GS525 Molecular Imager.

Chromosome walking. A gene bank consisting of genomic DNA fragments from *S. venezuelae* ISP5230 was prepared in λ GEM-11 (Facey *et al.*, 1996) and screened for plaques that hybridized with a [³²P]dCTP-labelled 1.7 kb *Pst*I-*Xho*I DNA

Table 1. Bacterial strains and plasmids

Strains	Genotype/phenotype	Source/reference
<i>S. venezuelae</i>		
ISP5230	Wild-type	Stuttard (1982)
VS1050	ISP5230 with 3.5 kb <i>NcoI</i> segment of chromosome upstream of <i>pabAB</i> replaced by 1.5 kb <i>Am^R</i> gene	This work
VS1051	ISP5230 with <i>cmlH</i> disrupted by 1.5 kb <i>Am^R</i> gene	This work
VS1052	ISP5230 with <i>cmlP</i> disrupted by 1.5 kb <i>Am^R</i> gene	This work
VS1053	ISP5230 with <i>cmlC</i> disrupted by 1.5 kb <i>Am^R</i> gene	This work
VS1054	ISP5230 with <i>cmlF</i> disrupted by 1.5 kb <i>Am^R</i> gene	This work
VS1055	ISP5230 with <i>cmlJ</i> disrupted by 1.5 kb <i>Am^R</i> gene	This work
<i>S. lividans</i>		
JG10	<i>pab-1 str-6 SLP2⁻ SLP3⁻</i>	Gil & Hopwood (1983)
<i>E. coli</i>		
DH5 α F'IQ	F' ϕ 80dlacZ Δ M15 Δ (lacZYA-argF) W169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_k⁻ m_k⁻</i>) <i>supE44 thi-1 gyrA96 relA1</i> F' <i>proAB⁺ lacI^qZ</i> Δ M15 <i>zzf::Tn5</i> (Km ^R)	Hanahan (1983)
ET12567	<i>dam dcm hsdM</i>	MacNeil <i>et al.</i> (1992)
Plasmids		
pBluescript II (SK +)	<i>E. coli</i> phagemid; Ap ^R , <i>lacZ'</i>	Stratagene
pDQ116	pIJ41 with 5.65 kb <i>S. venezuelae</i> insert	Brown <i>et al.</i> (1996)
pDQ502	pHJL400 with 5.4 kb <i>PstI</i> - <i>PstI</i> fragment of <i>S. venezuelae</i> chromosome including 3'-end of <i>pabAB</i>	This work
pHJL400	<i>E. coli</i> - <i>Streptomyces</i> bifunctional vector; Par ⁻ Ap ^R Ts ^R	Larson & Hershberger (1986)
pJV168	pBluescript II with 7.5 kb <i>S. venezuelae</i> DNA	This work
pJV225	pBluescript II SK + containing <i>Am^R</i> gene flanked by multicloning sites with inverted orientations	Chang (1999)
pJV326	<i>E. coli</i> - <i>Streptomyces</i> conjugal bifunctional vector; Par ⁻ Ap ^R Ts ^R	This work
pJV351	pUC18 with a 1.7 kb <i>KpnI</i> - <i>BamHI</i> fragment of <i>S. venezuelae</i> DNA	This work
pJV352	pJV351 with 1.5 kb <i>Am^R</i> gene inserted into <i>NotI</i>	This work
pJV353	pJV326 with disrupted <i>cmlC</i> recloned from pJV352	This work
pJV354	pHJL400 with 7.5 kb <i>BamHI</i> - <i>BamHI</i> fragment of <i>S. venezuelae</i> chromosome upstream of <i>pabAB</i>	This work
pJV355	pJV354 with 1.5 kb <i>NcoI</i> insert containing <i>Am^R</i> gene replacing a 3.5 kb <i>NcoI</i> - <i>NcoI</i> segment	This work
pJV357	pKC1218 with 7.5 kb <i>BamHI</i> - <i>BamHI</i> insert from <i>S. venezuelae</i> in its <i>BamHI</i> site	This work
pJV358	Same as pJV357 but inserted in opposite orientation	This work
pJV368	pJV326 with a 5.1 kb <i>BamHI</i> - <i>EcoRI</i> fragment containing disrupted ORF8 from pJV367	This work
pJV369	pBluescript II SK + containing 3.2 kb <i>BglII</i> - <i>BamHI</i> fragment from pJV168, blunted and inserted at <i>SmaI</i> site	This work
pJV370	pJV369 with a 1.5 kb <i>EcoRV</i> fragment containing the <i>Am^R</i> gene inserted at its <i>NruI</i> site	This work
pJV371	pJV326 with a 4.7 kb fragment containing disrupted ORF7 in the <i>XbaI</i> / <i>EcoRI</i> site	This work
pJV375	pBluescript II SK + with 3.6 kb <i>PstI</i> - <i>BamHI</i> fragment from pJV168 disrupted at <i>NcoI</i> with 1.5 kb <i>Am^R</i> gene from pUC120	This work
pJV501	pBluescript II SK + with 1.6 kb <i>MluI</i> - <i>BamHI</i> fragment containing ORF9 from pJV168	This work
pJV502	pUC18 with 1.6 kb <i>XbaI</i> - <i>KpnI</i> fragment containing ORF9 from pJV501	This work
pJV503	pJV502 with ORF9 disrupted at its <i>EcoRV</i> site by insertion of the <i>Am^R</i> gene	This work
pJV504	pJV326 with a 3.1 kb <i>XbaI</i> - <i>EcoRI</i> fragment from pJV503 containing disrupted ORF9	This work
pKC1218	<i>E. coli</i> vector containing <i>Am^R</i> and <i>oriT</i> genes	Biermann <i>et al.</i> (1992)
pOJ446	Conjugative vector with <i>oriT</i> between <i>PstI</i> - <i>PstI</i> sites	Biermann <i>et al.</i> (1992)
pUC18	<i>E. coli</i> vector with <i>lacZ</i> and Ap ^R	BRL
pUC120	pBluescript with <i>Am^R</i> gene from pKC462	Paradkar & Jensen (1995)
pUZ8002	<i>E. coli</i> plasmid: RK2 derivative mediating conjugative DNA transfer; Km ^R Cm ^R Tra	M. S. B. Paget, John Innes Institute, Norwich
Phages		
GEM-11	λ vector used for library construction	Promega
PB1	λ library clone containing <i>pabAB</i>	This work

