Accumulation of the angucycline antibiotic rabelomycin after disruption of an oxygenase gene in the jadomycin B biosynthetic gene cluster of *Streptomyces venezuelae*

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DNA from a region downstream of and overlapping the polyketide synthase (PKS) gene cluster for jadomycin B biosynthesis in *Streptomyces venezuelae* was cloned and sequenced. Analysis of the nucleotide sequence located one complete ORF (ORF6), an incomplete one representing the 3' region of ORF4 in the PKS cluster, and a second incomplete one (ORF7). The deduced amino acid sequences for ORFs 6 and 7 resemble those of oxygenases. Since a plausible biosynthetic pathway for jadomycin B includes an angular polyketide intermediate that undergoes oxidative ring fission before condensation with an amino acid, we subcloned one of the presumptive oxygenase genes (ORF6) in a segregationally unstable shuttle vector (pHJL400) and disrupted it by inserting the gene for apramycin resistance. Transformation of *S. venezuelae* with the disruption vector and selection for apramycin resistance gave mutants blocked in jadomycin biosynthesis. Southern hybridization confirmed that gene replacement had occurred. Cultures of the mutants accumulated a metabolite identified by comparison with an authentic sample as rabelomycin, a non-nitrogenous polyketide-derived antibiotic originally isolated from *Streptomyces olivaceus*.

Keywords: *Streptomyces venezuelae*, angucycline, gene disruption, jadomycin B, polyketide antibiotic

INTRODUCTION

Streptomyces venezuelae ISP5230 produces a group of novel angucycline antibiotics when cultures grown under nutrient limitation in a medium containing poorly assimilated sources of carbon and nitrogen are exposed to additional stress, such as heat shock, phage infection or exposure to toxic concentrations of ethanol (Doull *et al.*, 1993, 1994). Jadomycin B, the main component produced during growth in an isoleucine-containing medium, has an isoleucine moiety fused into the cyclic structure and is glycosylated (Fig. 1; Ayer *et al.*, 1991). Under less severe stress conditions, *S. venezuelae* ISP5230 produces chloramphenicol (Doull *et al.*, 1985).

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Abbreviation: PKS, polyketide synthase.

The GenBank accession number for the nucleotide sequence reported in this paper is U28382.

Although the biosynthesis of jadomycin B has not been examined in detail, the inference from inspection of the chemical structure that it is derived from a polyketide intermediate was supported by specific incorporation into the structure of isotopically labelled acetate (S. Ayer,

Fig. 1. Molecular structure of jadomycin B

unpublished data). Cloning and sequence analysis of a fragment of *S. venezuelae* ISP5230 genomic DNA that hybridizes with *act* genes from *Streptomyces coelicolor* A3(2) established the presence of a polyketide synthase (PKS) gene cluster in *S. venezuelae*. Selective loss of jadomycin B production when a plasmid containing part of the cloned sequence integrated into the chromosome indicated that the PKS genes are associated with jadomycin biosynthesis (Han *et al.*, 1994).

In this paper, we present evidence that a region of the *S. venezuelae* ISP5230 chromosome immediately downstream of the PKS gene cluster encodes an oxygenase involved in opening ring B of an angucycline (Rohr & Thiericke, 1992) likely to be a biosynthetic intermediate. Subsequent insertion of an isoleucine molecule at this site would account for the formation of jadomycins.

METHODS

Bacterial strains, plasmids and culture conditions. Sources and characteristics of the bacteria and plasmids used are listed in Table 1. Cultures of *Streptomyces venezuelae* and *Escherichia coli* were grown as described previously (Han *et al.*, 1994). The conditions used for jadomycin B production were as described by Doull *et al.* (1994). For selective growth of *S. venezuelae* mutant strains, apramycin was added at concentrations of 50 µg ml⁻¹ to agar media and 10 µg ml⁻¹ to liquid media.

DNA manipulation and transformation. Plasmid DNA was isolated from *E. coli* by the alkaline lysis method of Kieser (1984). Genomic DNA was isolated as described by Hopwood *et al.* (1985). DNA ligase and restriction enzymes were used according to manufacturers' instructions. Competent cells of *E.*

coli were prepared and transformed by standard methods (Sambrook *et al.*, 1989); protoplasts of *S. venezuelae* were prepared and transformed by modifying the methods of Hopwood *et al.* (1985) as follows: MYME medium, containing 10.3% (w/v) sucrose, 1% (w/v) maltose, 0.5% (w/v) peptone, 0.3% (w/v) yeast extract, 0.3% (w/v) malt extract, 5 mM MgCl₂ and 1% (w/v) glycine, was inoculated with *S. venezuelae* ISP5230 spores and incubated for 48 h at 27 °C; protoplasts were formed in modified P buffer (7.32% (w/v), mannitol, 70 mM NaCl, 10 mM MgCl₂, 20 mM CaCl₂ and 25 mM TES buffer at pH 7.2); protoplast regeneration medium contained 7.32% (w/v) mannitol and 1% (w/v) maltose in place of sucrose and glucose, respectively; modified T buffer contained 25% (w/v) PEG 4000 (Sigma) in modified P buffer. Transformants were selected by overlaying the agar with soft nutrient agar containing thiostrepton to give a plate concentration of 25 µg ml⁻¹.

