Cloning and functional analysis of a phosphopantetheinyl transferase superfamily gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP5230

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Sequence analysis of a *Xho*/*Sac* fragment of chromosomal DNA downstream of *jadL* in the *Streptomyces venezuelae* ISP5230 gene cluster for jadomycin biosynthesis detected a partial ORF similar in its deduced amino acid sequence to the *hetI* product involved in synthesizing a regulator of heterocyst spacing in *Anabaena*. By probing a phage library of *S. venezuelae* DNA with the *Xho*/*Sac* fragment, the authors identified and isolated a hybridizing clone. The nucleotide sequence of its DNA contained three complete ORFs (*jadM*, *N* and *X*) and one incomplete ORF (*jadO*). The *jadM* ORF lay immediately downstream of, and partially overlapped, *jadL*. It contained 786 nucleotides encoding an amino acid sequence like those of enzymes in the phosphopantetheinyl transferase family. The *jadN* ORF contained 1794 nucleotides and encoded an amino acid sequence resembling acyl-CoA decarboxylases, thus suggesting a role in polyketide condensation reactions. The *jadX* ORF was not identified, but the partial *jadO* showed marked similarities in its deduced amino acid sequence to NDP-hexose-2,3-dehydratases, indicating a role in forming the sugar component of jadomycin B. Expression of *jadM* in *Escherichia coli* and examination of the product by SDS-PAGE established that the ORF encoded a 29 kDa protein, corresponding in size to the 262 amino acid polypeptide deduced from the *jadM* sequence. Evidence from a Northern hybridization indicated that *jadM* expression is correlated with jadomycin B synthesis. Cultures of *S. venezuelae* ISP5230 disrupted in *jadM* produced only 2–5% of the wild-type titre of jadomycin B, but grew well and produced chloramphenicol normally. The authors conclude that *jadM* encodes a holo-ACP synthase needed primarily for jadomycin B biosynthesis.

**Keywords:** phosphopantetheinyl transferase gene, *jad* genes, polyketide antibiotic, acyl carrier protein synthase

INTRODUCTION

Polyketide synthases (PKSs) direct head-to-tail assembly of acyl precursors into intermediates that can then be processed to form a broad range of final products. The PKSs fall into two organizational groups: type I are multifunctional proteins whereas type II are multi-enzyme complexes. Genes for PKSs have been cloned and characterized from numerous bacteria, fungi and plants (Hopwood & Sherman, 1990; Hopwood, 1997). The actinomycete *Streptomyces venezuelae* ISP5230 produces, in addition to chloramphenicol, a second antibiotic, jadomycin B (Doull *et al.*, 1993, 1994), deduced from its chemical structure and the pattern of
its labelling by $[^{13}C]$acetate to be a member of the polyketide family (S. Ayer, personal communication). Molecular genetic evidence has confirmed the structure assignment (Han et al., 1994; Yang et al., 1995, 1996) and established that jadomycin is derived from a decapolyketide generated by an iterative type-II PKS complex encoded by a chromosomal gene cluster (Meurer et al., 1997; Kulowski et al., 1999). A gene encoding a biotin-dependent acyl-CoA carboxylase potentially able to synthesize the malonyl-CoA needed to supply the C-2 extender units for jadomycin biosynthesis has been identified within the jad gene cluster (Han et al., 2000).

The polyketide family of secondary metabolites shares a general biosynthetic mechanism with the fatty acids of primary metabolism. The carbon skeletons are formed by sequential condensation of activated low-molecular-mass acids, such as acetate, propionate and butyrate. The PKS condensation reaction is similar to that used by the fatty acid synthases (FAS) found in all organisms where fatty acids are required for the biosynthesis of lipids (Hopwood, 1997). Both FAS and FAS enzyme complexes require post-translational modification of their constituent acyl carrier proteins to become catalytically active. The inactive apo-proteins are converted to active holo-enzymes by esterifying a specific serine hydroxyl with the 4'-phosphopantetheine prosthetic group of coenzyme A (Lambalot et al., 1996). Genes for the phosphopantetheinyl transferases of fatty acid synthase (ACPS) and enterobactin synthase (EntD) in E. coli have been identified (Lambalot et al., 1996), as has the gene for Sfp, responsible for activating surfactin synthetase in Bacillus subtilis (Quadri et al., 1999).

The jad cluster of genes for jadomycin production in S. venezuelae ISP5230 is made up of a core group, jadABCDE, encoding the PKS that assembles the parent polyketide (Han et al., 1994) and auxiliary genes encoding supplementary enzymes with tailoring (Yang et al., 1996) or regulatory functions (Yang et al., 1995). Among the tailoring group are jadE, encoding an oxidoreductase that reduces the C-10 ketone of the incipient angucycline ring system in jadomycins (Kulowski et al., 1999), and jadF, which encodes another oxidoreductase that opens the angucycline B ring to facilitate insertion of isoleucine. Less clearly in the tailoring group is jadJ, which encodes an enzyme complex for carboxylating acetyl-CoA to malonyl-CoA and might be expected to serve a general metabolic purpose. However, the role of the gene in S. venezuelae appears to be linked to extension of the polyketide chain used for jadomycin B biosynthesis (Han et al., 2000).

