Cloning and characterization of polyketide synthase genes for jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230

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Hybridizing fragments in the genomic DNA of *Streptomyces venezuelae* ISP5230, which produces the jadomycin group of angucycline antibiotics, were detected by probing with *actI* DNA from *Streptomyces coelicolor* A3(2). The hybridizing regions were isolated from a 16.5 kb insert of *S. venezuelae* DNA recovered from a genomic library cloned in a λ replacement vector. Subcloning and sequencing of a 4.8 kb segment of the insert, containing regions hybridizing to *actIII* as well as *actI*, identified five open reading frames (ORFs). The deduced polypeptide products of the ORFs closely resemble in sequence the components of streptomycete type-II polyketide synthases (PKSs): the ORF1 product corresponds to the ketoacyl synthase, and the ORF2 product to a polypeptide closely related to the ketoacyl synthase and involved in determining chain length; the ORF3 product matches the acyl carrier protein; ORF4 encodes a bifunctional cyclase/dehydrase; and ORF5 encodes a ketoreductase. Integration into the chromosomal DNA of a plasmid containing a segment of the ORF2–ORF4 region severely depressed jadomycin B biosynthesis; since the integrant showed no change in growth or spore pigmentation, the cloned PKS genes are presumed to encode enzymes in the pathway for jadomycin biosynthesis.

Keywords: *Streptomyces venezuelae*, jadomycins, polyketide synthase gene cluster, gene disruption

INTRODUCTION

Compounds derived biosynthetically via polyketide intermediates represent one of the largest families of secondary metabolites, and include numerous antibiotics and pigments produced by streptomycetes (for references, see Hopwood & Sherman, 1990; O'Hagan, 1992). The polyketide synthases (PKSs) that assemble the structural skeletons of polyketides are organized either as large multifunctional enzymes (type-I PKSs) in which the biosynthetic activities are present as domains, or as multi-enzyme complexes in which the activities are distributed on individual proteins (type-II PKSs). Sequence analysis of cloned PKS genes has shown strong evolutionary

relationships among systems for the biosynthesis of polyketide-derived metabolites in micro-organisms, and between these systems and the fatty acid synthases (Hopwood & Khosla, 1992). Within the type-II PKSs, the amino acid sequences deduced for proteins of equivalent function are strikingly similar (McDaniel *et al.*, 1993; Shen & Hutchinson, 1993), and in streptomycetes the nucleotide sequences of the genes themselves have sufficient identity to hybridize under relatively stringent conditions.

Malpartida *et al.* (1987a, b) demonstrated that digests of genomic DNA from 14 of 18 streptomycetes known to produce polyketide-derived metabolites contained fragments hybridizing with DNA sequences from *actI* or *actIII*, the genes encoding ketoacyl synthase and keto-reductase activities, respectively, in the PKS associated with actinorhodin biosynthesis in *Streptomyces coelicolor* A3(2). In several species, the hybridizing fragments have been confirmed as PKS genes: thus the genomic frag-

Abbreviation: PKS, polyketide synthase.

The GenBank accession number for the nucleotide sequence reported in this paper is L33245.

Fig. 1. Jadomycin B.

ments in *Streptomyces violaceoruber* were shown to contain genes encoding the PKS for granaticin (Sherman *et al.*, 1989), and those in *Streptomyces halstedii* were identified with a PKS engaged in spore pigment biosynthesis (Blanco *et al.*, 1992).

In the survey by Malpartida *et al.* (1987a, b), three of seven streptomycetes not then known to produce polyketide-derived metabolites, unexpectedly gave genomic DNA fragments that hybridized with the *act* probes. One of these, *Streptomyces parvulus* was later found to produce the polyketide-derived antibiotic nonactin (cited by Malpartida *et al.*, 1987b), and *Streptomyces venezuelae* ISP5230 was subsequently reported to produce jado-

mycins (Ayer *et al.*, 1991), a group of pigmented compounds believed from the pattern of isotopic labelling with [^{13}C]acetate to be of polyketide origin (S. Ayer, personal communication).

Jadomycin B, the main component of the mixture produced during growth in an isoleucine-containing medium, is glycosylated and has an isoleucine moiety fused into the cyclic structure (Fig. 1). This and related products are formed when *S. venezuelae* ISP5230 is grown under stress conditions, and expression of the biosynthesis genes appears to be activated by heat shock (Doull *et al.*, 1994). Although strain ISP5230 differs from *S. venezuelae* UC2374, the strain reported by Malpartida *et al.* (1987a, b) to hybridize with *actI* and *actIII*, both strains produce chloramphenicol, and we have verified that both hybridize similarly to the *actI* probe. We report here the cloning and sequence analysis of the hybridizing DNA in *S. venezuelae* ISP5230. A selective, severe decrease in jadomycin B production when a plasmid containing part of the cloned sequence integrated into the chromosome indicates that the cloned PKS genes are associated with jadomycin biosynthesis, and are not involved in the formation of spore pigments.

METHODS

Bacteria, plasmids, phages and culture media. The bacterial strains, plasmids and phages used are listed in Table 1. All cultures in liquid media were grown on a rotary shaker at

Table 1. Bacterial strains, plasmids and phages used

Strain	Genotype/phenotype	Source/reference
<i>Streptomyces venezuelae</i>		
ISP5230	Wild type	Doull <i>et al.</i> (1985)
UC2374, 13s	Wild type, 13s is a natural variant	Ahmed & Vining (1983)
VS651, VS652	ISP5230 with integrated pJV63	This study
<i>Escherichia coli</i>		
TG1	<i>supE hsdΔ5 thiΔ(lac-proAB) F'(traD36 proAB⁺ lacI lacZΔM5)</i>	Carter <i>et al.</i> (1985)
LE392	<i>Δ(lacIZY)6 galK2 galT22 metB1 trpR55 λ⁻</i>	Sambrook <i>et al.</i> (1989)
ET12567	<i>dam⁻ dcm⁻ hsdM⁻</i>	MacNeil <i>et al.</i> (1992)
Plasmid		
pHJL400	<i>tsr, amp, lacZ'</i> (bifunctional)	Larson & Hershberger (1986)
pIJ2345	pBR329 with a 2.2 kb <i>actI</i> insert	D. A. Hopwood (John Innes Institute, Norwich)
pIJ2346	pBR329 with a 1.1 kb <i>actIII</i> insert	D. A. Hopwood
pJV62	pHJL400 with a 0.6 kb <i>jad</i> insert	This study
pJV63	pHJL400 with a 2.6 kb <i>jad</i> insert	This study
pBluescript II	<i>amp, lacZ'</i>	Stratagene
Phage		
VCSM13	Km ^R derivative of M13K07	Stratagene
λGEM-11	λ replacement vector	Promega
LH7	λGEM-11 with a 16.5 kb insert from <i>S. venezuelae</i> ISP5230	This study

240 r.p.m. *Escherichia coli* was incubated at 37 °C in Luria-Bertani medium (Sambrook *et al.*, 1989), supplemented for plasmid selection with ampicillin (100 µg ml⁻¹); strain TG1 was the host strain for all DNA manipulations, whereas strain ET12567 was used to generate unmethylated plasmid DNA suitable for transforming *S. venezuelae* ISP5230. Cultures of *S. venezuelae* were normally grown in MYM medium (Doull *et al.*, 1985) at 27 °C for 24–48 h. Thiostrepton (20 µg ml⁻¹) was added for selection when required. Cultures producing mycelium from which genomic DNA was extracted were grown in MYM medium supplemented with 5 mM MgCl₂. To prepare a vegetative inoculum for jadamycin B production, *S. venezuelae* was grown in MYM medium as usual for 24 h. Mycelium collected from the culture by centrifugation was incubated in a galactose-isoleucine medium (Doull *et al.*, 1994) for 6 h at 27 °C. For jadamycin B production, the culture was supplemented to 6% (v/v) with absolute ethanol, and incubated at 27 °C for a further 48 h.