Gene disruption. The apramycin-resistance gene in pKC462a (Stanzak *et al.*, 1986) was excised as a 1.5 kb *Bam*HI-*Pst*I fragment and blunt-ended by treatment with the Klenow fragment of DNA polymerase I. pJV66 was digested with *Sfi*I to remove a 130 bp segment of the insert, and the ends of the residual vector fragment were also blunted. Ligation of the two blunt-ended products, transformation of *E. coli* TG1 with the ligation mixture and selection for apramycin resistance (Apr^R) yielded strains containing the disruption vector pJV67. This was used to transform *E. coli* ET12567 (MacNeil *et al.*, 1992); pJV67 reisolated from transformants of this DNA-methylase-deficient strain was used to transform *S. venezuelae* ISP5230. Approximately 200 colonies (µg plasmid DNA)⁻¹ were obtained by initial selection for thiostrepton resistance (Thio^R). Samples of genomic DNA extracted from 10 of these were fractionated by agarose gel electrophoresis and screened for hybridization to pHJL400. Colonies containing chromosomal DNA that hybridized to the probe were transferred to MYM agar

Table 1. Bacterial strains and plasmids used

Strain	Genotype/phenotype	Source/reference
<i>Streptomyces venezuelae</i>		
ISP5230	Wild-type	Doull <i>et al.</i> (1985)
VS654a and b	ISP5230 with integrated pJV67	This study
VS655a and b	ISP5230 with ORF6 disrupted by a DNA fragment conferring Apr ^R	This study
<i>Escherichia coli</i>		
TG1	<i>supE hsdΔ5 thiΔ(lac-proAB) F' (traD36 proAB⁺ lacI lacZΔM15)</i>	Carter <i>et al.</i> (1985)
ET12567	<i>dam dcm hsdM</i>	MacNeil <i>et al.</i> (1992)
Plasmids and cosmid		
pKC462a	Apr ^R <i>amp</i> ; bifunctional cosmid	Stanzak <i>et al.</i> (1986)
pHJL400	<i>tsr amp lacZ'</i> (bifunctional)	Larson & Hershberger (1986)
pJV66	pHJL400 with the 2.4 kb <i>Sac</i> I insert of pJV60	This study
pJV67	pJV66 with the 130 bp <i>Sfi</i> I segment replaced by a fragment conferring Apr ^R	This study
pBluescript II SK(+)	<i>amp lacZ'</i>	Stratagene
pJV85	pBluescript II SK(+) with a 1.5 kb <i>Bam</i> HI- <i>Pst</i> I insert conferring Apr ^R	J.-Y. He, this laboratory
pJV60/61	pBluescript II SK(+) with a 2.4 kb <i>Sac</i> I insert of <i>S. venezuelae</i> DNA; orientations in pJV60/61 differ	This study

(Stuttard, 1982) containing 50 µg apramycin ml⁻¹, and the presumptive integrant colonies were subjected to two rounds of sporulation on MYM agar with apramycin but without thio-strepton. Plating of these spores yielded colonies sensitive to thio-strepton.

DNA sequencing and sequence analysis. The 2.4 kb fragment isolated from *SacI*-digested λ LH7 DNA (Han *et al.*, 1994) was subcloned in both orientations in the *SacI* site of pBluescript II SK(+). The recombinant phagemids (pJV60/61) were digested with exonuclease III and ligated to generate overlapping deletion clones. Single-stranded DNA templates were prepared using helper phage VCSM13 (Stratagene; Sambrook *et al.*, 1989); they were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 (US Biochemical) and [³⁵S]dATPαS. When necessary, dGTP in sequencing reactions was replaced with 7-deaza-dGTP or dITP to resolve compressions. Nucleotide sequences were analysed with version 7.3 software developed by the Genetics Computer Group, Madison, WI, USA.

Southern hybridization. Genomic DNA was completely digested with *SacI* and the fragments, separated by agarose gel electrophoresis, were transferred to nylon membranes (Southern, 1975). Digoxigenin (DIG)-dUTP was used to label DNA probes by the random priming method. Hybridization was carried out for 16 h at 65 °C in a solution containing 5 × SSC (1 × SSC is 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 2% (w/v) Hammarsten casein (blocking reagent), 0.1% (w/v) *N*-lauroylsarcosine and 0.02% (w/v) SDS. Membranes were washed twice at 65 °C with 1 × SSC containing 0.1% SDS, and then with 0.1 × SSC containing 0.1% SDS. The labelled DNA was detected by a chemiluminescence procedure (Boehringer Mannheim).