Here, we report evidence that another gene, jadM, located unambiguously in the jad cluster, encodes a phosphopantetheinyl transferase that is functionally associated with biosynthesis of the polyketide-derived antibiotic.

**METHODS**

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

**Culture conditions.** E. coli strains for plasmid isolation were grown as described by Sambrook et al. (1989). For plasmid DNA isolation, the protocol of Leder et al. (1977) was used. Jadomycin B was produced in galactose-isoleucine medium (Doull et al., 1994), but with isoleucine doubled to 78 mg ml$^{-1}$ (giving Gal2I medium). Recombinant E. coli DH5α strains were grown in LB medium containing appropriate antibiotics (Sambrook et al., 1989). To isolate genomic DNA, S. venezuelae strains were grown in MYM medium (Stuttard, 1982) at 30 °C for 24 h on a rotary shaker (220 r.p.m.).

**DNA manipulation and transformation.** Plasmid DNA was isolated from E. coli by the alkaline lysis method (Sambrook et al., 1989). Bacteriophage λ DNA was purified essentially as reported by Yamamoto et al. (1970). Genomic DNA was isolated as described by Hopwood et al. (1985). T4 DNA ligase and restriction enzymes were used as recommended by the suppliers. Competent cells of E. coli strains were prepared and transformed by the procedures of Sambrook et al. (1989). For conjugal transfer of plasmids from E. coli to streptomycetes, the protocol of Mazodier et al. (1989) was followed.

**Cloning and sequencing of jadM.** A 0.55 kb Xhol/SacI fragment of jadM was obtained from a segment of S. venezuelae ISP5230 genomic DNA cloned in Lambda LH7 (Han et al., 1994) and subcloned in pBluescript II SK(+) as pJV104 (McVey, 1998). The plasmid insert, labelled with 32P, was used to probe a genomic library prepared in Lambda GEM-11 from a partial Sau3AI digest of S. venezuelae ISP5230 (Facey, 1994) plated on LB agar in three 9 cm diameter Petri dishes, and incubated overnight to give approximately 109 plaques. Hybridization (Sambrook et al., 1989) yielded eight labelled clones (Lambda LW1–LW8), from which DNA was extracted, digested with XhoI and probed by Southern hybridization with the 0.55 kb Xhol/SacI fragment from the plasmid pJV104. A 7.0 kb labelled fragment was detected in DNA from six of the eight clones. This fragment from LW3 was inserted in the XhoI site of pBluescript II SK(+) to obtain the plasmid pJV401. From pJV401, a 3.3 kb XhoI/KpnI fragment containing jadM was subcloned in pBluescript II SK(+) to give pJV402. Nested overlapping deletions were introduced into the pJV402 insert with an ExoIII/S1 deletion kit (MBI Fermentas) and the cloned DNA was sequenced (GenBank accession numbers AF222693 and AY026363) by the dideoxynucleotide chain-termination method (Sanger et al., 1977).

**Sequence analysis and disruption.** The sequenced pJV402 insert was examined with ORF Finder (NCBI) and FramePlot 2.3 (Ishikawa & Hotta, 1999) to detect ORFs. BLASTX was used with individual ORF sequences to query GenBank nucleic acid and protein databases. Sequences were aligned and their relatedness was assessed with CLUSTAL W (Thompson et al., 1994). The preferred target for insertional inactivation was an NrdI site 85 bp downstream of the jadM start codon. However, this site was only 210 bp from the XhoI end of the cloned fragment, so to increase opportunities for double crossovers when the plasmid was introduced into S. venezuelae, the 3.4 kb XhoI/KpnI chromosomal sequence cloned in pJV402 was extended at the XhoI end by ligation to a 3.3 kb NrdI/EcoRV/XhoI fragment of pJV405 that contained S. venezuelae DNA adjoining jadM. The pJV405 insert had initially been subcloned in pBluescript II SK(+) as a 4.0 kb SacI fragment from Lambda LH7. One of the products (pJV404A) was digested with NrdI and XhoI, then subcloned as a 1.3 kb fragment between the EcoRV and XhoI sites of pBluescript II SK(+) to give pJV406. Digestion of pJV405 with XhoI/KpnI and ligation with the 3.4 kb XhoI/KpnI

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**Table 1. Bacterial strains, plasmids and phages**