Fig. 3. Restriction map of *S. venezuelae* chromosomal DNA containing the Cm biosynthesis gene cluster. (a). Cloned chromosomal DNA in pDQ116 (Brown *et al.*, 1996) extended by chromosome walking. (b). ORFs identified in genomic DNA fragments subcloned from λ GEM-11.

fragment isolated from pDQ116 (Brown *et al.*, 1996). The probe contained the 3' region of *pabAB* and an adjacent downstream segment of the *S. venezuelae* chromosome (Fig. 3). Phages eluted from 10 hybridizing plaques were purified to remove non-hybridizing background and their DNA was isolated. Agarose gel electrophoresis of samples digested with *Bam*HI, *Xho*I and *Pst*I was examined for fragment patterns that established the homogeneity of the plaque. The gels were blotted on nylon membranes and probed to ensure that the DNA from purified phages included a region hybridizing to the labelled *Pst*I-*Xho*I fragment. Comparing restriction fragments from the DNA of phage containing *pabAB* identified a representative phage (PB1) with a truncated end-fragment. The fragment was labelled and used as a probe for the next round of plaque hybridizations. Contiguous *Pst*I fragments from phage PB1 extended cloned chromosomal DNA into the region beyond the 3' end of *pabAB*. On the upstream side, the 2.4 kb *Bam*HI-*Pst*I fragment from pDQ116 was used to probe contiguous *Bam*HI fragments of phage PB1 DNA extending cloned chromosomal DNA beyond the 5' end of *pabAB* (see Fig. 3). Through successive digestions of phage PB1 DNA and subsequent walking steps, the cloned region of the *S. venezuelae* chromosome was increased by 15–20 kb on each side of *pabAB*.

Construction of a bifunctional vector for conjugal DNA transfer. The 5.8 kb *Streptomyces*-*E. coli* vector pHJL400 (Larson & Hershberger, 1986) was linearized at its multiple cloning site with *Pst*I and ligated to a 0.76 kb *Pst*I fragment

containing *oriT* excised from pOJ446 (Biermann *et al.*, 1992). The ligation mixture was used to transform *E. coli* DH5α and the *Streptomyces*-*E. coli* bifunctional vector pJV326 containing *oriT* was isolated.

Conjugal transfer of plasmid DNA from *E. coli* to *S. venezuelae*. Competent cells of *E. coli* ET12567(pUZ8002) on LB agar supplemented with Cm (25 µg ml⁻¹) and kanamycin (Km; 50 µg ml⁻¹) were transformed with the plasmid of interest (e.g. pJV354). To transfer the plasmid conjugally from the *E. coli* transformant to *S. venezuelae* ISP5230, the two organisms were grown together using culture conditions and protocols based on those described for other streptomycete recipients (Flett *et al.*, 1997; Mazodier *et al.*, 1989). Trans-conjugant *S. venezuelae* colonies with double-crossovers were selected on MS agar containing nalidixic acid (25 µg ml⁻¹) and Am (50 µg ml⁻¹), and were patched on MYM agar containing these antibiotics. Spores appeared after 3–4 d at 30 °C and were patched in duplicate on MYM agar containing either Am (50 µg ml⁻¹) or Am and Ts (25 µg ml⁻¹ each).

Construction of *S. venezuelae* deletion mutant VS1050. The mutant was constructed by recloning the 7.5 kb *Bam*HI insert from pJV168 in the bifunctional vector pHJL400, giving pJV354. Digestion of pJV354 with *Nco*I removed an internal 3.4 kb segment and the residual *Nco*I ends were ligated to a 1.5 kb *Nco*I cassette containing the Am^R gene from *E. coli* (Rao *et al.*, 1983). The resultant plasmid, pJV355, contained a 5.6 kb *Bam*HI insert in which the Am^R gene was flanked on the

side near ORF1 by 1.5 kb and on the other side by 2.5 kb of chromosomal DNA. Recloning the *Bam*HI insert from pJV355 in the conjugal vector pJV326 and transfer of the vector into *S. venezuelae* yielded the deletion mutant VS1050, in which homologous recombination had replaced native chromosomal DNA with the vector insert containing the 3.4 kb deletion. The predicted structures of plasmids and transconjugants were supported by Southern hybridizations with the pJV354 insert and the *Am*^R gene as ³²P-labelled probes.