Isolation of rabelomycin. Two cultures (1000 ml) of each *S. venezuelae* mutant were treated with 6.0% (v/v) ethanol at 6 h after inoculation and grown for a further 24 h under the conditions used for jadomycin production (Doull *et al.*, 1993). The pooled cultures, adjusted to pH 3.2 with HCl, were filtered (Whatman no. 5 paper) under vacuum and extracted twice with 500 ml ethyl acetate. The extract was evaporated *in vacuo* at 40 °C and chromatographed on a column (2.0 × 15 cm) of silicic acid (J. T. Baker). Yellow fractions (containing rabelomycin) were eluted with ethyl acetate/toluene (1:9, v/v) and evaporated to give 2.8 mg orange oil. Further purification of this material by preparative TLC on C₁₈ reverse-phase silica (E. Merck RP-18 F₂₅₄, 0.25 mm thickness) with water/acetonitrile (20:80, v/v) as the mobile phase yielded 1.6 mg rabelomycin (*R_F* 0.72).

Analytical and spectroscopic procedures. Jadomycin and rabelomycin were monitored in culture extracts and chromatographic eluates by HPLC using a C₁₈ reverse-phase silica column (80 × 4.0 mm) and a programmed gradient of acetonitrile (solvent A) in acetonitrile/water (1:1, v/v; solvent B); 0.1% (v/v) trifluoroacetic acid was included in both solvents A and B. The content of solvent A in the eluent changed linearly, and was 0, 25, 100, 100 and 0% at 0, 3, 6.5, 7.5 and 10 min, respectively. The retention times for jadomycin B and rabelomycin were 5.97 and 7.29 min, respectively. For analytical TLC, glass plates (5 × 20 cm) coated with a 250 µm thickness of silica gel 60 F₂₅₄ (E. Merck) were used. With toluene/ethyl acetate (1:1, v/v) as the solvent, the *R_F* values for jadomycin B and rabelomycin were 0.21 and 0.16, respectively.

¹H- and ¹³C-NMR spectra were obtained with a Bruker AMX 500 spectrometer (500 MHz for ¹H; 125 MHz for ¹³C). The ¹H- and ¹³C-NMR spectra (recorded in CDCl₃) of authentic rabelomycin and the product from *S. venezuelae* VS655a were

identical and agreed well with the published spectra, except that under our conditions both the authentic and *S. venezuelae* samples contained a ¹³C resonance at 72.2 p.p.m. in place of one at 76.95 p.p.m. in the ¹³C-NMR spectrum reported for synthetic (±)-rabelomycin by Krohn & Khanbabaee (1994). Imamura *et al.* (1982) reported a resonance at 71.6 p.p.m.

RESULTS

Sequence and analysis of the 2.4 kb *SacI* fragment from *S. venezuelae*

A 2.4 kb *SacI* fragment from the *S. venezuelae* DNA insert in λ phage LH7 (Han *et al.*, 1994) was subcloned in pHJL400 to give pJV66. The nucleotide sequence (Fig. 2) showed that one end of the pJV66 insert coincided with the 3'-end of the cyclase-dehydratase gene (ORF4) in the PKS gene cluster for jadomycin B biosynthesis (Han *et al.*, 1994). Analysis of the sequence of the entire 2.4 kb fragment with the FRAME (Bibb *et al.*, 1984) and CODON-PREFERENCE (Devereux *et al.*, 1984) programs showed, in addition to the incomplete ORF4, one complete ORF (ORF6) and a second incomplete one (ORF7). ORFs 6 and 7 lie downstream of, and are transcribed in the same direction as, the ORFs of the PKS cluster (Fig. 3). ORF6 is separated by only 1 bp of non-coding DNA from the 3'-end of ORF4; it starts with an ATG at nt 530–532, which is preceded 9 nt upstream, and thus within the ORF4 coding sequence (see Fig. 2), by a plausible ribosomal-binding site. The stop codon for ORF6 is the TGA at nt 2105–2107. ORF7 starts with an ATG at nt 2131–2133, and is thus separated by 22 nt from the stop codon of ORF6; a ribosomal-binding site is present 9 nt upstream. No in-frame stop codon was present in the sequenced region downstream of the ORF7 start codon.