<table>
<thead>
<tr>
<th>Strain, plasmid or phage</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tr>
<td><strong>Streptomyces venezuelae</strong></td>
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<tr>
<td>ISP5230</td>
<td>Wild-type, jadomycin B producer</td>
<td>Stuttard (1982)</td>
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<tr>
<td>VS1075</td>
<td>ISP5230 with jadM disrupted by DNA conferring Am&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>VS1076</td>
<td>Same as VS1075 but with Am&lt;sup&gt;+&lt;/sup&gt; in opposite orientation</td>
<td>This study</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<tr>
<td>BL 21(DE3)</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;ompT bsdSB (r&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;−&lt;/sup&gt;) gal dcm</td>
<td>Novagen</td>
</tr>
<tr>
<td>DH5αF′IQ</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;α80lacZα(lacZYA−argF) U169 deoR recA1 endA1 bsdR17 (r&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;−&lt;/sup&gt;) pboA supE11 i− thi−1 gyrA96 relA1/F' proAB&lt;sup&gt;+&lt;/sup&gt; lac&lt;sup&gt;−&lt;/sup&gt;VZAM15 zsf::Tn5[Km&lt;sup&gt;+&lt;/sup&gt;]</td>
<td>Gibco BRL</td>
</tr>
<tr>
<td>ET 12567(pUZ8002)</td>
<td>ET12567 containing pUZ8002</td>
<td>M. Paget, John Innes Centre, Norwich, UK</td>
</tr>
<tr>
<td>LE 392</td>
<td>Δ(lacIZY&lt;sup&gt;−&lt;/sup&gt;)6 galK2 galT22 metBI trpR55 i&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Maniatis et al. (1982)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
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<tr>
<td>pBluescript II</td>
<td>Phagemid derivative of pUC18 with SK+ and KS+, f1 ori, and primer-binding sites for T3 and T7</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pET 21+</td>
<td>amp, primer-binding sites for T7 promoter and terminator</td>
<td>Novagen</td>
</tr>
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<td>pHJL400</td>
<td>tsr amp lacZ, bifunctional</td>
<td>Larson &amp; Hershberger (1986)</td>
</tr>
<tr>
<td>pUZ8002</td>
<td>cmk km tra</td>
<td>M. Paget</td>
</tr>
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<td>pJV104</td>
<td>pBluescript II SK(+) containing 5′-region of jadM in a 0.55 kb Xhol/SacI insert sub-cloned from Lambda LH7</td>
<td>McVey (1998)</td>
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<tr>
<td>pJV105</td>
<td>pHJL400 with 4.0 kb SacI fragment from Lambda LH7</td>
<td>McVey (1998)</td>
</tr>
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<td>pJV225</td>
<td>pBluescript II SK(+) containing Am&lt;sup&gt;+&lt;/sup&gt; gene flanked by multiple cloning sites</td>
<td>Chang (1999)</td>
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<td>pJV326</td>
<td>pHJL400 containing 0.76 kb PsiI insert with oriT, bifunctional</td>
<td>J. Y. He (personal communication)</td>
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<td>pBluescript II SK(+) with 6.6 kb Xhol insert from Lambda LW3</td>
<td>This study</td>
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<td>pJV402</td>
<td>pBluescript II SK(+) with 3.4 kb Xhol/KpnI insert containing 5′-region of jadM and downstream genes</td>
<td>This study</td>
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<tr>
<td>pJV403</td>
<td>pBluescript II SK(+) with 3.0 kb EcoRV/Xhol insert containing 5′-region of jadM and downstream genes</td>
<td>This study</td>
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<td>pJV404A/B</td>
<td>pBluescript II SK(+) with 4.0 kb SacI fragment from pJV105 (two orientations)</td>
<td>This study</td>
</tr>
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<td>pJV405</td>
<td>pBluescript II SK(+) with 1.3 kb NruI/Xhol fragment from pJV404A in its EcoRV site</td>
<td>This study</td>
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<td>pJV406</td>
<td>pBluescript II SK(+) with 4.7 kb NruI/KpnI fragment (ligated pJV402 and pJV405 inserts) in its EcoRV site</td>
<td>This study</td>
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<tr>
<td>pJV407A/B</td>
<td>pBluescript II SK(+) with 6.3 kb NruI/KpnI fragment containing 1.6 kb Am&lt;sup&gt;+&lt;/sup&gt; gene in the NruI site (two orientations)</td>
<td>This study</td>
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<tr>
<td>pJV408A/B</td>
<td>pJV326 with blunted 5.6 kb EcoRI/EcoRV fragment from pJV407A/B in its blunted BamHI site (two orientations)</td>
<td>This study</td>
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<td>pJV409</td>
<td>pET-21(+) with a blunt-ended 1.0 kb Xhol/PvuII fragment from pJV402 in its blunted BamHI site</td>
<td>This study</td>
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<tr>
<td><strong>Phages</strong></td>
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<tr>
<td>Lambda LH7</td>
<td>Lambda GEM-11 with a 14 kb insert of S. venezuelae DNA</td>
<td>Han et al. (1994)</td>
</tr>
<tr>
<td>Lambda LW3</td>
<td>Lambda GEM-11 with an 11.5 kb insert of S. venezuelae DNA hybridizing with pJV104</td>
<td>This study</td>
</tr>
<tr>
<td>Lambda gene bank</td>
<td>Lambda GEM-11 with 9.0−23.0 kb inserts of S. venezuelae genomic DNA</td>
<td>Facey (1994)</td>
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</table>