Transformation procedures. Competent cells of *E. coli* were prepared and transformed with plasmid DNA as described by Hopwood *et al.* (1985). Protoplasts of *S. venezuelae* were prepared and transformed by the procedure of Aidoo *et al.* (1990). They were regenerated on R5 medium, and transformants were selected by overlaying the agar surface with soft nutrient agar containing sufficient thiostrepton to give a final plate concentration of 25 µg ml⁻¹.

DNA manipulation. The general procedures described by Sambrook *et al.* (1989) were followed. 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); phage DNA was isolated by the method of Meese *et al.* (1990). The genomic library of *S. venezuelae* DNA used initially consisted of *Bgl*II fragments ligated to *Bam*HI arms of λGEM-11 DNA (Promega). The second library contained sized (9–23 kb) fragments from a partial *Sau*3AI digest ligated to *Xho*I half-site arms of the vector.

DNA sequencing and sequence analysis. DNA fragments were subcloned in pBluescript II SK(+), and overlapping deletion clones were generated. Single-stranded DNA templates were isolated from cultures of *E. coli* TG1 phagemid transformants as described by Sambrook *et al.* (1989). They were sequenced by the dideoxy chain-termination method (Sanger *et al.*, 1977) using Sequenase version 2.0 (USB) and [α-³⁵S]dATP. Oligonucleotide primers were used for some regions, and to resolve anomalies where necessary, segments were resequenced with 7-deaza-dGTP in place of dGTP. Both strands of the *S. venezuelae* genomic DNA were sequenced, and the sequence was analysed with version 7.0 software developed by the Genetics Computer Group, Madison, WI, USA.

Hybridization. For plaque and colony hybridization, DNA was transferred to Hybond-N nylon membranes (Amersham) as described by Hopwood *et al.* (1985). Restriction enzyme digests were fractionated by electrophoresis in 0.7% agarose, and transferred to nylon membranes by the Southern procedure (Southern, 1975). DNA probes were labelled with [α-³²P]dCTP or with digoxigenin (DIG)-dCTP by the random priming procedure. Hybridization was carried out at 65 °C in a solution containing 5 × SSPE (1 × SSPE is 0.18 M NaCl, 10 mM sodium phosphate and 1 mM EDTA, pH 7.7), 5 × Denhardt's solution (Denhardt, 1966), 0.5% SDS and sheared herring sperm DNA (100 µg ml⁻¹). Membranes were washed twice at 65 °C with the following solutions in sequence: 2 × SSPE containing 0.1% SDS, 1 × SSPE containing 0.1% SDS, and 0.1 × SSPE containing 0.1% SDS. The stringency of the final wash should have removed DNA possessing less than 70% sequence identity with

the probe. ³²P-labelled DNA was detected by autoradiography; DIG-labelled DNA was detected by a chemiluminescence procedure (Boehringer-Mannheim).

Chromatographic analysis. Samples (50 ml) of *S. venezuelae* culture filtrate were passed through 40 µm particle size C₁₈ reverse-phase silica columns (1 g Chromosep, Chromatographic Separations); the adsorbed jadamycin B was eluted with methanol. The eluate, concentrated to 0.2 ml, was chromatographed (20 µl sample) on a 4.6 × 250 mm column of 5 µm particle size C₁₈ reverse-phase silica. The eluting solvent was a gradient of acetonitrile in 50% aqueous acetonitrile, with both solvents containing 0.1% trifluoroacetic acid (Ayer *et al.*, 1991). Authentic jadamycin B eluted from the column with a retention time of 11.6 min; the concentration of jadamycin B in culture filtrates was quantified by comparing chromatographic peak areas with those given by reference solutions.

RESULTS

Detection of *actI*-hybridizing DNA in *S. venezuelae*

Samples of genomic DNA from *S. venezuelae* strains ISP5230 and 13s (UC2374; see Table 1) were digested with *Bam*HI, and the fragments separated by electrophoresis were hybridized at high stringency with pIJ2345, which contained an insert from the *actI* of *S. coelicolor* A3(2). The DNA from each strain showed a hybridizing fragment within the size range (2.0–2.8 kb) of that detected by Malpartida *et al.* (1987a, b) in *S. venezuelae* UC2374.

Cloning of DNA hybridizing with *actI* and *actIII*

Using pIJ2345 to screen a genomic DNA library containing fragments from a *Bgl*II digest of *S. venezuelae* ISP5230 DNA in the replacement vector λGEM-11 yielded a recombinant phage containing a 1.8 kb *Bgl*II–*Sac*I fragment that hybridized with the probe. This fragment, which was located at one end of the insert DNA, was used to probe a second *S. venezuelae* ISP5230 library prepared in λGEM-11 from a partial *Sau*3AI digest. Three hybridizing λ clones (LH2, LH7 and LH24) were isolated; the DNA from each was shown by Southern analysis to contain a 2.3 kb *Sac*I fragment that hybridized with the 1.8 kb probe. Reprobing the same Southern blots with the 1.1 kb insert excised from pIJ2346 located 1.0 and 0.3 kb *Sac*I fragments that hybridized at high stringency. A restriction map of the region (Fig. 2a) showed that the 2.3, 1.0 and 0.3 kb *Sac*I fragments hybridizing to *actI* and *actIII* are adjacent. To isolate the 2.3 kb *Sac*I fragment, a mixture of the 2.3 and 2.4 kb fragments from a *Sac*I digest of DNA from λ clone LH7 was excised from an electrophoresis gel and subcloned in pHJL400. The plasmid mixture was used to transform *E. coli*, and transformants containing the 2.3 kb fragment were identified by colony hybridization.

Sequence analysis

The region of *S. venezuelae* ISP5230 DNA containing the 2.3, 1.0 and 0.3 kb *Sac*I fragments hybridizing with *actI* and *actIII* was subcloned from the insert in λ clone LH7.

Fig. 2. (a) A *SacI* restriction map of the *S. venezuelae* ISP5230 DNA insert in λ clone LH7. The 2.3 and 1.0 kb regions hybridizing with *actI* and *actIII* are indicated with double and single asterisks, respectively. The 4.8 kb region sequenced is shown as a thickened line. (b) An expansion of the 4.8 kb region showing significant restriction sites; pointed bars represent the extent and direction of the five ORFs detected. (c) The rectangular bars identify the two fragments used in gene disruption experiments.