Amino acid sequence deduced for ORF6

ORF6 encodes a protein of 525 amino acids, with an estimated *M_r* of 55126, and an isoelectric point of 6.41. Comparison of the deduced amino acid sequence of ORF6 with database sequences showed significant similarity to two groups of oxygenases: one catalyses the introduction of hydroxyl groups into polyketide antibiotics; the other participates in the catabolism of aromatic compounds. No similarity was detected to the sequence of P-450 cytochromes that hydroxylate erythromycin aglycone (Haydock *et al.*, 1991; Weber *et al.*, 1991; Stassi *et al.*, 1993); neither the oxygen-binding site nor the haem-ligand pocket characteristic of these monooxygenases was present (Nebert & Gonzales, 1987; Poulos *et al.*, 1987; Omer *et al.*, 1990). In the group of polyketide hydroxylases, the strongest resemblance [45.5% identity in comparisons with the GAP program (Devereux *et al.*, 1984)] was to the product of a putative oxygenase gene (*urdF*) involved in biosynthesis of the angucycline antibiotic urdamycin A in *Streptomyces fradiae* (Rohr *et al.*, 1989); other similar gene products were hydroxylases such as RdmE (27.5% identity; Niemi & Mäntsälä, 1995) of *Streptomyces purpurascens*, TcmG (25.0% identity) involved in tetracenomycin biosynthesis in *Streptomyces glaucescens* (Decker *et al.*, 1992), DnrF (22.7% identity) of

ORF4 end ORF6 --->

CGGGCCACCTCGGGTACGCCAAGGCCTACGCCGAGGGCAAGCGCTGACATGACGGAGCC 540
 R A T L G Y A K A Y A E G K R * M T E P
 CCGCCGGGAGGAGCGCCGACCGGAGTCCCCGGACGCCCGGACACCCCGGTCTCCT 600
 R R A G A P A P E S P D A P D T P V L L
 GGACGCCGACGTCGTCGTCATCGGCCCGGGGCCACCGCCTGATGCTCGCCGGCGAACT 660
 D A D V V V I G A G P T G L M L A G E L
 GCGGCTCGGGCGGGGACGTCGTCCTGGAGAGCGGGAGACCCCGACCGAGTC 720
 R L G G A D V I V L E S R E T P T T E S
 CCGGGCTCCACGCTGCACGCCCGACGATGGAAGTGTGACGACCGCGGGTGTCTAC 780
 R A S T L H A R T M E L L D D R G L L T
 CCCGCTCGGCACTCCGCGTCCGAGCCCGCGGGCGGCACTTCGGCGCATCCCGCTCGA 840
 P L G T P P S E P R G G H F G G I P L D
 CCTGACCTGCGGGTGGGACCCCGGGCAGTGAAGTGCAGCAGACCCGGACCGAGGC 900
 L T L P G R H P G Q W K V E Q T R T E A
 GCTGCTCCAGGAGTGGGCCACCGGCCTCGGGGCCGACGTCGACGCGGCCACACCTGCG 960
 L L Q E W A T G L G A D V R R G H T L R

SfiI

CTCCCTCACGGTGACGGAGACGTACGCCGAGGCCAGCGCCCGGGCGGCGCTGA 1020
 S L T V T E T Y A E A G A T G P G G R D
 CGTGCGCGTGGGGCCCGGTACGCCGTGGGCTGCGACGGGAGCGGACCGGTACGGGC 1080
 V R V R A V A V G C D G E R T T V R A