Fragment from pJV402 gave pJV406 (Fig. 1a). Digestion of pJV406 with NruI, dephosphorylation with calf intestinal alkaline phosphatase and ligation with the 1.6 kb EcoRV cassette containing apr (retrieved from pJV225) yielded constructs with apr in alternative transcriptional orientations (opposite to jadM in pJV407A; similar in pJV407B). Linearizing pJV407A/B with EcoRI/EcoRV, blunting the 5.6 kb linear products with S1 nuclease and ligation to the promiscuous vector pJV326 linearized with BamHI and blunted with S1 nuclease, gave pJV408A/B (with the apr
Fig. 1. (a) Restriction map of the 4.6 kb fragment of *S. venezuelae* ISP5230 genomic DNA subcloned from the recombinant λ vectors LH7 (Han et al., 1994) and LW3 (see text). Numbers below the line at the top indicate distances in kb. The 3.4 kb region retrieved from λ LW3 and subcloned in pBluescript II SK(+) is shown as a thickened line. Arrows above the restriction map show the locations and orientations of ORFs (*jadM*, *jadN*, *jadX*, *jadO*) in the *jad* cluster.

Restriction sites in the inserts of plasmids pJV402, pJV405 and pJV406 are aligned below the chromosomal map. Abbreviations: B, BamHI; Ev, EcoRV; K, KpnI; Nr, NruI; Pv, PvuII; X, XhoI; Am R, apramycin resistance.

(b) Disruption of *jadM*: a cassette containing the apramycin-resistance gene in alternative orientations (A or B) was inserted into the NruI site within *jadM* cloned in pJV408. Introduction of the plasmids into *S. venezuelae* ISP5230 gave strains (VS1075 and VS1076) with double crossovers selected for their AmR ThioS phenotype.

Hybridization. Procedures described by Hopwood et al. (1985) and Sambrook et al. (1989) were used to screen an *S. venezuelae* ISP5230 genomic DNA library in Lambda GEM-11 (Facey, 1994). Phage propagated in *E. coli* LE392 was suspended in 2.5 ml top agar and spread on LB agar in 9 cm Petri plates to form plaques. Plaque DNA was adsorbed on a nylon membrane, denatured, neutralized and bound to the nylon (80 °C for 2 h) before incubation at 65 °C. Hybridization solution (Sambrook et al., 1989) with a 32P-labelled probe. The membrane was washed twice by agitation with 1 × SSC/0.1% SDS at room temperature and once with 0.1 × SSC/0.1% SDS at 65 °C for 1 h. It was then exposed to a Bio-Rad CS phosphor-imaging screen for approximately 24 h. Hybridization signals on the screen were detected using the GS-525 Molecular Imaging system (Bio-Rad). In Southern hybridizations, plasmid DNA digested with restriction enzymes was fractionated by agarose gel electrophoresis, transferred to a nylon membrane and processed as above.

Jadomycin production and analysis. Cultures of *S. venezuelae* ISP5230 were grown from a vegetative inoculum obtained by incubating a spore suspension (20 µl) in 25 ml MYM medium (Stuttard, 1982) in a 125 ml Erlenmeyer flask on a rotary shaker for 24 h at 30 °C. Portions (1 ml) of this culture were used to inoculate 25 ml Gal2I medium in 125 ml Erlenmeyer flasks. The Gal2I cultures, after incubation as above for 6 h, were supplemented with 0–0.75 ml of absolute ethanol and incubation was resumed for 48 h. Filtered broths were extracted with ethyl acetate and assayed by HPLC using Beckman System Gold equipment and software. Culture
extracts (20 μl) in methanol were injected on a C18 reverse-phase silica column (50 x 4.6 mm) and eluted at a flow rate of 1 ml min⁻¹ with a linear gradient from 100% solvent A (acetonitrile/water, 1:1) to 25, 50, 100 and 0% solvent B (100% acetonitrile) programmed to change after 3, 6-5, 7-5 and 10 min, respectively. Both solvents contained 0.1% (v/v) trifluoroacetic acid. Jadomycins in the eluate were monitored at 313 nm; jadomycin B had a retention time of 7.5 min.

**Northern hybridization.** RNA was isolated from Gal2I cultures of *S. venezuelae* ISP5230 with the modified Kirby mix, followed by phenol/chloroform extraction and DNase I treatment (Kieser et al., 2000). It was fractionated and adsorbed on nylon membranes (GeneScreen & GeneScreen Plus, NEN Life Science) by the method of Sambrook et al. (1989). Northern hybridization followed NEN's protocol for their products.

**Bioassay of chloramphenicol.** The method was modified from that of Doull et al. (1986). Spores of an *S. venezuelae* strain spread evenly on MYM agar in a 9 cm Petri plate were incubated at 30 °C for 48–72 h. Plugs were removed aseptically with a cork borer, placed equidistantly on MYM agar in 9 cm Petri plates and incubated at 30 °C for 12–14 h. They were then overlaid (2.5 ml per plate) with soft GNY agar (Malik & Vining, 1970) seeded with 1.0% (v/v) of a *Micrococcus luteus* culture grown overnight in GNY liquid medium. The overlaid plates were incubated overnight and the plugs were examined for zones of inhibition.

