Biosynthesis of the dideoxysugar component of jadomycin B: genes in the jad cluster of Streptomyces venezuelae ISP5230 for L-digitoxose assembly and transfer to the angucycline aglycone

Liru Wang¹, Robert L. White² and Leo C. Vining¹

Author for correspondence: Leo C. Vining. Tel: +1 902 494 2040. Fax: +1 902 494 3736.
e-mail: leo.vining@dal.ca

Eight additional genes, jadX, O, P, Q, S, T, U and V, in the jad cluster of Streptomyces venezuelae ISP5230, were located immediately downstream of jadN by chromosome walking. Sequence analyses and comparisons implicated them in biosynthesis of the 2,6-dideoxysugar in jadomycin B. The genes were cloned in Escherichia coli, inactivated by inserting an apramycin resistance cassette with a promoter driving transcription of downstream genes, and transferred into Streptomyces venezuelae by intergeneric conjugation. Analysis by HPLC and NMR of intermediates accumulated by cultures of the insertionally inactivated Streptomyces venezuelae mutants indicated that jadO, P, Q, S, T, U and V mediate formation of the dideoxysugar moiety of jadomycin B and its attachment to the aglycone. Based on these results and sequence similarities to genes described in other species producing deoxysugar derivatives, a biosynthetic pathway is proposed in which the jadQ product (glucose-1-phosphate nucleotidyltransferase) activates glucose to its nucleotide diphosphate (NDP) derivative, and the jadT product (a 4,6-dehydratase) converts this to NDP-4-keto-6-deoxy-D-glucose. An NDP-hexose 2,3-dehydratase and an oxidoreductase, encoded by jadO and jadP, respectively, catalyse ensuing reactions that produce an NDP-2,6-dideoxy-D-threo-4-hexulose. The product of jadU (NDP-4-keto-2,6-dideoxy-5-epimerase) converts this intermediate to its L-erythro form and the jadV product (NDP-4-keto-2,6-dideoxyhexose 4-ketoreductase) reduces the keto group of the NDP-4-hexulose to give an activated form of the L-digitoxose moiety in jadomycin B. Finally, a glycosyltransferase encoded by jadS transfers the activated sugar to jadomycin aglycone. The function of jadX is unclear; the gene is not essential for jadomycin B biosynthesis, but its presence ensures complete conversion of the aglycone to the glycoside. The deduced amino acid sequence of a 612 bp ORF (jadR*) downstream of the dideoxysugar biosynthesis genes resembles many TetR-family transcriptional regulator sequences.

Keywords: antibiotic, gene disruption, polyketide glycoside

INTRODUCTION

Polyketide intermediates generated by sequential condensation of activated low-molecular-mass carboxylic acid metabolites catalysed by polyketide synthases (PKSs) are converted into many types of naturally occurring aromatic and macrocyclic ester structures. Among compounds of this type produced as secondary metabolites by actinomycetes is the antibiotic jadomycin B, discovered in Streptomyces venezuelae ISP5230. The

Abbreviations: Am, apramycin; NDP, nucleotide diphosphate; PKS, polyketide synthase.
The GenBank accession number for the sequence reported in this paper is AY026363.
deduced chemical structure (Ayer et al., 1991; Doull et al., 1993) and the pattern of labelling by \[^{14}C\]acetate (Crowell, 1993) indicate that jadomycin B contains polyketide aglycone and a 2,6-dideoxysugar moiety. A recent X-ray diffraction analysis of crystalline jadomycin B has refined stereochemical features of the structure (Fig. 1; T. S. Cameron, R. L. White, L. Wang & L. C. Vining, unpublished) and established that the glycosidic moiety is l-digitoxose. Molecular genetic evidence has established that the aglycone is biosynthesized from a decapolyketide generated by an iterative type-II PKS complex encoded by a chromosomal gene cluster (Han et al., 1994; Kulowski et al., 1999; Wang et al., 2001).