Overlapping fragments (2.3 kb *SacI*, 2.6 kb *PstI* and 2.4 kb *SacI*; see Fig. 2a) were inserted individually into pBluescript II SK(+), and sequenced (Fig. 3). Analysis of the data with the FRAME program (Bibb *et al.*, 1984) showed five ORFs, all transcribed in the same direction (see Fig. 2b). Their designations as ORFs 1–5 are based on the similarity of their deduced amino acid sequences to those of ORFs 1–5 in streptomycete gene clusters for type-II PKSs (Hopwood & Sherman, 1990). The translational start site for each ORF was predicted from the location of a potential start codon closely preceded by a plausible ribosome-binding site (RBS). The 3' end of ORF1 overlaps by 4 bp the predicted start codon (ATG) of ORF2, which begins at nucleotide 1412 and is preceded by a likely RBS located within the ORF1 sequence. This type of organization suggests translational coupling, and occurs frequently in ORFs 1 and 2 of other PKSs. ORF2 is separated from ORF3 by 42 bp of non-coding DNA, ORF3 and ORF5 are 73 bp apart, while 39 bp of non-coding DNA separates ORF4 and ORF5.

Deduced functions

The deduced amino acid sequence of ORF1 is strikingly similar to sequences deduced for ORF1 in the PKS gene clusters for actinorhodin in *S. coelicolor* A3(2) (Fernández-Moreno *et al.*, 1992), granaticin in *S. violaceoruber* (Sherman *et al.*, 1989), tetracenomycin C in *Streptomyces glaucescens* (Bibb *et al.*, 1989), an unidentified polyketide-derived metabolite in *Streptomyces cinnamomensis* (Arrowsmith *et al.*, 1992) and the *whiE* spore pigment in *S. coelicolor* A3(2)

(Davis & Chater, 1990). Over a region of 420 amino acids, the polypeptide sequences exhibit more than 60% identity. The similarity is particularly strong (almost 100% identity) in the region of the presumed active site, where the cysteine and serine residues responsible for substrate attachment are located (Fig. 4a). The deduced amino acid sequence of ORF2 shows 60% identity to the sequences deduced for ORF2 in known PKS clusters. The ORF2 from *S. venezuelae* ISP5230 also resembles other ORF2s in being very similar in sequence to ORF1, but lacking the two active site motifs. McDaniel *et al.* (1993) have recently proposed that the protein encoded by ORF2 in the *act*, *gra* and *tcm* PKS clusters influences the chain length of the PKS product.

The deduced product of ORF3 is a relatively small protein of 90 amino acids; its amino acid sequence shows 66% identity to the sequence deduced from ORF3 of the *S. cinnamomensis* PKS, and somewhat lower identity (40–50%) to the sequence of acyl carrier proteins (ACPs) of fatty acid and polyketide synthases. This similarity includes a conserved motif around the serine residue to which the pantetheinyl cofactor is presumably attached (Fig. 4b). The products of ORF1, ORF2 and ORF3 would be expected to form an enzyme complex able to condense acyl building units into a polyketide chain; thus the function analysis of this region indicates that the 2.3 kb *SacI* fragment from *S. venezuelae* encodes part of a PKS gene cluster.

The deduced product of ORF5 showed 82% sequence identity with the deduced products of ORF5 from the 'mon' PKS gene cluster of *S. cinnamomensis* (Arrowsmith *et al.*, 1992) and 60% identity to the ORF5 products from *S. coelicolor* A3(2) (Hallam *et al.*, 1988), and *S. violaceoruber* (Sherman *et al.*, 1989). It also shares with these proteins the conserved sequence motif Gly-X-Gly-X-X-[Gly/Ala] characteristic of nucleotide binding proteins (Fig. 4c). Thus ORF5 probably encodes a ketoreductase that catalyses the reduction of a specific keto group in the polyketide to a hydroxyl group that is subsequently eliminated during chain assembly.

Strong overall similarity (59% identity) was also apparent between the amino acid sequences deduced from ORF4 of *S. venezuelae* ISP5230 and *S. cinnamomensis*, and only slightly less (45% identity) between the ORF4 products from *S. venezuelae* and the *act* and *gra* PKS gene clusters. The ORF4 product was less similar to ORFVI in the *whiE* cluster, and resembled only the N-terminal half of ORF4 in the *tcm* PKS gene cluster, but overall the results indicated that ORF4 in each cluster encodes a protein with a similar function, believed to be the correct cyclization of a region in the polyketide backbone (Zhang *et al.*, 1990).

Gene disruption

A 0.6 kb *SmaI* fragment of *S. venezuelae* DNA internal to ORF1 (see Fig. 2c) was subcloned in the unique *SmaI* site of pHJL400, an *E. coli*–*Streptomyces* shuttle vector that is segregationally unstable in streptomycetes because it lacks