Gene disruptions. In general, the gene to be disrupted was cloned in an *E. coli* vector and inactivated by insertion of the *Am*^R gene from pUC120 (Paradkar & Jensen, 1995) or pJV225 (Chang, 1999). The insertionally inactivated gene was then excised from the vector and recloned in the multicloning site of the conjugal vector pJV326. The recombinant conjugal vector was passaged through *E. coli* ET12567(pUZ8002) to evade streptomycete restriction systems during intergeneric transfer. To disrupt *cmlC*, a 1.7 kb *Kpn*I–*Bam*HI fragment of *S. venezuelae* DNA containing the gene was cloned in pUC18 to give pJV351 and disrupted by insertion into its blunt-ended *Not*I site of a 1.5 kb *Eco*RV fragment carrying the *Am*^R gene from pJV225 (Chang, 1999). From this plasmid (pJV352), a 3.2 kb *Eco*RI–*Bam*HI fragment containing the disrupted *cmlC* was recloned in pJV326 to give pJV353 for conjugal transfer to *S. venezuelae* ISP5230. To disrupt *cmlP*, a 3.6 kb *Pst*I–*Bam*HI segment was excised from the 7.5 kb *Bam*HI insert in pJV168 and recloned in pBluescript II SK+. The resultant plasmid was linearized at its *Nco*I site and ligated with the 1.5 kb *Nco*I fragment containing the *Am*^R gene from pUC120 (Paradkar & Jensen, 1995) to give pJV375. Digestion of pJV375 with *Eco*RI and *Bam*HI generated a 5.1 kb fragment containing the disrupted *cmlP* sequence, which was ligated into the multicloning site of pJV326 to give pJV368. Transferring pJV368 from its *E. coli* host into *S. venezuelae* ISP5230 yielded the disrupted *S. venezuelae* strain VS1052. To disrupt the putative gene *cmlH*, a 3.2 kb *Bgl*II–*Nco*I segment of DNA containing ORF7 was excised from the 7.5 kb *Bam*HI fragment of *S. venezuelae* DNA in pJV168, blunt-ended and subcloned at *Sma*I in the multicloning site of pBluescript II SK+. The plasmid obtained (pJV369) was linearized at its *Nru*I site and ligated with the 1.5 kb *Am*^R gene excised with *Eco*RV from pJV225 (Chang, 1999) to give pJV370. Digestion of pJV370 with *Eco*RI and *Xba*I recovered the 4.7 kb insert containing *cmlH* disrupted by the *Am*^R gene; ligation of this fragment into the multicloning site of pJV326 gave pJV371. The pJV371 construct was transferred from its *E. coli* host to *S. venezuelae* ISP5230, generating the transconjugant VS1051. To disrupt *cmlJ*, the ORF9-containing linear plasmid that remained after deletion of a 4.6 kb *Mlu*I–*Sal*I fragment from pJV168 was blunt-ended and recircularized to give pJV501. The 1.6 kb *Mlu*I–*Bam*HI segment containing ORF9 was retrieved from pJV501 in an *Xba*I–*Kpn*I fragment and inserted into the multicloning site of pUC18 to give pJV502. The ORF9 sequence in pJV502 was disrupted at its *Eco*RV site to give pJV503 by introducing the *Am*^R gene from pJV225. Insertionally inactivated *cmlJ* was excised from the multicloning site of pJV503 as a 3.1 kb *Eco*RI–*Xba*I fragment. Ligation of this fragment into pJV326 gave pJV504 for conjugal transfer to *S. venezuelae* ISP5230. Selection with *Am* yielded the *Am*^R Ts^S transconjugant strain VS1055.

DNA sequencing and sequence analysis. DNA fragments were subcloned in both orientations in pBluescript II SK(+). Overlapping deletions were generated with an *Exo*III/S1 nuclease deletion kit (MBI Fermentas) and the religated phagemid DNA was used to transform *E. coli* DH5 α (BRL). Plasmid DNA from transformants was screened by agarose gel

electrophoresis for the desired size and sequenced (ABI 373 automated DNA sequencer). ORFs were detected with both Frameplot 2.3 (Ishikawa & Hotta, 1999) and the CODON-PREFERENCE program (version 8.1) developed by the Genetics Computer Group (GCG), University of Wisconsin (Devereux *et al.*, 1984). Frameplot 2.3 was accessed electronically (www.nih.go.jp/~jun/cgi-bin/frameplot.pl) to plot the mean G + C content of each reading frame and to calculate the G + C content at specific locations. The CODON-PREFERENCE program was used with the streptomycete codon table of Wright & Bibb (1992). The BLAST programs of Altschul *et al.* (1997) were used to search GenBank for proteins with sequence similarity to deduced ORF products. Multiple sequence alignments were generated with the PILEUP program (GCG) and the internet-based CLUSTAL W program (www.clustalw.genome.ad.jp/sit-bin/nph-clustalw).

Estimation of aromatic amines. Filtered supernatants (5 ml) of *S. venezuelae* cultures grown on a shaker (220 r.p.m.) for 7 d in GI medium at 30 °C were analysed for aromatic amino and nitro compounds by the procedure of Levine & Fischbach (1951). The 5 ml sample was applied to a 10 ml column (1.2 × 12.5 cm) of Dowex-50 × 8 (H+; 200 mesh particles) and the resin was washed with 20 ml water. Amines retained on the column were eluted with 4 M ammonium hydroxide and collected in 1 ml fractions; samples (50 μ l) of each absorbed on filter paper for evaporation were sprayed with 0.2% (w/v) ninhydrin in *n*-butanol/acetic acid (99:1, v/v) and heated at 80 °C. Fractions testing positively for amino acids were pooled, evaporated and redissolved in 6.5% aqueous methanol for HPLC. Analyses by HPLC were performed with a 4.6 × 100 mm C₁₈ reverse phase silica gel column (CSC-Sil 80A/ODSZ; 5 μ m particles) equilibrated with 0.1 M sodium acetate buffer, pH 4.75. After sample injection (20 μ l) the eluant gradient was applied (buffer to 50% methanol/buffer over 4 min, increasing to 100% methanol over the next 2 min). Aromatic amino acids in the eluate were detected at 260 nm. The retention times of PAPA and PAPS were 2.35 and 1.69 min, respectively. The column was re-equilibrated with a gradient from methanol to 100% buffer over 1 min, followed by buffer alone for 6 min.

Measurement of Cm production. Strains of *S. venezuelae* ISP5230 were bioassayed against *Micrococcus luteus* as described by Aidoo *et al.* (1990). Spores from single colonies on MYM agar were transferred in triplicate to give five equally spaced patches on MYM agar in 9 cm Petri plates. Spores from the wild-type and a Cm-non-producing mutant were patched as positive and negative controls. After 36–48 h at 27 °C, a thin layer of GNY soft agar (Malik & Vining, 1970) seeded with *Micrococcus luteus* was poured between the patches and after a further 18 h at 27 °C the mean width of each inhibition zone was measured. To determine Cm titres more precisely, shaken cultures were grown in 250 ml Erlenmeyer flasks containing 25 ml glucose/isoleucine medium (Doull *et al.*, 1985). Each culture received a 2% (v/v) vegetative inoculum prepared by incubating *S. venezuelae* spores at 30 °C on a shaker (220 r.p.m.) for 24 h in 10 ml GNY medium. Production cultures were sampled aseptically 2, 4 and 6 d after inoculation; the product extracted into ethyl acetate from 5 ml filtered broth was evaporated, taken up in 0.2 ml methanol/water (3:1, v/v) and analysed (20 μ l injection; 4.6 × 100 mm C₁₈ reverse-phase silica-gel column) by HPLC with stepped linear gradients of methanol/water: 0–25 (v/v)% for 1 min, 25–50% for 5 min, 50–100% for 1 min, 100% methanol for 1 min and then a gradient returning to 100% water over 1 min. The Cm titre was estimated by comparing the area of the sharp peak eluted at 6.05 min;

(detector at 273 nm) with areas given by Cm calibration samples (Brown *et al.*, 1996).