SfiI

CCTGGCCGGGGCGGAGTTCGCCGGCCAGGAGGCCCGCGCAACTGCTCCGGCGCGATGT 1140
 L A G A E F P G Q E A R R E L L R A D V
 GGCCGGGATCGAGTCCCCGACCGGCGCTTCAGCGGCTGCCCGCGGACTGGCCGTCGC 1200
 A G I D V P D R R F Q R L P G G L A V A
 CGCGTCCGCAACGGCGTACCCGGGTCTGTTCCACGAGTTCGGCAGGCCCGCGTGGC 1260
 A C R N G V T R V M V H E F G R P A V A
 CAGACCGGCGGAGCGGAGTTCGCGAGGTGTCGAGCGTGTGAAGCGGTCACGGGGGA 1320
 R T G E P E F R E V V D V W K R V T G E
 GGACATCACGGCGGGCAGCGCGTCTGGGTGAAGTCTTCCACGACGCCAACCGGCAGCT 1380
 D I T A G T P L W V N S F H D A N R Q L
 CACCCGCTACCGGACGGGCGCTCTGTGGCGGGGGACGCCGCCACCGAGCAGATGCC 1440
 T R Y R D G R V L W A G D A A H Q Q M P
 GATCGGCGGCGGAGCCCTCAACCTGGGGCTCCAGGACCGCGTCAACCTCGGCTGGAAGCT 1500
 I G G Q A L N L G L Q D A V N L G W K L
 CGCCGCGGTGTCGCGGCGGCGCGGCGGCGGCTGCTCGACACCTACCACGACGAGCG 1560
 A A V V R G T A P D G L L D T Y H D E R
 GCACGCGTCCGGCGCCAGGTCTCGGCAACATCAGGGCCCGAGCCCTGCTGCTCCTCGG 1620
 H A V G R Q V L G N I R A Q A L L L L G
 CGGGCCCGAGGCGGAGCCGCTCCCTCTCGGCGGGCCCGAGGCGGAGCCCGTCCG 1680
 G P E A E P V R S L L G G P E A E P V R
 CTCCCTCTCGGCGCGTCTCGCCCTGGACGACGTGCGGCGCACCTGGCCGCAAGGT 1740
 S L L G P L I A L D D V R A H L A G K V
 CTCAGGACTCGACATCCGCTACGGGGCGGGCGGCGGACGTGCTACCGCTGACCGGCAC 1800
 S G L D I R Y G A G A A D V H P L T G T
 CCGGCTGCCCCGACGGCCCTCGTGGACGACGGCGCGAGGGCTGCACCCGTCGCTGCG 1860
 R L P R T A L V D D G R E G L H P S L R
 GGCCGGCCAGGGCCTGTCTGACGCTGGACCGGAGCGGACCGGGCCCGGGACCGGAGC 1920
 A G Q G L V L T L D P S G T G P G T G A
 GGCGGCGGCCCGGCTGGGCGGCGGGTGGCACCGCGCTGGCCAGGCCCGTGCCCGG 1980
 A A A A A G R V G T A V A R P V P G
 CGGCGTCTCGACGGAAGTCTCGACGCGTCTCGTACGGCCGACGGCTACCTCGCTGGAC 2040
 G V L D G L D A V L V R P D G Y L A W T
 CTCGGCGGACGGCGGGAGCGGCGGCTGCACCGCTGGTTCGGCGCCCCCACCCA 2100
 S A D G A G P E A A L H R W F G A P T H

ORF7 --->

CCTCTGAACCCCGACGAGGACCGAGAACCATGCCTCTGATCAACCCCGAAGACGGCTAC 2160
 L * M P L I N P E D G Y
 CTGACCGTCTTCAACCTCTTCGAGACCGAGACCTACGACGGTCAGGCCCGCGCTCGTCGAC 2220
 L T V F N L F E T E T Y D G Q A R V D
 GAGATGAAGGACATCGTCGACACGCCACGTACCCCGGCTGGATCTCCTCCACCGTGCAC 2280
 E M K D I V D N A T Y P G W I S S T V H
 GCCGGCGTCGACACCCCGGGCACCTCAACTACATCCAGTGGCGCAGCCTCGCCGACCTG 2340
 A G V D T P G T L N Y I Q W R S L A D L
 GAGGCGGCTACGAGGGGAGAGTTCCAGAGAAGACCGTGCCGCTCTTCGACCGAGCTC 2400
 E A R Y Q G Q K F Q K K T V P L F D Q L

SacI

GCCACCTCCGTGAAGCTGCTCAAGACGGAGCTC 2433
 A T S V K L L K T E L

Fig. 2. Nucleotide sequence of the 2.4 kb *SacI* segment of *S. venezuelae* DNA excised from λ clone LH7, omitting the region previously sequenced (Han *et al.*, 1994). Deduced amino acid sequences are shown for ORF6 and a part of ORF7 (see Fig. 3). Putative ribosome-binding sites are doubly underlined. Significant restriction sites are underlined. Translational start codons locate the beginning of ORFs; asterisks identify stop codons.

Streptomyces peucetius, used for 11-hydroxylation of aklavinone in daunorubicin biosynthesis (Hong *et al.*, 1994), SchC (24.3% identity) involved in spore pigment formation in *Streptomyces balstedii* (Blanco *et al.*, 1993) and OtcC involved in oxytetracycline biosynthesis in *Streptomyces rimosus* (cited in Blanco *et al.*, 1993). The ORF6 product did not resemble any of the products of *actVA* implicated in ring hydroxylation of actinorhodin intermediates (Caballero *et al.*, 1991).