SacI
 GAGCTCATCGAGCGGGCCAAGACCGACCCCGCTTCGTGGGCATCAGCGAGGACCTGAAG 60
 CCGTTCATCGAGGCGCTACGACCCGGCCACCTGGCGCTCGCCCGCCGACGCCATGGCCACC 120
 RBS ORF1--->
 CGCTTCTACAACGGGAGGCGAACCGGTGACCGCGCAGCGCTCGTCATCACCGGCATCG 180
 M T A R R V V I T G I E
BamHI
 AGGTCCTCGCCCCCGGTGGCACCGGATCCAAGGCCTTCTGGAACCTGCTCAGCGAGGGCC 240
 V L A P G G T G S K A F W N L L S E G R
SmaI
 GCACGGCCACCCGGGGCATCACCTTCTTCGACCCGACGCCGTTCCGCTCCCGGGTCGCCG 300
 T A T R G I T F F D P T P F R S R V A A
 CCGAGATCGACTTCGACCCGGAGGCCACGGCCTCAGCCCGCAGGAGATCCGCCGCATGG 360
 E I D F D P E A H G L S P Q E I R R M D
 ACCGGGCCCGCCAGTTCGCGGTGGTCGCCGCGCGCGCGCTCGCCGACAGCGGCATCGACC 420
 R A A Q F A V V A A R A V A D S G I D L
 TGGCCGCCCGACCGCTACCGCGTCGGCGTCACCGTCGGCAGCGCGCTCGCGGCCACCA 480
 A A H D P Y R V G V T V G S A V G A T M
 TGGGCTCGACGAGGAGTACCGGGTCGTCAGCGACGGCGCGCGCTCGACCTCGTCGACC 540
 G L D E E Y R V V S D G G R L D L V D H
 ACGCCTACCGGTGGCGACCTCTACGACTACATGGTGCCAGCTCCTTCTCCCGCCAGG 600
 A Y A V P H L Y D Y M V P S S F S A E V
 TCGCCTGGGCGTGGCGCCGAGGGCCCCAACACCGTGGTCTCCACCGGCTGCACCTCCG 660
 A W A V G A E G P N T V V S T G C T S G
 GCCTCGACTCCGTCGGCTACGCGCGTGGGAGCTGATCCGCGAGGGCTCCGCCGACGTCA 720
 L D S V G Y A R G E L I R E G S A D V M
 TGATCGCCGGTTCCTCGGACGCCCCGATCTCCCCGATCAGATGGCCTGCTTCGACGCCA 780
 I A G S S D A P I S P I T M A C F D A I
 TCAAGCGACCAACCGGTACGACGACCCGGCGCACGCTCCCGGCCCTTCGACGGCA 840
 K A T T N R Y D D P A H A S R P F D G T
 CCCGCAACGGCTTCGTGCTCGGCGAGGGCGCGCCGCTCTTCGTCTGGAGGAGCTGGAGA 900
 R N G F V L G E G A A V F V L E E L E S
SmaI
 GCGCCCCGGCCCGCGCGCCACATCTACGCCGAGATCGCCGGCTACGCCACCCGACGCA 960
 A R A R G A H I Y A E I A G Y A T R S N
 ACGCGTACCACATGACCGGTCTGCGGCCGACGGCGCGGAGATGGCGGAGCGATCCGGG 1020
 A Y H M T G L R P D G A E M A E A I R V
 TCGCGTCGACGAGGCGGGATGAACCCACCGAGATCGACTACATCAACGCGCACGGCT 1080
 A L D E A R M N P T E I D Y I N A H G S
 CCGGCACCAAGCAGAACGACCGCCACGAGACCGCAGCGTTCAAGAAGAGCCTCGGGGACC 1140
 G T K Q N D R H E T A A F K K S L G D H
 ACGCGTACCGCACCCCGGTACGCTCCATCAAGTCGATGGTCGGGCACTCCCTCGGTGCCA 1200
 A Y R T P V S S I K S M V G H S L G A I
 TCGGCTCCATCGAGATCGCCGCTCGGCCCTGGCCATGGAGCACAACGTGGTCCCGCCGA 1260
 G S I E I A A S A L A M E H N V V P P T
 CGGGCAACCTGCACACCCCGGACCCGAGTGGCGACCTGGACTACGTCCGCTCGTGCCGCG 1320
 G N L H T P D P E C D L D Y V R S C R E
 AGCAGCTACGGACTCGGTCTGACCGTCGGCAGCGGATTCGGCGGCTTCCAGAGCGCCA 1380
 Q L T D S V L T V G S G F G G F Q S A M
 RBS ORF2--->
 TGGTCTGGCCCGTCCCGAGAGGAAGATCGCATGAGCGGTCCGTTGGTGGTGACCGGCCT 1440
 V L A R P E R K I A M* S A S V V V T G L
 CGGTGTCGCCGACCAACGGCCTGGGCGCGAGGACTTCTGGGCCTCGACCTCGGGCGG 1500
 G V A A P N G L G R E D F W A S T L G G
 GAAGAGCGGCATCGGCCGCTCACCCGCTTCGACCCACCGGCTACCCGGCGCGCCTCGC 1560
 K S G I G P L T R F D P T G Y P A R L A
 CGGCGAGGTGCCCGGCTTCGCCGCCGAGGAGCACCTGCCGAGCCGGCTGCTGCCGACAG 1620
 G E V P G F A A E E H L P S R L L P Q T
 CGACCGGATGACCCGCTCGCGCTGGTCGCCGCGGACTGGGCGCTGGCCGACGCGGGCGT 1680
 D R M T R L A L V A A D W A L A D A G V
 CCGCCCCGAGGAGCAGGACGACTTCGACATGGGCGTGGTCACGGCCAGCGCCTCCGGCGG 1740
 R P E E Q D D F D M G V V T A S A S G G
PstI
 CTTTCGAGTTTCGGCCAGGGCGAGCTGCAGAAGCTGTGGAGCCAGGGCAGCCAGTACGTCAG 1800
 F E F G Q G E L Q K L W S Q G S Q Y V S
BglII
 CGCGTACCAGTCCTTCGCTGGTTCTACGCCGTCAACAGCGGCCAGATCTCCATCCGCAA 1860
 A Y Q S F A W F Y A V N S G Q I S I R N
 CGGCATGAAGGGCCCCAGCGGCGTGGTCGTACGACGACGAGCGCGGCGTCTGGACGCGGT 1920
 G M K G P S G V V V S D Q A G G L D A V

Fig. 3. For legend see page 3385.

CGCGCAGGCCCCGCCACAGATCCGCAAGGGCACCCGGCTCATCGTCTCCGGTGGCGTCGA 1980
 A Q A R R Q I R K G T R L I V S G G V D
 CGCCTCGCTCTGCCCCGTTGGGTGGGTGGCGACGTCGCCTCCGACCGGCTCAGCACCAG 2040
 A S L C P W G W V A H V A S D R L S T S
 CGAGGAGCCCCCGGGTACCTGCCCTTCGACCGCGAAGCCCAGGGCCACGTGCCCGG 2100
 E E P A R R G Y L P F D R E A Q G H V P G
 CGAGGGTGGGGCGATCCTCGTCATGGAGGCCGCCGAGGCGGCCGGGAGCGGGGGCCCG 2160
 E G G A I L V M E A A E A A R E R G A R
 GATCTACGGCGAGATCGCCGGATACGGCTCCACCTTCGACCCCCGGCGGGCAGCGGGCG 2220
 I Y G E I A G Y G S T F D P R P G S G R
 CGAGCCCCGGCTGCGCAAGGCCATCGAACTCGCCCTGGCCGACGCCGGAGCGGCACCCGG 2280
 E P G L R K A I E L A L A D A G A A P G
SacI
 TGACATCGACGTCGTCTTCGCGGACGCGGCCCGCTCCCGGAGCTCGACCGGTCGAGGC 2340
 D I D V V F A D A A A V P E L D R V E A
 CGAGGCCCTCAACGCCGTCTTCGGCACCGGCCCGCTCCCGGTCACCGCGCCCAAGACGAT 2400
 E A L L N A V F G T G A V P V T A P K T M
 GACCGGCGGGCTACTCCGGCGCGCGCCGCTCGACCTGGCCGCGCGTTCCTCGCGAT 2460
 T G R L Y S G A A P L D L A A A F L A M
 GGACGAGGGCGTCATCCCGCCACCGTCAACGTCGAGCCGACGCCCGGTACGGCCTCGA 2520
 D E G V I P P T V N V E P D A A Y G L D
 CCTGGTCGTCGGGCGGCCCGTACCGCGAGGTGAACACCGCCCTGGTGATCGCCCGCGG 2580
 L V V G G P R T A E V N T A L V I A R G
 TCACGGCGGCTTCAACTCCGCGATGGTCGTCCGTTCCGCGAACTGACCTTTCCCCCGCAG 2640
 H G G F N S A M V V R S A N *
 RBS ORF3--->
 TACCTCACAGTCACCCAGGAGAAGAACCATGAGCAGCAAGACCTTCACCCTCGACGACCT 2700
 M S S K T F T L D D L
BamHI
 CAAGCGGATCCTGCGCGAGGGCGCCGGCGCCGACGAGGGCGTCGACCTCGACGGCGACAT 2760
 K R I L R E G A G A D E G V D L D G D I
 CCTCGACACCCACTTCGAGACGCTCGGCTACGAGTCGCTCGCCCTGCTGGAGACCGGCAG 2820
 L D T H F E T L G Y E S L A L L E T G S
 CCGCATCGAGCGCGAGTACTCCATCACCTCGACGACGACGTGCTGTCCGACGCCGACAC 2880
 R I E R E Y S I T L D D D V L S D A D T
 CCGCGCGCCCTCATCGAGGCCGTCAACGCGACCTGTGCGCCGTGCGCCCGCCTGACC 2940
 P R A L I E A V N A H L S A V A A A *
 RBS
 CACCCGACCGACACCGGCGCCCGTACCCGCCCGCTACCGCCGCCCTGGCAAGAGAAGA 3000
 ORF5--->
 GACGACACCGCATGTCCCAGCAGGAGAAGCAGGAGAAGAGGGTCGCCCTCGTCACCGGAG 3060
 M S Q Q E K Q E K R V A L V T G A
 CCACCACTGGCATCGGCCTCGCCGTGCGCCGGCTCCTGGCGTCCCAGAACCACCGGGTGT 3120
 T S G I G L A V A R L L A S Q N H R V F
 TCCTCGGTGCCGCAACGCCGAGAACGTGCGCCGCCACCGTCAAGCAGCTCCAGGACGAGG 3180
 L G A R N A N V A A T V K Q L Q D E G
 GCCTTGAGGTGACGGCACCACTTCGACGTGCGCTCCGGCGAGGACGTCAAGGCCTTCG 3240
 L E V D G T T L D V R S G E D V K A F V
SacI
 TCCAGCGCCCGTCGACCGCTTCGGCACCGTCGAGCTCCTCGTGAACAACGCGGGCCGCA 3300
 Q A A V D R F G T V E L L V N N A G R S
 GCGGCGCGGGCGTACCGCCGACATCGCCGACGAGCTGTGGAACGACGTCATCGACACCA 3360
 G A G V T A D I A D E L W N D V I D T N
 ACCTCAACTGCGTCTTCGGCTACCCGCGAGGCCCTGACCACCGGCGCATGCGCGAGA 3420
 L N C V F R L T R E A L T T G G M R E K
 AGTCCCGCGGCGGATCATCAACATGGCCTCGACCGCCGCAAGCAGGGCGTCGTCTCG 3480
 S R G R I I N M A S T A G K Q G V V L G
 GCGCCCCCTACTCGGCTCGAAGCACGGCGTCTGGCTTCACCAAGGCCCTCGGCAACG 3540
 A P Y S A S K H G V V G F T K A L G N E
SacI
 AGCTCGCCCCGACCGGCATCACCGTCAACGCGGTCTGCCCCGGTTACGTGAGACGCCGA 3600
 L A P T G I T V N A V C P G Y V E T P M
 TGGCCAGCGCGTCCGCCAGGGCTACGCCGCCGCTACGAGACCTCCGAGGACGCCATCC 3660
 A Q R V R Q G Y A A A Y E T S E D A I L
XhoI
 TCGAGAAGTTCCAGTCGAAGATCCCGCTCGGCCGCTACTCCACGCCGAGGAGGTGCGCCG 3720
 E K F Q S K I P L G R Y S T P E E V A G
 GTCTCGTGGCTACCTGGCCTCCGACACCGCGCCTCCATCACTCGCAGGCGCTCAACG 3780
 L V G Y L A S D T A A S I T S Q A L N V
 RBS
 TCTGCGGCGGCTGGGCAACTTCTGATCCCCGCACCCCTCTCGCACCGTCCCGAGGAGT 3840
 C G G L G N F *