RESULTS

Cm biosynthesis genes downstream of *pabAB*

The region of the *S. venezuelae* chromosome adjacent to the 3' end of *pabAB* was present in a 1.7 kb *Pst*I-*Xho*I segment of DNA cloned in pDQ116 (Brown *et al.*, 1996). By subcloning this segment in pBluescript II (SK+) and deleting internal fragments, the DNA immediately downstream of *pabAB* was sequenced from flanking vector primers. Examining the sequence with the Frameplot and CODONPREFERENCE programs detected ORF3 (*cmlC*), which overlapped the end of *pabAB* by 1 bp and contained 987 bp. Its ATG start codon was separated by 12 bp from the putative RBS AGTGG ($\Delta G -5.0$ kcal mol⁻¹; Tinoco *et al.*, 1973). A partial ORF4 (*cmlD*) was also present. To obtain the complete sequence of *cmlD* and genes further downstream, the 5.4 kb *Pst*I fragment adjacent to the 3' end of *pabAB* (see Fig. 3) was recovered from phage PB1 DNA, subcloned in pBluescript II SK+ to give pDQ502 and sequenced. Frameplot and CODONPREFERENCE analysis of the pDQ502 insert detected three potential ORFs (4–6). That for *cmlD* began at a GTG 75 bp downstream of the ORF3 stop codon and was preceded 5 bp upstream by a putative RBS (GGACC; $\Delta G -7.3$ kcal mol⁻¹). An in-frame TGA was located only 240 bp downstream of the GTG start codon and was followed 84 bp later by an ATG for the start of ORF5 (*cmlE*). A plausible RBS (GGAGG; $\Delta G -16.6$ kcal mol⁻¹) was present 10 nt before the ORF5 start codon and the first in-frame stop codon (TGA) in the sequence defined a 1179 bp gene encoding 392 aa. The ORF6 (*cmlF*) sequence was transcribed in the reverse direction from ORFs 1–5 (see Fig. 3); it spanned 1212 bp and its ATG start codon was preceded with a 9 bp separation by a putative RBS (CGAGG; $\Delta G -13.8$ kcal mol⁻¹). The ORF5 and ORF6 stop codons were separated from the each other by 100 bp DNA. The regions upstream of ORF3, ORF4 and ORF5 did not contain sequences recognizable as -10 or -35 consensus hexamers of *E. coli* *s*⁷⁰-type promoters.

Searching GenBank with BLASTP for proteins matching the deduced amino acid sequences of ORFs 3–6 showed a close resemblance between the ORF3 (*cmlC*) product and PapC from *Streptomyces pristinae-spiralis* (48% identical and 59% similar amino acids in a 319 aa overlap). PapC is a monofunctional prephenate dehydrogenase involved in biosynthesis of the *p*-dimethylaminophenylalanine component of pristinamycin I (Blanc *et al.*, 1997). Consistent with a relationship to prephenate dehydrogenase, the deduced amino acid sequence of ORF3 also resembled (24% identical and 44% similar aa in a 251 aa overlap) the *tyrA* product of *Methanococcus jannaschii* (Bult *et al.*, 1996). The translated product of ORF4 (*cmlD*) contained only 79 aa (see Fig. 3), but showed a high overall resemblance (61% identical amino acids) to PapB, a monofunctional

chorismate mutase used by *S. pristinae-spiralis* to biosynthesize the *p*-dimethylaminophenylalanine component of pristinamycin I. The deduced amino acid sequence of ORF5 (*cmlE*) was markedly similar (41–44% identity) to class II deoxy-*arabino*-heptulosonate-7-phosphate (DAHP) synthases encoded by genes within clusters for ansamycin biosynthesis in *Streptomyces collinus* Tu1892 (Chen *et al.*, 1999) and for rifamycin biosynthesis in *Amycolatopsis mediterranei* (August *et al.*, 1998). Other DAHP synthase genes associated with the biosynthesis of shikimate-derived secondary metabolites have been reported in *Pseudomonas aeruginosa*, where PhzC (43% identity to CmlE) contributes to pyocyanine biosynthesis (Mavrodi *et al.*, 1998) and in *Streptomyces hygroscopicus* ATCC 29253, where a DAHP synthase gene accompanies a type-I polyketide synthase gene cluster (Ruan *et al.*, 1997). The deduced amino acid sequence of ORF6 (*cmlF*) was strikingly similar to proteins encoded by the Cm efflux genes *cmrA* from *Rhodococcus erythropolis* (Nagy *et al.*, 1997), *cmlR* from *Rhodococcus fascians* (Desomer *et al.*, 1992) and *cmlG* from *S. lividans* (Dittrich *et al.*, 1991). It also resembled the product of *cmlV*, which is located in a region of the *S. venezuelae* ISP5230 genome distant from *pabAB*, but containing genes expressing Cm resistance (Mosher *et al.*, 1995). Topological analysis of the deduced *cmlF* product using the TMPRED program of Hofmann & Stoffel (1993) (http://www.ch.embnet.org/software/TMPRED_form.html) showed 12–13 membrane-spanning domains like those in other Cm efflux proteins. The similarity suggested a role in exporting Cm that might be associated with resistance of the producing species to its toxic metabolite.

In separate experiments, *cmlC* and *cmlF* were disrupted by inserting an *E. coli* Am^R gene (Rao *et al.*, 1983) into the cloned sequences. The disrupted *cmlC*, recloned in the conjugal vector pJV326, was introduced into *S. venezuelae* ISP5230 to generate the transconjugant VS1053. This insertionally inactivated strain grew prototrophically on minimal agar, but produced only 4% of the wild-type Cm titre. Cultures of strain VS1054, containing the disrupted *cmlF* transconjugant, also grew prototrophically, but unlike VS1053 produced 70–108% of the wild-type Cm titre.