**Expression of jadM in *E. coli*.** A 1.0 kb *XhoI/PvuII* fragment containing *jadM* was retrieved from pJV406 (see Fig. 1a), blunt-ended with S1 nuclease and ligated (T4 DNA ligase) to pET-21(+), also linearized with *BanHI* and blunt-ended with S1 nuclease. Transforming *E. coli* BL21(DE3) with the ligation mixture yielded a colony from which pJV409 was isolated. Cultures of the transformant and *E. coli* BL21(DE3) harbouring pET-21(+) were grown to late-exponential phase in LB medium; the T7 polymerase promoter in pET-21(+) was induced with isopropylthio-β-galactopyranoside (1 mM final concentration) and cells harvested by centrifugation were resuspended in gel loading buffer (62.5 mM Tris/HCl, pH 6.8; 2%, w/v, SDS; 5%, w/v, 2-mercaptoethanol; 10%; w/v, glycerol; 0.025% bromphenol blue), lysed by heating at 100 °C for 3–5 min and analysed by SDS-PAGE (Laemmli, 1970). Total proteins electrophoresed on a 4% (w/v) polyacrylamide stacking gel and a 15% (w/v) separating gel were visualized by staining the gel with 0.1% Coomassie brilliant blue R-250.

**RESULTS**

**Sequence of jadM and downstream genes.** Sequencing and analysis of the 3.4 kb *XhoI/KpnI* insert in pJV402 detected three complete ORFs and one partial ORF (see Fig. 1a). Their 77-3, 70-0, 72-3 and 71-2, mol% G + C contents were in the range expected for streptomycetes. The putative ATG start codon of the ORF containing *jadM* was located 26 bp inside the 3′-end of the preceding ORF (*jadL*; McVey, 1998). A putative RBS (AAGG) for *jadM* could be recognized 10 bp upstream of the start codon, and an in-frame downstream stop codon (TGA) predicted a gene of 786 bp encoding a protein with 262 amino acids. The next ORF (containing *jadN*) was oriented for transcription in the same direction as *jadM*, and shared two nucleotides of its ATG start codon with the *jadM* stop codon. The *jadN* ORF was predicted to terminate 1578 bp downstream of its starting ATG. In a *blastx* search, the deduced amino acid sequence of *jadN* showed strong similarity to several malonyl-CoA decarboxylases and putative propionyl-CoA carboxylases. Malonyl-CoA is one of the most common chain-extender units for the biosynthesis of polyketide antibiotics in streptomycetes (Hopwood & Sherman, 1990), and its use in the synthesis of jadomycin would be expected. Therefore *jadN* may function as a malonyl-CoA decarboxylase in polyketide condensation reactions. The supply of malonyl-coenzyme A needed for these reactions is potentially available as the product of another *jad* cluster gene, *jadJ*, that encodes a biotin carboxylase–biotin carboxyl carrier protein able to convert acetyl-CoA to malonyl-CoA (Han et al., 2000). Possibly *jadN* functions in conjunction with *jadJ* to support polyketide chain extension in jadomycin biosynthesis. The third complete ORF in pJV402 (*jadX*) is transcribed in the same direction as *jadN*, but is separated from the 3′-end of *jadN* by 268 bp. A *blastp* search of GenBank for protein sequences resembling the deduced *jadX* amino acid sequence showed similarity to several proline-rich plant cell wall proteins, but the function of the gene in *S. venezuelae* is not known. Immediately downstream of *jadX*, and transcribed in the same direction, is the partial ORF containing a putative *jadO*. It begins with an ATG that shares the last letter in the *jadX* stop codon and its deduced amino acid sequence strongly resembled several NDP-hexose 2,3-dehydratases. As jadomycin B contains a 2,6-dideoxyhexose component in addition to the aromatic jadomycin aglycone, *jadO* is probably involved in biosynthesis of the sugar moiety.

**Function of jadM**

Database searches with *blastx* showed that the deduced amino acid sequence of *jadM* is 36% similar and 24% identical to the 237 amino acid sequence of HetI in *Anabaena PCC7120*. In this cyanobacterium, *hetI*, *hetM* and *hetN* have been implicated in the production of an unidentified secondary metabolite regulating heterocyst spacing (Black & Wolk, 1994). Sequence analysis indicates that HetI is an NAD(P)H-dependent enzyme similar to oxidoreductases associated with polyketide and fatty acid biosynthesis, and in addition suggests that HetM contains an ACP domain. Similarities between HetI and members (Spf, Gsp and Ent) of the phosphopantetheinyl transferase superfamily (Lambalot et al., 1996; Walsh et al., 1997; Silakowski et al., 1999) led Lambalot et al. (1996) to propose that HetI is a HetM-specific PPTase involved in synthesis of the secondary metabolic product influencing heterocyst formation. In addition to resembling the HetI sequence in *Anabaena*, the deduced amino acid sequence of *jadM* resembles the sequence of MtaA in *Stigmatella aurantiaca* (29% similar and 22% identical amino acids). This protein is a PPTase activating biosynthesis of the electron-transport inhibitor myxothiazol, probably by post-translational modification of MtaB, MtaG and the unique combination of PKs and nonribosomal peptide
synthetases (NRPSs) forming the biosynthetic machinery for making myxothiazol (Silakowski et al., 1999). MtaA may also be responsible for transferring Ppan to proteins involved in the biosynthesis of a variety of secondary metabolites in S. aurantica.