Many polyketide-derived secondary metabolites exhibit biological activity and are important pharmaceutical agents. The medical applications of these compounds have fostered programmes to obtain new structures with enhanced or modified properties. Novel metabolites can be generated by interchanging secondary metabolic biosynthesis genes among a variety of micro-organisms, or by creating hybrid genes that direct the synthesis of unique and hitherto unavailable structures. Since Hopwood & Sherman (1990) reviewed polyketide antibacterial synthesis with a focus on PKS genes, there has been substantial progress in recombining genes to construct novel polyketide aglycones (Hutchinson 1999; McDaniel et al., 1999; David et al., 1998; Shen et al., 1999); recent extensions of this approach to include dideoxysugar biosynthesis genes expand opportunities to genetically engineer new microbial metabolites with medical applications (Olano et al., 1999; Gaissert et al., 2000).

Dideoxysugars are found in nature as constituents of glycoproteins, bacterial cell walls and glycosidic secondary metabolites. The 2,6- and 4,6-dideoxyhexoses, in particular, are present in a broad range of bioactive compounds. The precise function of the carbohydrate moiety in most glycosylated metabolites has not been elucidated, but the presence of a dideoxysugar seems to alter responses at the interface between a compound and its surroundings: daunosamine in daunorubicin and doxorubicin confers antineoplastic activity (Krugel et al., 1993), glycosylation detoxifies oleandomycin during its biosynthesis (Quiro et al., 1998) and oligosaccharide moieties stabilize antibiotic binding in the minor groove of DNA (Walker et al., 1990). The existence of a large variety of dideoxysugars suggests selection for diversity in their biosynthesis, and an influence of structural differences on the properties of the final product. However, contributions to mechanisms of action are still poorly understood, and except for the 3,6-dideoxysugar ascaryllose (Liu & Thorson, 1994; Kirshning et al., 1997), knowledge of biosynthetic pathways is also incomplete. In the route to jadomycin B, many genes associated with assembly of the aglycone have been identified, but knowledge of the genes responsible for synthesizing and attaching the sugar moiety is limited to detection in the \textit{Streptomyces venezuelae} jad cluster of a partial ORF with sequence similarity to dideoxysugar biosynthesis genes (Wang et al., 2001). Here we report the discovery by chromosomal walking of a group of adjacent genes, \textit{jadXOQRSTUV}, occupying approximately 9 kb of the chromosome downstream of the \textit{jad} PKS cluster. We show by disruptions that the genes are required to form or attach the dideoxysugar in jadomycin B and propose from sequence comparisons with dideoxysugar biosynthesis genes in other organisms that \textit{jadXOQRSTUV} encodes the pathway synthesizing the l-digitoxose component of the antibiotic.

**METHODS**

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

**Culture conditions.** \textit{Escherichia coli} strains used for plasmid isolation and \textit{\lambda} phage propagation were grown as described by Sambrook et al. (1989). Jadomycin B was produced in galactose-isoleucine medium (Doull et al., 1993), but with isoleucine doubled to 7.8 g l\(^{-1}\) (giving Gal2I medium). Recombinant \textit{E. coli} DH5\(\alpha\) strains were grown in LB medium containing appropriate antibiotics (Sambrook et al., 1989). Strains of \textit{Streptomyces venezuelae} from which genomic DNA was isolated were grown in MYM medium (Stuttard, 1982) at 30 °C for 24 h on a rotary shaker (220 r.p.m.). To assess promoter activity by measuring catechol 2,3-dioxygenase in \textit{Streptomyces venezuelae} cell extracts, strains transformed with pXE4 or pXE4::Am\(^{R}\) were grown for 2 days at 30 °C on a rotary shaker (220 r.p.m.) in either MYM or Gal2I culture medium.