Fig. 3. For legend see facing page.

ORF4--->	
TTCACGATGACGACCCGTGAGGTCGAGCACGAGATCACCGTCGCGGCCCCGCCCCAGGCC	3900
M T T R E V E H E I T V A A P A Q A	
GTGTACCGGCTGATCGCCGAGGTGGAGAACTGGCCGCGGATCTTCCCGCCACCCCTCTAC	3960
V Y R L I A E V E N W P R I F P P T L Y	
GTCGACCACGTCGAGCGCGGCGAGCGGGAGGAGCGCATCCGGATCTGGGCGACCGCCAAC	4020
V D H V E R G E R E E R I R I W A T A N	
GGCGCCCCGAAGAACTGGACGTCCAAGCGGACCCCTGGACCCGGACAACCTCCGGATCACC	4080
G A P K N W T S K R T L D P D N L R I T	
TTCCGCCAGGAGGTCTCCACCCCGCCGTCGCCCATGGCGACCTGGATCATCGAGCCG	4140
F R Q E V S T P P V A A M A T W I I E P	
GTCTCCGCGACCGAGTCCGGATCCGCCTTGCTGCACGACTACCGGGCGATCGACGACGAC	4200
V S A T E S G S A L L H D Y R A I D D D	
SacI	
CCCAGGGGCTGAAGTGGGTGACGAGGCCGTCGACCGCAACTCGCGCTCCGAGCTCGCC	4260
P E G L K W V D E A V D R N S R S E L A	
GCCCTCAAGACCAACGTGCAACTCGCCACGCCCTCCGAGGAGATCACCTTCTCCTTCGAG	4320
A L K T C N V E L A H A S E E I T F S F E	
GACACCGTGCAGATCGACGGCTCCGCCAAGGACGCCTACGACTTCGTCAACGAGGCGAAC	4380
D T V Q I D G S A K D A Y D F V N E A N	
CTCTGGCCGGAGCGGCTGCCGCACGTGCCACCGTGCCTTCGAGGAGGAGACCCCGGGC	4440
L W P E R L P H V A T V R F E E E T P G	
PstI	
CTGCAGAGCCTGGAGATGGACTCCCAGGCCAAGGACGGCTCCACCCACACGACGAAGTCG	4500
L Q S L E M D S R A K D G S T H T T K S	
TACCGGGTGACCTTCCCGCACCACCGGATCGCTACAAGCAGATCACCTGCCGGCGCTC	4560
Y R V T F P H H R I A Y K Q I T L P A L	
ATGACCGTGCACACCGGCTACTGGACGTTTCGAGGAGAACGAGGACGGCGTCGCCGCCTCC	4620
M T L H T G Y W T F E E N E D G V A A S	
TCGCAGCACACGGTCGTCTCAACACCGAGAATCATGCCCGGATCCTCGGCGCCGACGCC	4680
S Q H T V V L N T E N I A R I L G A D A	
ACCGTCGCCGACGCCCGGAGTACGTGCAGACGGCGCTCAGCACCAACAGCCGGGCCACC	4740
T V A D A R E Y V Q T A L S T N S R A T	
CTCGGGTACGCCAAGGCCTACGCCGAGGGCAAGCGCTGACATGACGGAGCCCCGCCGGGC	4800
L G Y A K A Y A E G K R *	
AGGAGCGCCCGCACCGGAGTCCCCGACGCCCGGACACCCCGGTCTCCTGGACGCCGA	4860
AatII	
CGTC	4864

Fig. 3. Nucleotide and deduced amino acid sequences of the 4.8 kb segment of DNA containing ORFs 1–5. Putative ribosome-binding sites and significant restriction enzyme recognition sequences are underlined. Translational start codons are identified as the beginning of ORFs; asterisks identify stop codons.