Comparison of JadM with the phosphopantetheinyl transferase superfamily

Phosphopantetheinyl transferase activity was first detected in EntD from E. coli and Spf from B. subtilis. Acyl carrier protein synthetase (ACPS), which catalyses conversion of the inactive apo-form of a fatty acid synthase complex to the functional form, was the first PPTase for which the gene was cloned and characterized. Several genes involved in peptide secretion (entD in E. coli, sfp in B. subtilis and gsp in Bacillus brevis) have since been identified, and their products appear to represent a new class of proteins (Borchert et al., 1994). Through refinement of sequence alignments that indicated 12–22% similarity with the ACPS peptide sequence, a PPTase superfamily that included the Spf group was identified (Lambalot et al., 1996; Gehring et al., 1997). Gsp is present in a locus required for gramicidin biosynthesis; EntD and Spf were originally reported to activate enterobactin and surfactin synthetase, respectively, and Spf was recently reported to modify the apo-form of heterologous recombinant proteins, including the PCP domain of Saccharomyces cerevisiae Lys2 (involved in lysine biosynthesis; Ehmann et al., 1999) and the E. coli ACP domain (Quadri et al., 1998a; Gokhale et al., 1999). Lambalot et al. (1996) identified two consensus motifs shared by PPTase family members, and further study implicated the conserved residues in enzymatic reactions transferring the phosphopantetheinyl moiety of coenzyme A to the hydroxyl of conserved serines in the ACP domain of PKS and the PCP domain of NRPS (Reuter et al., 1999).

**Fig. 2.** Multiple sequence alignment with CLUSTAL W. Highly conserved amino acids are shown in bold letters; conserved sequences in the PPTase superfamily are in bold and underlined. As an aid to locating key regions, the residues at positions 90, 107 and 151 are marked with a vertical arrow. The organisms involved are: Sfp, *Bacillus subtilis*; Lpa-14, *B. subtilis*; Psf-1, *B. pumilus*; Gsp, *B. brevis*; EntD*, *Salmonella typhimurium*; HetI, *Anabaena PCC7120*; JadM, *Streptomyces venezuelae*; MtaA, *Stigmatella aurantiaca*; CELT04G9, *Caenorhabditis elegans*; Nsh-orfC, *Streptomyces actuosus*; LYS5, *Saccharomyces cerevisiae*; 131454, *Schizosaccharomyces pombe*.
To determine whether JadM contained the PPTase consensus, CLUSTAL W was used to align the sequence with members of the PPTase superfamily (Fig. 2). Highly conserved amino acids in the superfamily were present in JadM, and also in some residues considered important in Sfp-type PPTases (Reuter et al., 1999). The crystal structure of Sfp enzymes indicates that the active site accommodates a magnesium ion that complexes with the pyrophosphate group in coenzyme A, the side chains of three acidic amino acids and one water molecule (Reuter et al., 1999). These are highly conserved regions that may interact with PCP substrates. In Sfp the Mg$^{2+}$-liganding residues Asp107 and Glu151 are highly conserved in every enzyme listed in Fig. 2, including JadM. The $\alpha$-phosphate of coenzyme A binds to Lys155 and His90. In JadM and all PPTases investigated, except 131454, Lys150 and His90 were highly conserved, while position 105 was invariably glycine for spatial reasons. The sequence motif Gly74-Lys75-Pro76 involved in binding the adenine base of coenzyme A was preserved in JadM, but with the Lys75 replaced by arginine. This is an acceptable substitution because Lys75 forms only hydrogen bonds and not a salt bridge with the main-chain carboxyls of Ile104, Lys153 and Gln156 (Reuter et al., 1999). A CLUSTAL W alignment of the deduced amino acid sequence of JadM with the sequences of PPTases from other organisms supported assignment of the gene to the PPTase superfamily. The phylogenetic tree in Fig. 3 indicates that JadM, Hetl and MtaA have a common ancestor, and that these three genes are more closely related to one another than to other members of the family. Comparisons of the deduced amino acid sequence of JadM with database sequences are consistent with such a conclusion.