**DNA manipulation and transformation.** Bacteriophage \textit{\lambda} DNA was purified as described previously (Wang et al., 2001). Plasmid DNA was isolated from \textit{E. coli} by the alkaline lysis method (Sambrook et al., 1989). Streptomyces 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 \textit{E. coli} cells were prepared and transformed by the procedures of Sambrook et al. (1989). In transformations of \textit{Streptomyces venezuelae} by the protoplasting procedure, the methods of Kieser et al. (2000) for preparing and transforming protoplasts were modified as follows: spores were grown in MYME medium (Yang et al., 1996) at 30 °C on a rotary shaker (220 r.p.m.) for 24 h.
**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>
<td>Wild-type, jadomycin B producer</td>
<td>Struttard (1982)</td>
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<tr>
<td>ISP5230</td>
<td>ISP5230 with jadO disrupted by Am&lt;sup&gt;R&lt;/sup&gt; gene in opposite orientation</td>
<td>This study</td>
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<td>VS1079/1080</td>
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<td>VS1081/1082</td>
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<td>VS1093/1094</td>
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<td>ET 12567 containing pUZ8002</td>
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<td>DH5αΔI&lt;sup&gt;T&lt;/sup&gt;IQ</td>
<td>F'&lt;sup&gt;r&lt;/sup&gt;80lacZA(lacZYA-argF) U169 deoR recA1 endA1 bsdR17(ri&lt;sup&gt;−&lt;/sup&gt;, m&lt;sup&gt;−&lt;/sup&gt;) phoA supE&lt;sup&gt;11&lt;/sup&gt; 2&lt;sup&gt;−&lt;/sup&gt; thi-1 gyrA96 relAI&lt;sup&gt;F&lt;/sup&gt; proAB&lt;sup&gt;+&lt;/sup&gt; lacUV5&lt;sup&gt;Z&lt;/sup&gt;AM15 zzf&lt;sup&gt;+&lt;/sup&gt;::Tn5[Km&lt;sup&gt;−&lt;/sup&gt;]</td>
<td>Gibco-BRL</td>
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<td>dam&lt;sup&gt;−&lt;/sup&gt; dcm&lt;sup&gt;−&lt;/sup&gt; bsdM&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>ET12567 containing pUZ8002</td>
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<td><strong>Phagemid</strong></td>
<td>Phagemid derivative of pUC18, SK&lt;sup&gt;−&lt;/sup&gt; and KS&lt;sup&gt;−&lt;/sup&gt;, fl ori, and primer-binding sites for T3 and T7</td>
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<td>pHL400</td>
<td>tsr amp lacZ, bifunctional</td>
<td>Larson &amp; Hershberger (1986)</td>
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<td>ori lacZ amp</td>
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<td>pJV225</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; containing Am&lt;sup&gt;R&lt;/sup&gt; gene flanked by multiple cloning sites</td>
<td>Chang et al. (2001)</td>
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<td>pJV326</td>
<td>pHL400 with 0.76 kb PstI insert containing oriT, bifunctional</td>
<td>Chang et al. (2001)</td>
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<td>pJV401</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; with 6.6 kb Xbal insert from phage LW3</td>
<td>Wang et al. (2001)</td>
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<td>pJV410</td>
<td>pUC18 with 6.0 kb PstI-BamHI insert from pJV401 containing jadMNXO and downstream genes</td>
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<td>pJV411</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; with 3.5 kb KpnI-XbaI fragment from pJV401 containing 5' region of jadO and downstream genes</td>
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<td>pJV413A/B</td>
<td>pUC18 with 6.0 kb XbaI-BamHI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in EcoRV site; pJV413A/B in opposite orientation</td>
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<td>pJV414A/B</td>
<td>pUC18 with 6.0 kb XbaI-BamHI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in KpnI site; pJV414A/B in opposite orientation</td>
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<td>pJV415A/B</td>
<td>pJV326 with 7.6 kb EcoRI-Xbal fragment subcloned from pJV414A/B</td>
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<td>pJV416A/B</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; with 3.5 kb XbaI-BamHI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in blunted BstEII site; pJV416A/B in opposite orientation</td>
<td>This study</td>
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<td>pJV417A/B</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; with 2.2 kb NotI-XbaI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in blunted Mael site; pJV417A/B in opposite orientation</td>
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<td>pJV418A/B</td>
<td>pUC18 with 2.0 kb BamHI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in NruI site; pJV418A/B in opposite orientation</td>
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<td>pJV419A/B</td>
<td>pUC18 with 2.3 kb Stul-KpnI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in blunted BamHI site; pJV419A/B in opposite orientation</td>
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<td>pJV420A/B</td>
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<td>pJV421A/B</td>
<td>pUC18 with 3.8 kb BamHI fragment containing 1.6 kb Am&lt;sup&gt;R&lt;/sup&gt; gene in NruI site; pJV421A/B in opposite orientation</td>
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<td>pJV423</td>
<td>pBluescript II SK&lt;sup&gt;−&lt;/sup&gt; with 2.0 kb BamHI fragment from LW12</td>
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<td>pJV424</td>
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<td>pJV425</td>
<td>pUC18 carrying in its Smal site a 5.6 kb Stul fragment containing jadS and downstream genes subcloned from λ LW18</td>
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<td>pJV427</td>
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<td>λ GEM-11 with 11.5 kb insert of Streptomyces venezuelae DNA</td>
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<td>λ LW3</td>
<td>λ GEM-11 with 9.5 kb insert of Streptomyces venezuelae DNA</td>
<td>This study</td>
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<td>λ LW12</td>
<td>λ GEM-11 with 15.5 kb insert of Streptomyces venezuelae DNA</td>
<td>This study</td>
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<td>λ gene bank</td>
<td>λ GEM-11 with 9.0−23.5 kb inserts of Streptomyces venezuelae genomic DNA</td>
<td>Facey (1994)</td>
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mycelium was regenerated on M2YE mannitol/maltose agar (Yang et al., 1996) and transformants were selected by overlaying with soft nutrient agar containing 50 μg thioestrepton or apramycin (Am) ml⁻¹. For conjugal transfer of plasmids from E. coli to streptomycetes, the protocol of Mazodier et al. (1989) was followed.