the partition function of the SCP2* parent (Larson & Hersherberger, 1986). The recombinant plasmid (pJV62) isolated from *E. coli* TG1 transformed *S. venezuelae* ISP5230 to thiostrepton resistance (the marker in pHJL400 used for selection) with very low efficiency. This difficulty was exacerbated by a relatively high rate of spontaneous mutation to thiostrepton resistance. All presumptive integrants selected on thiostrepton after passage of 100 putative Thio^R transformants through thiostrepton-free medium to promote plasmid loss failed to hybridize to pHJL400. However, the efficiency with which pJV62 transformed *S. venezuelae* ISP5230 could be markedly improved by isolating the plasmid from *E. coli* ET12567 (*dam*[−] *dcm*[−] *hsdM*[−]) (MacNeil *et al.*, 1992), thereby avoiding the restriction barrier to methylated DNA apparently present in this and other streptomycetes (MacNeil, 1988). Transformation of *S. venezuelae* ISP5230 with pJV62 then yielded approximately 70 Thio^R colonies per µg of plasmid DNA. Extraction and electrophoresis of DNA from 10 of these showed plasmid clearly present in two. However, the remaining eight harboured free

plasmid in low concentration, since transformation of *E. coli* TG1 with extracts gave, in each case, ampicillin-resistant colonies containing pJV62. An attempt to eliminate free pJV62 from 10 Thio^R transformants by two rounds of subculturing in the absence of thiostrepton selection, and then to select strains in which pJV62 had integrated into the *S. venezuelae* chromosome through a single cross-over between the 0.6 kb *Sma*I fragment and the homologous DNA region on the chromosome was not successful. Southern analysis of the subcultured colonies using pHJL400 and the 2.3 kb *Sac*I fragment as probes detected free pJV62 in each culture and showed that the chromosomal PKS gene was still intact.

Transformants containing integrated vector DNA were obtained by using a larger homologous insert. Because unfavourable secondary structure might also have contributed to the failure of pJV62 to yield stable integrants, the 2.6 kb *Pst*I–*Pst*I insert in the new plasmid (pJV63) was from a different region of the PKS gene cluster; see Fig. 2c). It was ligated into the *Pst*I site of pHJL400, and

(a)	Ketoacylsynthase site	Acyltransferase site
<i>actI</i> ORF1	GPVTMVSTGCTSGLDVS	----- SMVGH8LGAIGS
<i>gra</i> ORF1	GPVTMVSDGCTSGLDVS	----- SMGGH8LGAIGS
<i>tcm</i> ORF1	GPVTVSTGCTSGLDVS	----- SMIGH8LGAIGS
<i>whiE</i> ORFIII	GPVQTSTGCTSGLDVS	----- SMVGH8LGAIGS
<i>S. cinnamonensis</i> ORF1	GPSTVSTGCTSGIDAV	----- SMVGH8LGAIGS
<i>S. venezuelae</i> ORF1	GPNTVSTGCTSGLDVS	----- SMVGH8LGAIGS

(b)	Acyl carrier protein
<i>actI</i> ORF3	EDIGYD8LALMETA
<i>gra</i> ORF3	EELGYD8LALMESA
<i>tcm</i> ORF3	QDLGYD8LALLEIS
<i>whiE</i> ORFV	DTFGLD8LGLLGIV
<i>S. cinnamonensis</i> ORF1	ALLGYE8LALLETG
<i>S. venezuelae</i> ORF3	ETLGYE8LALLETG

(c)	NAD(P) binding domain
<i>actIII</i> ORF5	VNNAGRPPGGATAELAD
<i>gra</i> ORF5	VNNAGRSGGGATAEID
<i>S. cinnamonensis</i> ORF5	VNNAGRSGGGVTADLTD
<i>S. venezuelae</i> ORF5	VNNAGRSAGVTADIAD

Fig. 4. Alignment by the PILEUP program (Genetics Computer Group, University of Wisconsin, Madison, WI, USA) of segments of the amino acid sequences deduced from ORFs in the *S. venezuelae* ISP5230 and other streptomycete PKS gene clusters (see text for references). (a) ORF1 sequences around the presumed β -ketoacylsynthase active site cysteine and the presumed acyltransferase active site serine. (b) ORF3 sequences around the active site serine in putative ACPs. (c) ORF5 sequences around the presumed NAD(P)-binding domain of β -ketoreductases.

unmethylated plasmid DNA isolated from an *E. coli* ET12567 transformant was used to transform *S. venezuelae* ISP5230; approximately 30 Thio^R colonies were obtained per μ g of plasmid DNA. Agarose gel electrophoresis and Southern hybridization with pHJL400 as a probe failed to detect free plasmid in DNA extracted from the transformants, but only two of five samples examined gave hybridization signals. In each of these, the signal corresponded to chromosomal DNA in mobility. Integration of pJV63 into the two transformants VS651 and VS652 was confirmed by Southern analysis of restriction digests of genomic DNA using the 2.6 kb *PstI*-*PstI* fragment and pHJL400 as probes. Wild-type DNA digested with *MluI* or *EcoRI* and probed with the 2.6 kb fragment gave signals at 7.0 kb and approximately 30 kb, respectively. In contrast, VS651 and VS652 digested with *EcoRI* gave signals at 5.0, 8.4 and about 35 kb, and after digestion with *MluI* at above 30 kb (Fig. 5a). When the membrane was stripped to remove the probe and rehybridized with pHJL400, each of the signals in the wild-type DNA digests, and that at 5.0 kb in the *EcoRI* digests of the integrants were absent. The sizes and intensities of the signals, and the results with the different probes, were all consistent with integration into the 2.6 kb region of the chromosome of at least three copies of pJV63 (Fig. 5b).

No differences between the wild type and VS651 or VS652 were detected in growth, the abundance of spores on MYM medium, or the blue-green pigmentation of the spores. In contrast, production of jadomycin B in strains VS651 and VS652 was severely depressed. Whereas

Fig. 5. Southern hybridization of genomic DNA from *S. venezuelae* ISP5230 and transformants VS651 and VS652; the 2.6 kb *PstI*-*PstI* fragment from λ LH7 was used as the probe. (a) Autoradiogram of labelled DNA fragments after hybridization. Lanes: 1, *EcoRI*-digested pJV63; 2, *MluI*-digested VS652 DNA; 3, *MluI*-digested VS651 DNA; 4, *MluI*-digested ISP5230 DNA; 5, *EcoRI*-digested VS652 DNA; 6, *EcoRI*-digested VS651 DNA; 7, *EcoRI*-digested ISP5230 DNA. Fragment sizes were measured against *HindIII*-digested λ DNA. (b) Schematic diagram postulating tandem integration of three copies of pJV63 into the *S. venezuelae* chromosome and accounting for the products of digestion with *MluI* (M) and *EcoRI* (E). The numbers are restriction map distances in kb.

cultures of the wild type and of control strains transformed with pHJL400 or pJV62 showed strong orange pigmentation in the supernatant broth 48 h after ethanol

treatment, VS651 and VS652 cultures remained unpigmented even after 72 h. Extraction and HPLC analysis of the cultures confirmed the presence of jadomycin B at the expected concentration (30 mg l^{-1} ; Doull *et al.*, 1993) in the wild-type and control cultures. In cultures of VS651 and VS652, a small peak corresponding to jadomycin B represented 6% of that in the positive controls. A control not treated with ethanol gave no detectable peak for jadomycin B.