Cm biosynthesis genes upstream of *pabAB*

The *S. venezuelae* chromosomal DNA cloned in pDQ116 included *pabAB* and upstream DNA containing an incomplete ORF1 that appeared to be co-transcribed with *pabAB* (Brown *et al.*, 1996; see Fig. 3). The ORF1 sequence was completed by isolating a *Bam*HI fragment of phage PB1 DNA that hybridized with the *Pst*I-*Bam*HI probe from pDQ116. The fragment was subcloned in pBluescript II (SK+) and sequenced.

(i) *cmlA*. Analysis of codon usage and third-letter nucleotide bias (95.9 mol% G+C) in the completed ORF1 sequence indicated that the gene (*cmlA*) began with an ATG 196 bp upstream of the *Bam*HI site at nt 7281 and contained 1599 bp of DNA encoding 532 aa

Core Sequences																	
1		2		3		4		5		6		7		8		9	
TyrII (2570)	MSFREL-116-LAYIIYTS	GTTGKPKG-50-FDAS-89-YGPTE-49-GELCISGLSLARGYW-37-YRTGD-17-LGRIDHQVKIRGHRVEL-91-KIDR-53-GGDS															
Grs (65)	LTYPHEL-119-LAYVIYTS	GTTGNPKG-50-FDAS-89-YGPTE-49-GELCIGEGGLARGYW-37-YKTGD-17-LGRIDNQVKIRGHRVEL-91-KIDR-53-GGDS															
Saf (1623)	LTYAEL-119-LAYLIYTS	GSGTGTPKA-50-FDPS-95-YGPTE-49-GELFVGGVGLARGYH-39-YRTGD-17-LGRVDEQVKVSGYRVEL-94-KVNR-54-GGTS															
CDA (6321)	VSYAEL-139-LAYVMYTS	GSGTGVPKG-48-FDVS-95-YGPTE-49-GELFISGSLARGYL-38-YRTGD-17-LARADDQVKIRGFRVEL-93-KLDR-53-GGDS															
CmlP (27)	WTYEEL-121-VAYSIFTSG	SGTGDPKL-50-FDAS-85-YGPTE-49-GELVIVGDGVALGYA-34-YATGD-17-LGRIDEQIKRLGHRVNL-86-KVDR-54-GGSS															
Consensus																	
LT/sYxEL	LAYxxYTS	GS/TTGXPKG	FDxS	YGPTE	GELxIxGxGV/LARGYL	YR/KTGDL	GRxDxQVKIRGxRIEL	NGKV/L	GGD/HS								

Fig. 4. Comparison between core sequences in the putative adenylation and thiolation domains of CmlP, and motifs associated with adenylation and thiolation in non-ribosomal peptide synthetases. Core sequences 1–5, 7 and 8 are associated with ATP binding and motif 6 is associated with ATPase activity in the adenylation domain. Core sequence 9 contains the serine of the thiolation domain to which 4'-phosphopantetheine attaches. Bold letters identify invariant amino acids in the five sequences compared. The number in parentheses following the name of the NRPS indicates the position of the first core sequence amino acid in the overall sequence. The numbers interpolated between core sequences give the number of amino acids separating adjacent motifs. TyrII, tyrocidin synthetase II of *Brevibacillus brevis*; Grs, gramicidin S synthetase I of *Brevibacillus brevis* (Hori *et al.*, 1989); Saf, saframycin Mx1 synthetase A of *Myxococcus xanthus* (Pospiech *et al.*, 1996); CDA, calcium-dependent antibiotic synthetase I of *S. coelicolor* A3(2) (Chong *et al.*, 1998); CmlP, product of ORF8 in *S. venezuelae*. The consensus core sequence based on GenBank NRPS sequences is shown below the alignments.

(see Fig. 3). Its ATG start codon was preceded with a 14 bp separation, by a putative RBS (GGAG; $\Delta G - 15.2 \text{ kcal mol}^{-1}$). Comparison of its deduced amino acid sequence with proteins in the GenBank and *S. coelicolor* genome databases using BLASTP and TBLASTN (<http://www./Projects/S.coelicolor>) did not detect any close homologues, although the central portion of *cmlA* showed weak resemblance (30% identity, 50% similarity in a 94 aa overlap) to a protein (RomA) from *Enterobacter cloacae* regulating outer-membrane synthesis (Komatsu *et al.*, 1990). A search of the deduced amino acid sequence for possible membrane-binding sites by assessing topology with the TMPRED program of Hofmann & Stoffel (1993) predicted a strong *trans*-membrane helix from aa 425 to 447. Its full extent could not be determined in pDQ116 because the 5' end of *cmlA* was truncated. To investigate the region beyond the 5' end by chromosome walking, BamHI-digested phage PB1 DNA fragments that hybridized with the 2.4 kb BamHI-PstI fragment of pDQ116 were cloned in pBluescript II SK(+). Sequencing the fragments identified a plasmid (pJV168) that contained a 7.5 kb BamHI insert of cloned DNA extending the 5' end of *cmlA*. The sequence data did not implicate *cmlA* in Cm biosynthesis, but further examination of the plasmid (see below) indicated that *cml* genes were present elsewhere in the cloned DNA fragment carried by pJV168.

(ii) Evidence for *cml* genes upstream of *cmlA*. To test for *cml* genes in pJV168, the plasmid insert was recloned in the conjugal plasmids pJV357 and pJV358, then transferred into a series of previously characterized blocked mutants of *S. venezuelae* (Doull *et al.*, 1985). Plasmids pJV357 and pJV358 had been constructed in the *oriT*-containing vector pKC1218 (Bierman *et al.*, 1992) and carried the pJV168 insert in opposite orientations. The two recombinant plasmids were passaged through *E.*

coli ET12567(pUZ8002) before conjugal transfer to the *S. venezuelae* mutants *cml*-1, -2, -3, -4, -6, -8, -10, -11 and -12, each of which had been obtained by mutagenesis with nitrosoguanidine and was blocked at a genetically mapped step in Cm production (Doull *et al.*, 1986). Screening transconjugant cultures for restoration of antibiotic biosynthesis showed that *cml*-4 now produced $48 \mu\text{g Cm ml}^{-1}$, a concentration close to that in the wild-type; none of the other mutations was complemented. Cultures containing pJV358 and pJV357 gave similar Cm titres, whereas cultures with pJV357 subcloned to delete various portions of the 7.5 kb insert failed to produce Cm. The results implicated the 7.5 kb segment of *S. venezuelae* DNA in Cm biosynthesis. Supporting these results, no Cm was produced by the deletion mutant VS 1050 where an internal 3.4 kb NcoI fragment had been excised from pJV168 (see Fig. 3) and the construct, recloned in pJV326, had been transferred into *S. venezuelae* ISP5230 by intergeneric conjugation and allele exchange.