Cloning and sequencing deoxysugar genes. The 3.5 kb KpnI–XhoI fragment of the pJV401 insert (Wang et al., 2001) was subcloned in pBluescript II SK(+) to give pJV411 (Fig. 2). Nested overlapping deletions were introduced into the pJV411 insert with an ExoIII/I S deletion kit (MBI Fermentas) and the cloned DNA was sequenced by the dideoxynucleotide chain-termination method. A 0.6 kb BamHI–XhoI fragment from the XhoI end of pJV411 (see Fig. 2) was subcloned as pJV422 and used to probe a genomic library prepared in λ EGM-11 from a partial Sau3AI digest of Streptomyces venezuelae ISP5230 (Facey, 1994). A library fragment (λ LW12) that hybridized with the probe was cloned in pBluescript II SK(+) as pJV423 (see Fig. 2). For further chromosome walking, a 0.6 kb NrdI–BamHI fragment of pJV423, subcloned in pJV424, was labelled with [³²P] dCTP and used to probe the Streptomyces venezuelae genomic library. A library fragment (LW18) that hybridized with the probe was recloned as a 5.6 kb Stul fragment in pUC18, giving pJV425. The inserts in pJV423 and pJV425, sequenced as described for pJV411, contained an overlapping region; the pJV423 sequence also overlapped pJV401 (see Fig. 2).

Sequence analysis. The sequenced pJV411, pJV423 and pJV425 inserts were examined with ORF Finder (NCBI) and FramePlot 2.3 (Ishikawa & Hotta, 1999) to detect ORFs. BLASTX was used with individual ORF sequences and BLASTP with their translated sequences, to query GenBank. Sequences were aligned and their relatedness was assessed with CLUSTAL W (Thompson et al., 1994).