DISCUSSION

Cloning and sequence analysis of the fragment of *S. venezuelae* ISP5230 DNA that hybridizes with *actI* from *S. coelicolor* A3(2) has confirmed the initial indication from hybridizations by Malpartida *et al.* (1987a, b) that *S. venezuelae* possesses genes for the biosynthesis of polyketide-derived metabolites. The results add yet further evidence of the value of *act* probes in detecting strains producing type-II polyketide metabolites. The *S. venezuelae* strain probed by Malpartida *et al.* (1987a, b) differs from ISP5230 in several characteristics (Ahmed & Vining, 1983; Stuttard, 1982), but both fall within the species *venezuelae* (Stuttard, 1982), and both are known to produce chloramphenicol (Vining & Westlake, 1984). The ISP5230 strain has only recently been reported to form the polyketide-derived jadomycins (Ayer *et al.*, 1991), and indeed the earlier isolation of mutants blocked in chloramphenicol production (Doull *et al.*, 1985) was consistent with this antibiotic being the sole active product; failure to detect the jadomycins is attributed in a large measure to the unusual culture conditions required

for their production. Under conditions optimized for jadomycin biosynthesis, strain 13s (UC2374) produces these compounds in yields similar to ISP5230 (Singh, 1992).

The five ORFs located in the 4.8 kb of *S. venezuelae* ISP5230 DNA sequenced show marked similarity to genes in streptomycete type II PKS clusters. A tentative assignment of their functions based on comparisons of derived amino acid sequences suggests that ORF1 encodes a ketoacyl synthase, ORF2 a closely related polypeptide that influences the chain length of the PKS product, ORF3 an ACP, ORF4 a bifunctional cyclase/dehydrase, and ORF5 a ketoreductase. The presence of ORF5 among the *jad* (jadomycin biosynthesis) genes is consistent with the absence from the jadomycin B structure of a hydroxyl group at C-10 (see Fig. 1). In containing the ORF5 ketoreductase function, the *jad* PKS differs from those of *tcm* (Bibb *et al.*, 1989), *whiE* (Davis & Chater, 1990) and *sch* (*S. halstedii* spore pigment; Blanco *et al.*, 1993; whereas the *tcm* metabolite (tetracenomycin C) retains an oxygen substituent at C-3, jadomycin B at the corresponding position (nine carbons from the carboxyl end of the polyketide chain; Bartel *et al.*, 1990) is unsubstituted. The arrangement of ORFs 1, 2 and 3 in the group of *jad* PKS genes has features in common with most type-II streptomycete PKS genes (Fig. 6), but the location of ORF5 places *jad* in the subset represented by *otc* (Kim *et al.*, 1994), '*fren*' (Bibb *et al.*, 1994), *gris* (Yu *et al.*, 1994) and the *mon* cluster of *S. cinnamomensis* (Arrowsmith *et al.*, 1992). In these groups, ORF5 is located between ORF3 and ORF4, although with various spacings; the *jad* arrangement most closely resembles that of *gris*, the PKS gene cluster for griseusin biosynthesis in *Streptomyces griseus*. Griseusin (Tsuji *et al.*, 1976) and jadomycin are probably each derived from C_{20} polyketide intermediates that fold in a similar pattern. The arrangement of the *mon* gene cluster differs from those of *jad* and *gris* only in the closer spacing of ORFs 3 and 5. Although the *mon* metabolite has not yet been chemically characterized, the polyether antibiotic monensin and spore pigments are excluded as candidates (Arrowsmith *et al.*, 1992) and a structural relationship to jadomycin is possible.

No attempt was made to determine whether the size or DNA structure of the insert accounted for the failure of pJV62, containing the 0.6 kb *Sma*I fragment of ORF1, to integrate into the *S. venezuelae* ISP5230 chromosome, whereas integration occurred when the vector carried the 2.6 kb *Pst*I fragment containing the C-terminal portion of ORF2, the complete sequence of ORF3 and ORF5, and the N-terminal portion of ORF4. Information on expression of the *jad* PKS genes is lacking, but the corresponding *act* PKS genes all lie within a single transcriptional unit (Fernández-Moreno *et al.*, 1992). The isolation of *S. venezuelae* transformants disrupted in jadomycin B production not only indicates that the PKS gene cluster is involved in jadomycin B biosynthesis, but also supports the assumption that ORFs 2–4 are co-transcribed. Production of a small amount of jadomycin B by the disruptants suggests that during growth of the culture, some of the mycelium had excised pJV63, thereby

Fig. 6. A diagrammatic comparison of gene organization in the PKS clusters cloned from *S. coelicolor* A3(2) (*act* and *whiE*), *S. violaceoruber* (*gra*), *S. glaucescens* (*tcm*), *S. rimosus* (*otc*), *S. roseoflavus* (*fren*), *S. griseus* (*gris*), *S. cinnamomensis* (*mon*), and *S. venezuelae* ISP5230 (*jad*): see text for references.

restoring jadomycin B synthesis. Jadomycin production increased as expected when disruptant cultures were supplemented with 50 µg thiostrepton ml⁻¹, which would select for strains with high-level thiostrepton resistance resulting from excision of the multi-copy vector. Excision of integrated plasmids is not unexpected (MacNeil *et al.*, 1992).