(iii) *cmlP* and *cmlH*. A 4.87 kb BamHI-NcoI segment of the 7.5 kb DNA insert in pJV168 contained the region of the *S. venezuelae* chromosome near the 5' end of *cmlA* (see Fig. 3). This segment was excised from pJV168 and sequenced (GenBank accession no. AF262220). Frame (Ishikawa & Hotta, 1999) and codon usage (Devereux *et al.*, 1984) analyses of the nucleotide sequence detected two similarly oriented ORFs (see Fig. 3). The one nearest to *cmlA* (ORF8, *cmlP*) was preceded by a plausible RBS (GGAG; $\Delta G - 15.2 \text{ kcal mol}^{-1}$) and started with a GTG at nt 4185–4187. It ended with a TGA at nt 7078–7080 and the 96.2 mol% mean G + C bias in the third codon position was in the range predicted for streptomycete genes. The 2907 bp nucleotide sequence of *cmlP* encoded 968 aa which, in a BLASTP search of GenBank, showed marked sequence similarity to typical non-ribosomal peptide synthetases

CmlP	(607)	PGAADGDREVL ⁻⁻⁻ LTGASGFTGGHVLDRLLAAGR
SafA3	(2215)	LANYLAPSSVLLTGATGYLGAFLLLEQLLKRRP
Lys2	(964)	SAEGKTTINVFVTGVTGFLGSYILAGLLDRSP
		*:***: * :*

Fig. 5. Alignment of regions containing NAD(P)H-binding sites in the amino acid sequences of: CmlP, ORF8 from *S. venezuelae*; SafA3, NRPS from *Myxococcus xanthus* (Pospiech *et al.*, 1996); Lys2, α -amino adipate reductase of *Saccharomyces cerevisiae* (Morris & Jinks-Robertson, 1991). The position in the overall sequence of the first amino acid in each line is given in parentheses. The binding site is overlined; invariant and similar amino acids are marked beneath the alignment with asterisks and colons, respectively.

(NRPSs), including both gramicidin S synthetase I and tyrocidine synthetase II of *Brevibacillus brevis* (Hori *et al.*, 1989; 32% overall amino acid identity), saframycin of *Myxococcus xanthus* (Pospiech *et al.*, 1996) and the calcium-dependent antibiotic (CDA) of *Streptomyces coelicolor* A3(2) (Chong *et al.*, 1998). A CLUSTAL W alignment of the deduced amino acid sequences of *cmlP* and the peptide synthetases revealed motifs associated with predicted functional domains (Fig. 4; Konz & Marahiel, 1999). Notable in *cmlP* were highly conserved ATP-binding and ATPase-binding sequences and a phosphopantetheine-binding site. An N-terminal adenylation domain and a central thiolation domain were apparent, whereas there was no indication of the condensation domain that links amino acids of sequential modules and normally completes the three-domain structure of a peptide synthetase (Marahiel *et al.*, 1997). The C-terminal region of *cmlP* contained an NADH-binding site found in relatively few peptide synthetases (Fig. 5). Such motifs are postulated (Konz & Marahiel, 1999) to be integral components of reductase domains that terminate peptide-chain elongation. Tailoring domains for epimerization or methylation are notably lacking in *cmlP* and thus the principal features that define the function of this monomodular NRPS are its amino acid activation (adenylation), thiolation and reductase domains (see Fig. 1).

To disrupt *cmlP*, the DNA insert in pJV168 was subcloned in pBluescript II SK+ and the Am^R gene from pUC120 (Paradkar & Jensen, 1995) was inserted at an *Nco*I site to give pJV375. Ligation of a 5.1 kb fragment containing the disrupted *cmlP* sequence into the conjugal vector pJV326 gave pJV368, which after transfer from its *E. coli* host into *S. venezuelae* ISP5230, integrated into the chromosome. Allele exchange replaced the native *cmlP* with the disrupted copy, generating the *S. venezuelae* mutant strain VS1052. Cultures of VS1052 accumulated neither Cm nor PAPA under growth conditions that allowed wild-type *S. venezuelae* ISP5230 to accumulate 68–74 μg Cm ml^{-1} and 12–15 μg PAPA ml^{-1} . By comparison, similarly grown cultures of *S. venezuelae* mutant *cml-4*, postulated to carry a mutation blocking conversion of PAPA to Cm, accumulated 48 μg amino acid ml^{-1} .

The second ORF (ORF7, *cmlH*) in the 4.87 kb *Bam*HI–*Nco*I segment of chromosomal DNA subcloned from the 7.5 kb insert in pJV168 (see Fig. 3) was presumed to begin with an ATG at nt 3007–3009, although no strong RBS candidate was identified. The first in-frame TGA was at nt 4185–4187, giving an ORF overlapping the start of ORF8 by 13 bp and containing 1179 bp of DNA encoding 392 aa. A BLASTX search of GenBank with the ORF7 sequence did not detect any matching protein sequences. To disrupt the putative *cmlH*, ORF7 was excised from pJV168 and subcloned in pBluescript II SK+. The subcloned DNA was disrupted by introducing the Am^R gene from pJV225 (Chang, 1999). The 4.7 kb disrupted copy of *cmlH* was recloned in the conjugal vector pJV326 and transferred conjugally from its *E. coli* host to *S. venezuelae* ISP5230. Vector integration and allele exchange between the native and disrupted alleles generated the transconjugant VS1051, cultures of which accumulated 13.5 μg PAPA ml^{-1} , but no Cm. Cultures of wild-type *S. venezuelae* ISP5230 grown under the same conditions accumulated 15 μg PAPA ml^{-1} and produced 45–55 μg Cm ml^{-1} . The results imply that the *cmlH* product has a role in the conversion of PAPA to Cm and indicate that specifically preventing its function does not cause abnormal accumulation of the intermediate.