Fig. 2. Restriction map of the 13 kb region of Streptomyces venezuelae ISP5230 genomic DNA subcloned in the three recombinant λ vectors LW3, LW12 and LW18. Arrows above the restriction map show the locations and orientations of the ORFs in the jad cluster. The filled arrows represent genes involved in biosynthesis or attachment of the deoxysugar in jadomycin B. Unfilled arrows represent jad cluster genes flanking the deoxysugar pathway genes. Vertical arrows below the restriction map point to sites where an Am⁰ cassette was inserted in each orientation to disrupt a deoxysugar pathway gene. Restriction sites defining the inserts in plasmids pJV401, pJV410, pJV411, pJV422, pJV423, pJV424, pJV425 and pJV426/pJV427 are aligned below the map. Abbreviations: B, BamHI; Bi, BstEI; Ev, EcoRV; K, KpnI; Ma, MaEll; Ml, MluI; Nc, Ncol; Nt, NruI; Nt, NotI; St, Stul; X, XhoI.

Construction of pXE4::Am⁰. The Am⁰ gene was excised from pJV225 (Chang et al., 2001) as a HindIII fragment and ligated into the HindIII site of pXE4 (Ingram et al., 1989) with the same transcriptional orientation as xylE. The pXE4::Am⁰ construct and a pXE4 control plasmid were passed through E. coli ET12567 before being used to transform protoplasts of Streptomyces venezuelae ISP5230.

Gene disruption. Plasmid inserts containing jadX, O, P, Q, S, T, U and V were mapped with restriction enzymes and a central site in each was targeted for insertional inactivation. Thus to disrupt jadO, pJV410 was linearized with KpnI. The linear fragment was blunt-ended by treatment with SI nuclease and ligated with the 16 kb EcoRV cassette containing apr gene was excised from pJV225. The constructs (pJV414A/B) contained apr in opposite transcriptional orientations. Fragments containing the disrupted genes were retrieved from an agarose gel and ligated with the oriT-containing vector pJV326, giving pJV415A/B. To avoid restriction in Streptomyces venezuelae ISP5230, the plasmid constructs were passaged through the DNA methylation-deficient E. coli strain ET12567(pUZ8002) before being transferred from E. coli to Streptomyces venezuelae ISP5230 by intergeneric conjugation using the procedure described by L. Wang et al. (1989). Single colonies of the transconjugant strains VS1079 and VS1080, each resistant to apramycin but sensitive to thioestrepton, were selected. Using similar methods, jadX/jadP, jadQ/jadS, jadT/jadU and jadV cloned in either pBluescript II SK(+) or pUC18 were disrupted by inserting the Am⁰ cassette in alternative directions at the EcoRV, BstEI, MaEll, NrdI, BamHI, BstEI and NrdI sites, respectively (see Fig. 2), to give pJV413A/B, pJV416A/B, pJV417A/B, pJV418A/B, pJV419A/B, pJV420A/B and pJV421A/B, respectively. In each pair of plasmids, the member designated A carried the Am⁰ gene in the opposite transcriptional orientation to the.
gene disrupted. The inserts were excised and ligated to pJV326, then passaged through E. coli ET12567(pUZ8002) and transferred conjugally from E. coli to Streptomyces venezuelae ISP5230 as in the procedure above for jadO disruption. Transconjugant strains VS1081/1082, VS1083/1084, VS1085/1086, VS1087/1088, VS1089/1090, VS1091/1092 and VS1093/1094, each of which was resistant to apramycin but sensitive to thiostrepton, were selected. The first member of each pair contained the A series of plasmids (Amr* gene in opposite orientation to the disrupted gene).

Construction of merodiploids for gene complementation. The 3·6 kb BamHI fragment containing jadT, U, V and R* was ligated to the BamHI end of the fragment cloned in pBluescript II as pJV424 to give pJV426. The 4·2 kb NruI–BamHI insert in pJV426 was retrieved by EcoRI–HindIII digestion, end-filled by incubation with the Klenow fragment of DNA polymerase I and inserted into EcoRI-digested and end-filled pJV326. The plasmid obtained (pJV427) was passaged through ET12567 (pUZ8002) and transferred conjugally into the following Streptomyces venezuelae strains: VS1087 (jadS disrupted), VS1089 (jadT disrupted), VS1091 (jadU disrupted) and VS1093 (jadV disrupted).