**Temporal expression of jadM**

Cultures of *S. venezuelae* ISP5230 grown in Gal2I medium for 24, 48, 72, or 96 h, either with or without ethanol supplementation at 6 h, were assayed for jadomycin B, and total RNA extracted from the mycelium was used in Northern hybridizations. Gels from electrophoresis of 40 $\mu$g RNA samples were probed with the [32P]dCTP-labelled, 3.4 kb, *XhoI/KpnI* insert from pJV402 containing jadM. Expression of jadM mRNA was strongest 24 h after ethanol supplementation, and was still detectable at 48 h, but could not be detected at 96 h. The jadomycin B titre was highest 48 h after ethanol treatment, as reported by Doull et al. (1994), and had fallen to near zero in 72 h cultures. At all stages of growth, neither jadomycin B nor jadM mRNA was detected in cultures not treated with ethanol.

**Disruption of jadM**

To avoid restriction in *S. venezuelae* ISP5230, plasmid constructs pJV408A/B were passaged through the DNA methylation-deficient *E. coli* strain ET12567 (pUZ8002) before being transferred to *E. coli* to *S. venezuelae* by interspecies conjugation (Mazodier et al., 1989). pUZ8002 is a large (about 70 kb) plasmid carrying the *tra* genes that facilitate conjugal transfer of a plasmid containing the *oriT* sequence. Single colonies of VS1075 and VS1076 transconjugants resistant to apramycin but sensitive to thioestreptone were selected. When genomic DNA was extracted from *S. venezuelae* ISP5230, VS1075 and VS1076, digested with EcoRV/ BamHI, and probed by Southern hybridization with a [32P]-labelled 3.2 kb BamHI/EcoRV fragment of pJV406 containing jadM, the *S. venezuelae* ISP5230 digest gave a single strong signal at 3.2 kb, whereas VS1075 and VS1076 digests gave a comparable signal at 4.8 kb (Fig. 4). Use of the 1.6 kb *apr* cassette as a probe gave a single

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**Fig. 4.** Southern hybridization of genomic DNA digested with BamHI/EcoRV. Lane 1, *S. venezuelae* ISP5230; lane 2, VS1075; lane 3, VS1076; lane M, DNA size marker. Panel (a) probed with labelled 1.6 kb *Amr* gene; panel (b) probed with labelled 3.2 kb BamHI/EcoRV fragment.
strong hybridization signal at 4.8 kb from the disrupted strains VS1075 and VS1076, as expected for double-crossover mutants. Cultures of VS1075 and VS1076 grew normally on minimal agar, but produced only 2–5% of the wild-type jadomycin B titre when grown in Gal2I liquid medium optimized for jadomycin B production and supplemented with ethanol. The low titre in both strains indicated that insertion of the apr cassette interfered with jadM expression, irrespective of apr orientation. A polar effect of the insertion cannot be excluded, but the Am<sup>W</sup> gene is accompanied by its promoter (Kaster et al., 1983), and the apr cassette is not known to include a terminator that would interrupt transcription in both mutants. To determine if jadM was essential for chloramphenicol biosynthesis, the disrupted and wild-type strains were bioassayed after growth on MYM agar under conditions suitable for production and supplemented with ethanol. The low titre in both strains indicated that insertion of the apr cassette interfered with jadM expression, irrespective of apr orientation. A polar effect of the insertion cannot be excluded, but the Am<sup>W</sup> gene is accompanied by its promoter (Kaster et al., 1983), and the apr cassette is not known to include a terminator that would interrupt transcription in both mutants. To determine if jadM was essential for chloramphenicol biosynthesis, the disrupted and wild-type strains were bioassayed after growth on MYM agar under conditions suitable for chloramphenicol production (Doull et al., 1986). No decrease in the inhibition zone size was detected in strains VS1075 and VS1076, indicating that the jadM-disrupted mutants retained their ability to produce chloramphenicol.

**Expression of jadM in E. coli**

To confirm that the RBS (AAGG), ATG start codon and TGA stop codon detected in the cloned sequence correctly defined jadM, a 1.0 kb XhoI/PvuII pJV402 fragment expected to contain the gene (see Fig. 1a) was cloned in pET-21(+), giving pJV409. Extracts of E. coli BL21(DE3) cells harbouring pJV409 contained substantial amounts of a protein, close to 29.1 kDa in size (Fig. 5), that was absent from extracts of E. coli BL21(DE3) transformed with the pET-21(+) vector alone, and also from extracts of the transformed but uninduced E. coli host. These results imply that jadM supplied the RBS and start codon needed to synthesize the 29.1 kDa protein that was overexpressed after induction of the T7 polymerase promoter. Overall, they confirm that the jadM gene cloned from *S. venezuelae* ISP5230, and assigned to the PPTase superfamily by comparing the sequence of its product with database proteins, encodes a protein of the size estimated from the deduced amino acid sequence.