(iv) *cmlI* and *cmlJ*. The 2.63 kb *Nco*I–*Bam*HI segment of *S. venezuelae* DNA adjoining the 4.87 kb *Bam*HI–*Nco*I DNA segment of the pJV168 insert, and sharing its *Nco*I site, was subcloned from phage PB1 into pBluescript II SK(+), giving pJV372. Analysis of the insert sequence revealed previously undetected ORFs (see Fig. 3). Nearest to ORF7 and similarly oriented, ORF10 (*cmlI*) began with an ATG at nt 1994, which was preceded 11 bp upstream by a plausible RBS (GGAAG; $\Delta G = -7.2$ kcal mol^{-1}). The downstream ORF10 sequence extended into the adjacent 4.87 kb *Bam*HI–*Nco*I segment of pJV168 and terminated with a TGA stop codon at nt 3010–3012. The 1017 bp coding sequence showed a 91.7 mol% G+C bias in the third codon position, typical of streptomycete genes (Wright & Bibb, 1992). A gapped BLASTX search of GenBank for sequences resembling that of the 338 aa in the ORF10 product found similarity (29% identity in a 114 aa overlap) to the product of the chloroplast gene *mpbX* of *Marchantia polymorpha*, which belongs in the superfamily containing ATP-binding cassettes for ABC membrane transporters. The coding sequence for ORF9 (*cmlJ*) in the pJV372 insert was oppositely oriented from that of ORF10 and its GTG start codon (1365–1367) was 627 bp distant from the ORF 10 start codon. The 693 bp ORF9 sequence (84.8 mol% G+C bias in the third codon position) terminated with a TGA at nt 675. Searching GenBank with BLASTP for proteins with sequences similar to that of the translated 229 aa ORF9 sequence detected matches in the family of short-chain oxidoreductases that use NAD(P)H as a cofactor. A CLUSTAL W alignment of the deduced amino acid sequences of ORF9 with ketoreductases and dehydrogenases of bacterial short-to-medium-chain polyhydroxyalkanoate

synthases and short-chain fatty acid synthases (Morita & Okuyama, 1999) demonstrated a conserved NAD(P)H binding site near the N terminus of each of these sequences. Also conserved in the central region of each sequence was an active site representing the protein family signature.

Frame analyses of the pJV168 DNA sequence at the end furthest from *pabAB* detected a partial ORF11 (293 bp), with the same transcriptional orientation as ORF9. The ORF began with an ATG at nt 293–291 and extended beyond the cloned sequence. Using the initial 96 translated amino acids as a query sequence in a BLASTP search of GenBank gave matches with actinomycin synthetase I from *Streptomyces chrysomallus* (Keller & Schlumbohm, 1992) and with 4-coumarate coenzyme A ligase from plants. Extending the cloned *S. venezuelae* chromosomal DNA beyond the 3' end of the pJV168 insert yielded the complete sequence of ORF11 (data not shown). Analysis of this region and a BLAST search of GenBank for proteins with matching sequences indicated that the product of the putative gene (*cmlK*) closely resembled pristinamycin I synthetase of *S. pristinae-spiralis* (de Crecy-Lagard *et al.*, 1997) and confirmed its similarity to actinomycin synthetase I and a variety of plant 4-coumarate coenzyme A ligases.

DISCUSSION

Cloning DNA fragments recovered from chromosome walking in regions flanking the ADC synthase gene *pabAB* has revealed a cluster of genes involved in Cm biosynthesis. Molecular genetic analysis of the cloned DNA supports earlier biochemical evidence that Cm biosynthesis begins in a secondary metabolic branch of the shikimate pathway generating PAPA and provides evidence for involvement of a non-ribosomal peptide synthetase in the activation and protection of aromatic amino acid intermediates derived from PAPA. These new insights into the biosynthetic pathway are consistent with evidence from genetic mapping (Vats *et al.*, 1987) that Cm biosynthesis genes in *S. venezuelae* ISP5230 are clustered in a discrete region of the chromosome. The first specific reaction in Cm biosynthesis is catalysed by an ADC synthase, encoded by the distinctive secondary metabolic gene *pabAB* (*cmlB*) (Brown *et al.*, 1996). The ADC formed in the reaction is channeled towards the formation of aminodeoxy-prephenic acid by a monofunctional ADC mutase (see Fig. 2), the gene for which (*cmlD*; see Fig. 1) has now been located in the *S. venezuelae* ISP5230 chromosome downstream of *pabAB*. Aminodeoxy-prephenic acid may be converted to PAPA by either of two routes. The first would generate *p*-aminophenylpyruvic acid by the action of an enzyme analogous to the prephenate dehydrogenase that catalyses a similar primary metabolic reaction in tyrosine biosynthesis (Pittard, 1996; see Fig. 2). In *S. venezuelae* the enzyme responsible for the conversion is likely to be the monofunctional cyclohexadienyl dehydrogenase encoded by *cmlC*. The CmlC product could subsequently be converted to PAPA by a

non-specific aromatic aminotransferase found in *S. venezuelae* (Jones *et al.*, 1978; see Fig. 1). In the alternative route, 4-amino-4-deoxy-prephenic acid would be acted upon initially by the aminotransferase to form 4-amino-4-deoxyarogenic acid. This intermediate would be converted to PAPA by the monofunctional cyclohexadienyl dehydrogenase encoded by *cmlC*. Similar reactions are probably responsible for synthesis of PAPA in *S. pristinae-spiralis*, where this amino acid is a precursor of pristinamycin I and related peptidolactones (Blanc *et al.*, 1997).