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REFERENCES

- Ahmed, Z. U. & Vining, L. C. (1983). Evidence for a chromosomal location of the genes coding for chloramphenicol production in *Streptomyces venezuelae*. *J Bacteriol* **154**, 239–244.
- Aidoo, D. A., Barrett, K. & Vining, L. C. (1990). Plasmid transformation of *Streptomyces venezuelae*: modified procedures used to introduce the genes for *p*-aminobenzoate synthetase. *J Gen Microbiol* **136**, 657–662.
- Arrowsmith, T. J., Malpartida, F., Sherman, D. H., Birch, A., Hopwood, D. A. & Robinson, J. A. (1992). Characterization of *actI*-homologous DNA encoding polyketide synthase genes from the monensin producer *Streptomyces cinnamonensis*. *Mol & Gen Genet* **234**, 254–264.
- Ayer, S. W., McInnes, A. G., Thibault, P., Walter, J. A., Doull, J. L., Parnell, T. & Vining, L. C. (1991). Jadomycin, a novel 8H-benz[b]oxazolo[3,2-f]phenanthridine antibiotic from *Streptomyces venezuelae* ISP5230. *Tetrahedron Lett* **32**, 6301–6304.
- Bartel, P. L., Zhu, C.-B., Lampel, J. S., Dosch, D. C., Connors, N. C., Strohl, W. R., Beale, J. M., Jr & Floss, H. G. (1990). Biosynthesis of anthraquinones by interspecies cloning of actinorhodin biosynthetic genes in streptomycetes: clarification of actinorhodin gene functions. *J Bacteriol* **172**, 4816–4826.
- Bibb, M. J., Findlay, P. R. & Johnson, M. W. (1984). The relationship between base composition and codon usage in bacterial genes and its use in the simple and reliable identification of protein-coding sequences. *Gene* **30**, 157–166.
- Bibb, M. J., Biró, S., Motamedi, H., Collins, J. F. & Hutchinson, C. R. (1989). Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcmI* genes provides key information about the enzymology of polyketide antibiotic biosynthesis. *EMBO J* **8**, 2727–2736.
- Bibb, M. J., Sherman, D. H., Ōmura, S. & Hopwood, D. A. (1994). Cloning, sequencing and deduced functions of a cluster of *Streptomyces* genes probably encoding biosynthesis of the polyketide antibiotic frenolicin. *Gene* **142**, 31–39.
- Blanco, G., Pereda, A., Mendez, C. & Salas, J. (1992). Cloning and disruption of a fragment of *Streptomyces halstedii* DNA involved in the biosynthesis of a spore pigment. *Gene* **112**, 59–65.
- Blanco, G., Brian, P., Pereda, A., Mendez, C., Salas, J. A. & Chater, K. F. (1993). Hybridization and DNA sequence analysis suggest an early evolutionary divergence of related biosynthetic gene sets encoding polyketide antibiotics and spore pigments in *Streptomyces* spp. *Gene* **130**, 107–116.
- Carter, P., Bedovelle, H. & Winter, G. (1985). Improved oligonucleotide site-directed mutagenesis using M13 vectors. *Nucleic Acids Res* **13**, 4431–4443.
- Davis, N. K. & Chater, K. F. (1990). Spore colour in *Streptomyces coelicolor* A3(2) involves the developmentally regulated synthesis of a compound biosynthetically related to polyketide antibiotics. *Mol Microbiol* **4**, 1679–1691.
- Denhardt, D. T. (1966). A membrane filter technique for the detection of complementary DNA. *Biochem Biophys Res Commun* **23**, 641–646.
- Doull, J. L., Ahmed, Z., Stuttard, C. & Vining, L. C. (1985). Isolation and characterization of *Streptomyces venezuelae* mutants blocked in chloramphenicol biosynthesis. *J Gen Microbiol* **131**, 97–104.
- Doull, J. L., Ayer, S. W., Singh, A. K. & Thibault, P. (1993). Production of a novel polyketide antibiotic, jadomycin B, by *Streptomyces venezuelae* following heat shock. *J Antibiot* **46**, 869–871.
- Doull, J. L., Singh, A. K., Hoare, M. & Ayer, S. W. (1994). Conditions for the production of jadomycin B by *Streptomyces venezuelae* ISP5230: effects of heat shock, ethanol treatment and phage infection. *J Ind Microbiol* **13**, 120–125.
- Fernández-Moreno, M. A., Martínez, E., Boto, L., Hopwood, D. A. & Malpartida, F. (1992). Nucleotide sequence and deduced functions of a set of co-transcribed genes of *Streptomyces coelicolor* A3(2) including the polyketide synthase for the antibiotic actinorhodin. *J Biol Chem* **267**, 19278–19290.
- Hallam, S. E., Malpartida, F. & Hopwood, D. A. (1988). Nucleotide sequence, transcription and deduced function of a gene involved in polyketide antibiotic synthesis in *Streptomyces coelicolor*. *Gene* **74**, 305–320.
- Hopwood, D. A. & Khosla, C. (1992). Genes for polyketide secondary metabolic pathways in microorganisms and plants. *CIBA Found Symp* **171**, 88–112.
- Hopwood, D. A. & Sherman, D. H. (1990). Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu Rev Genet* **24**, 37–66.
- Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P. & Schrepf, H. (1985). *Genetic Manipulation of Streptomyces: a Laboratory Manual*. Norwich, UK: John Innes Foundation.
- Kieser, T. (1984). Factors affecting the isolation of ccc DNA from *Streptomyces lividans* and *Escherichia coli*. *Plasmid* **12**, 290–296.
- Kim, E.-S., Bibb, M. J., Butler, M. J., Hopwood, D. A. & Sherman, D. H. (1994). Sequences of the oxytetracycline polyketide synthase-encoding *otc* genes from *Streptomyces rimosus*. *Gene* **141**, 141–142.
- Larson, J. L. & Hershberger, C. L. (1986). The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. *Plasmid* **15**, 199–209.
- Malpartida, F., Hallam, S. E., Kieser, H. M. & Hopwood, D. A. (1987a). Organization of the actinorhodin genes of *Streptomyces coelicolor* and their use as probes to isolate other polyketide biosynthetic genes: implications for 'hybrid' antibiotic production. In *Proceedings of the 5th International Symposium Genetics of Industrial Microorganisms*, 1986, pp. 29–39. Edited by M. Alačević, D. Hranueli & Z. Toman. Zagreb, Yugoslavia: Pliva.
- Malpartida, F., Hallam, S. E., Kieser, H. M., Motamedi, H., Hutchinson, C. R., Butler, M. J., Sugden, D. A., Warren, M., McKillop, C., Bailey, C. R., Humphreys, G. O. & Hopwood, D. A. (1987b). Homology between *Streptomyces* genes coding for synthesis of different polyketides used to clone antibiotic biosynthetic genes. *Nature* **325**, 818–821.
- McDaniel, R., Ebert-Khosla, S., Hopwood, D. A. & Khosla, C. (1993). Engineered biosynthesis of novel polyketides. *Science* **262**, 1546–1550.

- MacNeil, D. J. (1988).** Characterization of a unique methyl-specific restriction system in *Streptomyces avermitilis*. *J Bacteriol* **170**, 5607–5612.
- MacNeil, D. J., Gewain, K. M., Rudy, C. L., Dezeny, G., Gibbons, P. H. & MacNeil, T. (1992).** Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61–68.
- Meese, E., Olson, S., Leis, L. & Trent, J. (1990).** Quick method for high yields of lambda bacteriophage DNA. *Nucleic Acids Res* **18**, 1923.
- O'Hagan, D. (1992).** Biosynthesis of polyketide metabolites. *Nat Prod Rep* **9**, 447–479.
- Sambrook J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Shen, B. & Hutchinson, C. R. (1993).** Enzymatic synthesis of a bacterial polyketide from acetyl and malonyl coenzyme A. *Science* **262**, 1535–1540.
- Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M., Bibb, M. J. & Hopwood, D. A. (1989).** Structure and deduced function of the granaticin producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tü22. *EMBO J* **8**, 2717–2725.
- Singh, A. K. (1992).** *Production of the antibiotic jadomycin B by S. venezuelae ISP5230: effects of heat and ethanol*. MSc thesis, Dalhousie University, Halifax, Canada.
- Southern, E. M. (1975).** Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* **98**, 503–517.
- Stuttard, C. (1982).** Temperate phages of *Streptomyces venezuelae*: lysogeny and host specificity shown by phages SV1 and SV2. *J Gen Microbiol* **128**, 115–121.
- Tsuji, N., Kobayashi, M., Terui, Y. & Tori, K. (1976).** The structures of griseusins A and B, new isochromanquinone antibiotics. *Tetrahedron* **32**, 2207–2210.
- Vining, L. C. & Westlake, D. W. S. (1984).** Chloramphenicol: properties, biosynthesis and fermentation. In *Biotechnology of Industrial Antibiotics*, pp. 387–409. Edited by E. J. Vandamme. New York: Marcel Dekker.
- Yu, T.-W., Bibb, M. J., Revill, W. P. & Hopwood, D. A. (1994).** Cloning, sequencing and analysis of the griseusin polyketide synthase gene cluster from *Streptomyces griseus*. *J Bacteriol* **176**, 2627–2634.
- Zhang, H. L., He, X. G., Adeferati, A., Gallucci, J., Cole, S. P., Beale, J. M., Keller, P. J., Chang, C. J. & Floss, H. G. (1990).** Mutactin, a novel polyketide from *Streptomyces coelicolor*: structure and biosynthetic relationship to actinorhodin. *J Org Chem* **55**, 1682–1684.

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