**DISCUSSION**

Although searching the GenBank database with BLASTX failed to identify *E. coli* ACP synthase or the Sfp/Gsp/EntD group of PPTases as homologues of JadM, and showed only 22–24% sequence identity between JadM and the two most closely related PPTases (HetI and MtaA), the deduced amino acid sequence of JadM contained residues highly conserved in the PPTase family; thus sequences present in the Sfp/Gsp/EntD group of PPTases were also present in the jadM product. Information now available on the crystal structure of Sfp indicates that these residues are most probably involved in interactions with the pantetheinylation substrate (Reuter et al., 1999), and suggests a similarity in reaction mechanism. We conclude that JadM contains a Ppan transferase domain and is a member of the PPTase superfamily. Its most probable function is to convert the inactive apo-enzyme form of JadC (the jad cluster ACP; Han et al., 1994) to the functional holo-enzyme by transferring the 4’-phosphopantetheinyl moiety from coenzyme A to the β-hydroxy group of the conserved serine in JadC. The newly introduced –SH group of the Ppan prosthetic group would act as a nucleophile in acylations by coenzyme A esters catalysed by a PKS (Lambalot et al., 1996). The severely decreased jadomycin B titre after insertional inactivation of jadM with an apr cassette indicates that the jadM product has a major role in jadomycin biosynthesis. The residual
production in jadM disruptants might arise from the activity of a FAS pantetheinyl transferase. The preliminary evidence obtained from a Northern hybridization indicated that jadM is expressed only in cultures stressed by ethanol treatment, and that expression reaches its maximum in 24 h, then decreases rapidly to become undetectable. This is consistent with previous results (Doull et al., 1993, 1994) showing that jadomycin B is produced by S. venezuelae ISP5230 only during a limited period after exposure to stress. Under our experimental conditions, peak expression of jadM preceded by 24 h the maximum jadomycin B titre, a lag suggesting that stress activation may be followed by relatively slow production and excretion of the antibiotic product. Nevertheless, the correlation between jadM transcription and jadomycin B production implies that JadM has a specific function in the pathway.

For the biosynthesis of type-II polyketide antibiotics in streptomycetes, each PKS complex must have a dedicated holo-ACP, which in turn requires a specific holo-ACP synthase. The latter enzymes function as integral components of the antibiotic biosynthesis pathway and differ from those that participate in fatty acid biosynthesis (Hopwood & Sherman, 1990; Hutchinson, 1995). However, both fren and gra apo-ACPs could be phosphopantetheinylated in vitro by purified E. coli ACPS. When combined with ACP-deficient act keto-synthase and chain-length factor isolated from Streptomyces coelicolor A3(2), the holo-ACPs formed in vitro were fully functional in polyketide synthesis (Carreras et al., 1997). Moreover, co-expression of actinorhodin and griseusin ACPs with ACPS in E. coli gave high titres of active holo-ACPS (Cox et al., 1997), and E. coli ACPS efficiently modified post-translationally the apo-ACPs involved in biosynthesis of granaticin, frenolicin, oxytetracycline and tetracenomycin (Gehring et al., 1996). These results imply that E. coli ACPS has broad substrate specificity. However, it will not recognize the apo-forms of several PCP and ArCP domains, including the apo-PCP domain of E. coli EntF and the apo-ArCP domain of E. coli EntB (Lambalot et al., 1996; Gehring et al., 1996; Quadri et al., 1998a). Our evidence that an S. venezuelae ISP5230 mutant disrupted in jadM is unaffected in the production of chloramphenicol, an antibiotic now known to be biosynthesized via a non-ribosomal peptide synthetase (J. Y. He, N. Magarvey, M. Pirae, K. A. Aidoo & L. C. Vining, unpublished results) indicates that JadM is not required for this process, and could mean that it is not recognized by the PCP domain in the NRPS. There may be a separate PPTase in the cm1 cluster catalysing the conversion of apo-PCP to its holo-form. Since the jadM-disrupted mutants VS1075 and VS1076 grew normally on minimal agar, jadM is also not essential for fatty acid biosynthesis, and thus appears to be a jadomycin B pathway-specific PPTase.

Although this report is the first to describe the cloning and characterization of a streptomycete of a gene encoding a PPTase, more than 20 examples of PPTases, including ACPS, EntD and O195 of E. coli, Sfp of B. subtilis and Gsp of B. brevis, have been added to the group on the basis of sequence similarity since the superfamily was first recognized (Lambalot et al., 1996; Walsh et al., 1997). However, only a few have been extensively characterized. Among these are PPT1 from the type-II fatty acid synthase of Brevibacterium ammoniagenes (Stuible et al., 1997), PPT2, which activates mitochondrial ACP in Saccharomyces cerevisiae (Stuible et al., 1998), Lys5 from the lysine biosynthesis system in S. cerevisiae (Ehmann et al., 1999), MtaA from the polyketide synthase/polypeptide synthetase complex synthesizing mycothiol in Stigmatella aurantiaca (Gaitatzis et al., 2001) and PptG, which is required in the assembly of mycobactin, the peptide-polyketide siderophore of Mycobacterium tuberculosis (Gehring et al., 1997; Quadri et al., 1998b). The crystal structure of Sfp has indicated regions likely to be involved in interactions with the substrate of this PCP (Reuter et al., 1999), and since most of these regions are highly conserved among PPTase family members, directed mutations may provide insights into substrate recognition and specificity by PCP, ACP and NRPS enzyme complexes. Already the crystallographic investigations have allowed a catalytic mechanism to be proposed (Parris et al., 2000).

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