The second group of oxygenases to which the ORF6 product showed sequence similarity participates in the degradation of aromatic compounds. Here the extent of the similarity matched that shown to most polyketide hydroxylases: e.g. 26.2% identity with pentachlorophenol 4-monooxygenase (PcpB) from a *Flavobacterium* species (Orser *et al.*, 1993); 22.2% identity with 2,4-dichlorophenol 6-monooxygenase (TfdB) of *Alcaligenes eutrophus* (Perkins *et al.*, 1990); and 20.7% identity with

(a)

		***** * * * * * * * *	
ORF6	27	DVVVIGAGPTGLMLAGELRLGGADVIVLESRETPTTESR	65
TcmG	21	PVLIVGGGLTGLSAALFLSQHGVSCRLVEKHRGTTVLTR	59
SchC	16	PVLVVGGSVLGLSTSVFLGRLGVRHMLVERHAGTSVHPR	54
OtcC	4	DVIAGAGPTGLMLACELRLAGARTLVLERLAERVDFSK	39
DnrF	9	DVLVGGGLGGLSTALFLARRGARVLLVERHASTSVLPK	48
PCLO	16	AVLIVGGGTGLIAANELRRGVSCRMIDRLPVAHQTSK	54
TFDB	8	DVLVGTGPAGASAGALLARYGVRTMLINKYNWTAPTTPR	46
PHEA	37	DVLTVGSGPAGSSAAMFLSTQGISNIMITKYRWTANTPR	75

(b)

		* ***** * * * * * ***** * * * * *	
ORF6	288	RDGRVLWAGDAAHQMPIGGQALNLGLQDAVNLGWKLAADVVRGTAPDGLLDITYHDERH	345
TcmG	327	RSGRVFLAGDAAHVHPPAGAFGANGGIQDAHNLAWKLAAVLKGTAGDALLDITYEQERL	384
SchC	304	RAGRVFLAGDSAHEMSPTGAFGSNTGIQDAHNLAWKLAAVLGGWAGDGLLDITYDAERR	361
OtcC	279	RDGRVLLAGDACHIHLPPAGGQGLNLGFQDAVNLGWKLGIAGTAPPELLDITYEAERR	336
DnrF	301	REGPVLLVGDAAKVTPPTGGMGGNTAIGHGFDVAWKLAAVLRGEAGERLLDSYGADGS	358
PCLO	289	RKGNVFLAGDAAHCHSPSGSGMNVGMQDAFNLGWKIAMVERGEAKPDLLDITYHTERT	346
TFDB	302	QQGRVFCAGDAVHRHPPTNGLGSNTSIQDSFNLAWKIAMVLNGTADSLDITYTIERA	359
PHEA	320	QKGRVCCAGDAIHKHPPSHGLGSNTSIQDSYNLCWKLACVLKGQAGPELLETYSTERA	377

Fig. 4. Alignment by PILEUP (Genetics Computer Group, University of Wisconsin, Madison, WI, USA) of segments of the amino acid sequences deduced from ORF6 and database hydroxylases. The similarity around two motifs present in FAD- and NADPH-dependent enzymes is shown. (a) A putative ADP-binding domain in the N-terminal region. (b) A putative ribityl-binding domain in the C-terminal region. Highly conserved amino acids are indicated by asterisks. The aspartic acid residue important for binding the ribityl chain of FAD is indicated by an arrow and is in boldface. TcmG, tetracenomycin C hydroxylase from *S. glaucescens*; SchC, a putative hydroxylase involved in forming spore pigment in *S. halstedii*; OtcC, anhydroxytetracycline monooxygenase from *S. rimosus*; DnrF, aklavinone 11-hydroxylase from *S. peucetius* subsp. *caesius*; PCLO, pentachlorophenol 4-monooxygenase from a *Flavobacterium* species; TFDB, 2,4-dichlorophenol hydroxylase from *Alcaligenes eutrophus*; PHEA, phenol monooxygenase from a *Pseudomonas* species (see text for references).

Among all sequences showing similarity, the resemblance was particularly strong around two motifs present in FAD- and NADPH-dependent hydroxylases. One of the motifs was the β - α - β fold associated with binding of the ADP moiety of FAD (Wierenga *et al.*, 1985, 1986; Eggink *et al.*, 1990); it was located near the N-terminus of the polypeptide (Fig. 4a). The second motif contains an aspartic acid that participates in binding the ribityl substituent of the flavin moiety of FAD (Fig. 4b; Russel & Model, 1988; Eggink *et al.*, 1990). The extent of the sequence similarity between the ORF6 product and both

From comparisons with the database, the amino acid sequence deduced from the incomplete ORF7 is similar to only two known proteins: TcmH of *S. glaucescens*, and *acrVA*-ORF6 of *S. coelicolor* A3(2). TcmH encodes an anthraquinol oxidase catalysing hydroxylation at C-5 of a tetracenomycin C biosynthetic intermediate, TcmF1, to generate the corresponding quinone, TcmD3 (Shen & Hutchinson, 1993b; Hutchinson, 1994). The product of