The presence of *cmlE* in the Cm biosynthesis cluster was unexpected. The DAHP synthase encoded by this gene condenses phosphoenolpyruvate and erythrose 4-phosphate, initiating the shikimate pathway; the reaction precedes formation of chorismic acid and the subsequent chorismate branching reaction that begins the specific route to Cm (Vining & Westlake, 1984; see Fig. 2). The presence of *cmlE* might be accounted for by a role in regulation of the shikimate pathway. In many bacteria, flow of precursors into the pathway is determined by feedback control of isozymic class I DAHP synthases individually sensitive to aromatic amino acids (Pittard, 1996). However, the class II enzyme in *S. venezuelae* would be expected to show the characteristic properties of plant DAHP synthases, which are activated by tryptophan and not inhibited by aromatic amino acids (Dyer *et al.*, 1990). In *S. venezuelae*, formation and excretion of Cm normally begins when the growth rate declines; the presence of a DAHP synthase insensitive to protein digestion products could support the secondary metabolic shikimate pathway activity producing an antibiotic that shields the producer from competitors.

Substantial and similar decreases in Cm production were caused by disrupting the gene for either ADC synthase (*pabAB*) or cyclohexenyl dehydrogenase (*cmlC*). Since the products of each of these genes participate in the biosynthesis of PAPA, the drop in Cm production can be attributed to interruption of the supply of shikimate pathway precursors. In contrast to the severe effect of inactivating *pabAB* and *cmlC*, the growth of cultures or their ability to produce Cm was only marginally affected by disrupting *cmlF*, which is postulated to be a Cm efflux gene releasing the antibiotic into the environment and protecting intracellular protein biosynthesis from inhibition. The apparent insensitivity to loss of *cmlF* function may reflect the presence in *S. venezuelae* of other genes mediating Cm efflux. Mosher *et al.* (1995) described such a gene in a cloned DNA fragment that also contained a Cm 3'-O-phosphotransferase gene conferring Cm resistance by inactivating the antibiotic. Chromosome walking experiments (K. A. Aidoo & L. C. Vining, unpublished) placed this separate set of Cm resistance genes at least 30 kb from *pabAB* and thus distinct from the auto-resistance genes in the Cm biosynthesis cluster.

Conversion of PAPA to PAPS is a key reaction in Cm biosynthesis; the presence in the *cml* cluster of a gene encoding this activity has been inferred from the

accumulation of PAPA in an *S. venezuelae* mutant (*cml-4*) blocked in Cm production after treatment with nitrosoguanidine (Doull *et al.*, 1985). Restoration of Cm production in the mutant by introducing the 7.5 kb fragment of *S. venezuelae* DNA cloned in pJV168 implies that the *cml-4* gene is located in this region of the chromosome. Among sites in the pJV168 insert where the *cml-4* function might be located, ORF7 was a potential candidate because Cm production was lost when the putative *cmlH* in ORF7 was disrupted. However, BLAST searches of GenBank and the *S. coelicolor* A3(2) genomic library (<http://www.Sanger.ac.uk/Projects/S.coelicolor/>) with the translated *cmlH* sequence failed to detect homologues from which the function of the gene product could be deduced. Another potential location of the DNA that restored Cm production in mutant *cml-4* was the truncated ORF11 (*cmlK*) detected at the 3' end of the chromosomal insert in pJV168. The deduced amino acid sequence of the partial ORF matched proteins (e.g. actinomycin synthetase I; Keller & Schlumbohm, 1992) that generate adenylated amino acids in the activating domains of enzyme systems. Among these systems may be a PAPA β -hydroxylase of the type recently characterized in *Streptomyces spheroides* by Chen & Walsh (2001). This enzyme system activates tyrosine by forming the adenylate and tethers the aminoacyl component by a thioester linkage to the active site; a cytochrome P450-type monooxygenase then hydroxylates the tethered component to form the β -hydroxy-tyrosyl derivative.

In contrast to the absence of matching proteins detected when GenBank was searched with the *cmlH* product in BLAST searches, numerous NRPSs were identified in similar searches with the *cmlP* product as the query sequence. The close similarity of conserved regions in the CmlP adenylation domain to the consensus sequences of adenylation domain motifs implicates an initial reaction activating an amino acid as its adenylate (see Fig. 1). We suggest that PAPS formed from PAPA in the preceding β -hydroxylation step is the amino acid activated by ATP and tethered to the adenylation domain. The reactive aminoacyl derivative is then transferred to the thiolation domain in CmlP, where it attaches to a peptidyl carrier protein (PCP) containing the conserved serine that forms a thioester with the phosphopantetheinyl group of coenzyme A. A noteworthy feature of the CmlP NRPS is the lack of condensation and thioesterase domains. Their absence implies that the reactive *p*-aminophenylseryl group is not transferred either to a second amino acid by forming a peptide bond or to a terminal substrate from which it is subsequently released by thioesterase activity. A conserved NAD(P)H binding site (Fig. 5) in CmlP suggests the presence of an NRPS reductase domain similar to that described by Pospiech *et al.* (1996) in SafA3, an NRPS involved with saframycin Mx1 biosynthesis in *Myxococcus xanthus*. Reductase domains recognized in this and other NRPSs are postulated to terminate peptide synthesis by releasing tethered

aminoacyl groups from their thioester linkage by forming linear aldehydes. (Konz & Marahiel, 1999) In the CmlP NRPS, the reductase domain may reduce the carrier-bound PAPS thioester to *p*-aminophenylserinal and release the product. Alternatively, reductase domain activity may be delayed until the thioester has undergone further modification. Close association of the activated and anchored PAPS thioester with short-lived reactants such as dichloroacetyl-coenzyme A, possibly generated *in situ* by halogenase-mediated reactions, with the products of intermediary metabolism, may be needed to efficiently introduce the dichloroacetyl substituent into Cm. Association of the aminoacyl thioester with this and other tailoring enzymes, such as a dichloroacetyl *p*-aminophenylserinal reductase and a dichloroacetyl-*p*-aminophenylserinol-4-amino oxidase (see Fig. 1), may avoid exposing the intermediates to extraneous catabolic reactions during completion of the α -N-dichloroacetyl-*p*-nitrophenylserinol structure of Cm.

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