Fig. 5. (a) Diagram showing the disruption of ORF6 by insertion of an apramycin-resistance gene (*apr^r*). Wild-type *S. venezuelae* was transformed with the pJV67 and Thio^R Apr^R colonies generated by the first crossover between homologous DNA in the plasmid and chromosome were propagated in the presence of apramycin. Those in which a second crossover occurred were selected by their Thio^S Apr^R phenotype. The filled arc in pJV67 represents the apramycin-resistance gene introduced into ORF6 by replacing an *SfiI*–*SfiI* fragment; adjacent open arcs represent cloned *S. venezuelae* DNA containing ORF6. The corresponding regions in chromosomal DNA are represented by filled and open bars. S, *SacI*; E, *EcoRI*; Sf, *SfiI*. The *SfiI* sites lost during construction of pJV67 are in parentheses. (b) Southern hybridization of genomic and pJV67 DNA digested with *SacI* and probed with pJV67. Lanes: 1, VS654a; 2, VS654b; 3, VS655a; 4, VS655b; 5, ISP5230; 6, pJV67. Numbers are the sizes (in kb) of hybridizing *SacI* fragments. Chemiluminescent bands were detected on X-ray film and recorded with a Hewlett Packard ScanJet IIcx/T using Adobe Photolab software.

actVA-ORF6 probably has a similar function (Caballero *et al.*, 1991; Fernandez-Moreno *et al.*, 1994).

Targeted disruption of ORF6 in the chromosome

A gene disruption vector was constructed from pHJL400, a bifunctional *E. coli*–*Streptomyces* plasmid that lacks the partition function of the SCP2* parent and is thus segregationally unstable in streptomycetes (Larson & Hershberger, 1986). The 2.4 kb *SacI* fragment of *S. venezuelae* DNA cloned in pHJL400 to furnish pJV66 contained a 130 bp *SfiI*–*SfiI* segment lying within the confines of ORF6; this was replaced with a 1.5 kb fragment containing the apramycin-resistance gene from

pKC462a (Stanzak *et al.*, 1986), thereby forming the ORF6 disruption vector pJV67 (Fig. 5a). To circumvent a restriction system recognizing methylated DNA apparently present in *S. venezuelae* ISP5230 (Han *et al.*, 1994), pJV67 was first used to transform a DNA-methylation-deficient strain of *E. coli* (MacNeil *et al.*, 1992), and reisolated for use with the streptomycete. *S. venezuelae* transformants propagated without thiostrepton selection yielded Thio^R Apr^R and Thio^S Apr^R colonies. *SacI* digests of genomic DNA from two colonies of each type were probed by Southern hybridization with pJV67 to detect single and double crossover events (Fig. 5b). Wild-type genomic DNA gave the expected signal at 2.4 kb, whereas the DNA from VS654a and VS654b (Thio^R Apr^R) gave a

signal at 5.8 kb for vector DNA, as well as signals at 0.8, 1.2 and 1.8 kb for the apramycin-resistance gene and flanking DNA segments. The signals were consistent with an initial integration of pJV67 into the chromosome of *S. venezuelae* ISP5230 by a single crossover. The absence of a signal at 5.8 kb in VS655a and VS655b (Thio^S Apr^R) indicated that a second crossover had excised the vector DNA. As a result, the ORF6 oxygenase gene in the chromosome was replaced with the sequence disrupted by insertion of the apramycin-resistance gene.

Characterization of metabolites

Analysis by HPLC of wild-type and mutant *S. venezuelae* ISP5230 strains grown under conditions that elicit jadomycin B production (Doull *et al.*, 1994) showed low yields of the antibiotic (10% of wild-type levels) in strains VS654a and VS654b carrying integrated pJV67. Accompanying the jadomycin B was a second product not encountered in the wild-type strain. The replacement mutants (VS655a and VS655b) produced only this product; no jadomycin B could be detected. Further analysis of cultures by TLC, and recovery of the products by chromatography of culture extracts on silicic acid columns, established that the substance accumulated by replacement mutants was a yellow antibiotic indistinguishable by direct comparison from an authentic specimen of (–)-rabelomycin (Liu *et al.*, 1970). The presence of a mixture of jadomycin B and rabelomycin in strains with pJV67 integrated by a single crossover was also confirmed by TLC.

DISCUSSION

Considerable progress has been made recently in clarifying the molecular genetics of polyketide antibiotic biosynthesis in actinomycetes, and many of the important principles have been established (McDaniel *et al.*, 1993; Shen & Hutchinson, 1993a; Hopwood *et al.*, 1994). One of the areas that remains to be fully explored is the molecular basis for the remarkable diversity of the polyketide family of secondary metabolites. Even within the subgroup distinguished by its use of acetate starter and malonate extender units, there is a wide range of structural options; these depend not only on the number of malonate units introduced, but also on the pattern in which the growing chain is folded, and ancillary reactions that modify specific sites on the chain. Although the roles of some PKS genes can be predicted from their sequence similarity to those of fatty acid biosynthesis (Hopwood & Sherman, 1990), others can not. The availability of sets of cloned PKS genes for a variety of metabolites has therefore been exploited to establish the rules for 'programming' polyketide-based structures. By determining the biochemical consequences of precisely replacing individual genes in well-defined PKS gene clusters with their counterparts from other clusters, plausible roles have been assigned to several PKS genes (Sherman *et al.*, 1992; Khosla *et al.*, 1993; Kim *et al.*, 1994).

Fig. 6 outlines the steps that might lead to the jadomycin

B structure. Assembly of a polyketide from acetyl-CoA and nine molecules of malonyl-CoA would be catalysed by a PKS complex encoded by ORFs 1–3. Correct cyclization of the polyketide chain probably requires the cyclase-dehydratase product of ORF4; an additional enzyme analogous to TcmI (Summers *et al.*, 1993; Shen & Hutchinson, 1993b) may be needed to complete the cyclization. The structural features of particular interest in jadomycin B are the angular ring fusion pattern and the interruption of a predicted polyketide carbon chain by a nitrogen atom that seems likely to originate biosynthetically from isoleucine supplied in the medium (Doull *et al.*, 1994). The component (or components) in the *jad* cluster that influences the chain folding and cyclization to generate an angular, rather than the more common linear ring fusion pattern, has yet to be determined. Introduction of an isoleucine moiety can plausibly be accounted for by ring scission in a polyketide-derived intermediate, followed by a reaction inserting isoleucine at the reactive centre thus created.

To open ring B of a cyclized polyketide intermediate, monooxygenase or dioxygenase activity, or both acting sequentially as in the metabolism of phenolic compounds by micro-organisms (Perkins *et al.*, 1990), might be predicted. The similarity between the ORF6 product and numerous monooxygenases that modify polyketide and aromatic compounds suggests that this ORF encodes a protein involved in the scission of ring B. If so, disruption of ORF6 would be expected to block ring opening, and cause an accumulation of earlier intermediates or their shunt products. The isolation of rabelomycin, an angucycline antibiotic discovered in cultures of *Streptomyces olivaceus* (Liu *et al.*, 1970) and later identified along with angucycline antibiotics of the vineomycin group as a product of *Streptomyces matensis* subsp. *vineus* (Imamura *et al.*, 1982), supports the presumption that jadomycin B is biosynthesized via an angular polyketide intermediate. It is noteworthy that in the comparison with database sequences, the deduced ORF6 product showed closest identity to *urdF*, which encodes an oxygenase implicated in the biosynthesis of urdamycin A, an angucycline antibiotic (Rohr *et al.*, 1989) with structural similarities to jadomycin B. Presumably the gene clusters for the two biosynthetic pathways have a close evolutionary relationship.

The absence of a hydroxyl group at C-10 in rabelomycin indicates that the carbonyl group at the corresponding position in the polyketide is reduced by a ketoreductase (presumably the product of ORF5; Han *et al.*, 1994) and lost by dehydration before ring B is opened. Similar timing for a decarboxylation step is implied by the absence of a carboxyl group at C-2 in rabelomycin. In contrast, loss of the C-3 hydroxyl in a dehydration reaction aromatizing ring A may occur after ring B is opened, and the lack of sugar substituents on rabelomycin suggests that glycosylation is also a late reaction. Similarities in the amino acid sequences of anthrone oxidases and that deduced for the incomplete ORF7 product implicates this ORF in the introduction of oxygen at C-12 to generate a quinone group in rabelomycin. A conserved histidine in

Fig. 6. Outline of a tentative pathway for the biosynthesis of jadomycin B.

the amino acid sequences deduced for *tcmH*, *actVA*-ORF6 and ORF7 of *S. venezuelae* is consistent with the essential role predicted for this amino acid in TcmH activity (Shen & Hutchinson, 1993a). Preliminary data indicate that ORF7 encodes a large protein containing more than 200 amino acids. It is, therefore, substantially bigger than either TcmH (81 amino acids) or the product of *actVA*-ORF6 (113 amino acids). Although ORF7 is downstream of ORF6, the presence of the quinone group in rabelomycin implies that the ORF7 product acts earlier than that of ORF6 in the biosynthetic pathway. Accumulation of the quinonoid intermediate rather than the corresponding anthrone in the replacement mutants is consistent with evidence from restriction analysis that the

apramycin-resistance gene in pJV67 has the same orientation as ORF6, in which it is inserted (see Fig. 5a). Thus expression of ORF7, which has the same orientation as ORF6, is not prevented; it may continue by readthrough from a promoter in the PKS gene cluster, or be initiated from the promoter of the apramycin-resistance gene.

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