Hybridization. Procedures described by Hopwood et al. (1985) and Sambrook et al. (1989) were used to screen a Streptomyces venezuelae ISP5230 genomic DNA library in λ GEM-11 (Facey, 1994). Phage hybridizations and Southern hybridizations were performed as described by Wang et al. (2001).

Jadomycin production and analysis. Cultures of Streptomyces venezuelae were analysed for jadomycin production by HPLC; the retention times for jadomycin B and its aglycone were 7·5 and 8·4 min, respectively (Wang et al. 2001). Ethyl acetate extracts of cultures from strains with disrupted genes were evaporated in vacuo and the residues (20–25 mg), redissolved in chloroform, were fractionated by flash chromatography on silica gel H (Merck); green-coloured eluate fractions, which gave an HPLC peak at 8·1 min, contained the aglycone. They were evaporated in vacuo and the residues (1·0–1·1 mg), taken up in CDCl₃, were examined by a Bruker AMX 400 spectrometer at 400·1 and 100·6 MHz to obtain ¹H and ¹³C NMR spectra, respectively.

Measurement of catechol 2,3-dioxygenase activity. Transformants selected on MYM agar supplemented with thiostrepton or apramycin were patched on the appropriate medium and incubated at 30 °C for 2–3 days. The patches were then sprayed with 0·5 M aqueous catechol (Zukowski et al., 1983; Ingram et al., 1989) and examined for the formation of a yellow product. To measure catechol 2,3-dioxygenase activity quantitatively, transformants were grown, along with the residues (1·0–1·1 mg), taken up in CDCl₃, were examined by a Bruker AMX 400 spectrometer at 400·1 and 100·6 MHz to obtain ¹H and ¹³C NMR spectra, respectively.

RESULTS
Sequence of jad genes downstream of jadN
In a previous investigation, Wang et al. (2001) obtained a DNA fragment from a genomic library of Streptomyces venezuelae ISP5230 and cloned it in λ LW3. The insert from LW3 was subcloned as 7·0 kb XhoI fragment in pJV401. Sequencing a 3·4 kb XhoI–KpnI segment at the left-hand end of the pJV401 insert (see Fig. 2) identified ORFs for jadM, encoding a phosphopantetheinyl transferase, and jadN, encoding an acylcoenzyme A decarboxylase, both associated with biosynthesis of jadomycin aglycone. In addition, the sequence included an unidentified gene (jadX) and a partial gene (jadO) potentially involved in biosynthesis of the sugar component in jadomycin B. The sequence between jadN and jadX contained an in-frame TGA 268 bp distant from the start of jadX and assumed to be a stop codon; however, subsequent FramePlot analyses showed that transcription of jadN did not terminate until it reached a second in-frame TGA only 22 bp away from jadX. Thus the 268 bp separation between jadN and jadX reported previously (Wang et al., 2001) is now considered incorrect. The remainder of jadO was obtained by subcloning (as pJV411) the 3·6 kb KpnI–XhoI segment from the right-hand end of the pJV401 insert. In addition, adjacent jad genes were obtained from the genomic library by chromosome walking. Probing with a BamHI–XhoI segment of DNA subcloned in pJV422 from the right-hand end of pJV411 (see Fig. 2) identified λ LW12 and the overlapping λ LW18. The LW12 insert was recloned as a BamHI fragment in pJV423 and the LW18 insert as a Stul fragment in pJV425. Sequencing and analysis of the inserts in pJV411, JV423 and pJV425 detected nine complete ORFs (jadX, O, P, Q, S, T, U, V and R*; see Fig. 2). Their properties are summarized in Table 2. Eight of the ORFs (jadX–V) were transcribed in the same direction. They were followed by the oppositely oriented jadR*, encoding 204 aa. The jadV and jadR* stop codons were separated by 16 bp.

Functions of jadX, O, P, Q, S, T, U, V and R*

jadX. A BLASTp search showed the sequence of the jadX product to be 26 % identical to that of MmyY (GenBank accession no. AJ276673), a protein of undetermined function encoded by a gene in the methylenomycin biosynthesis cluster located in the large linear plasmid SCP1 of Streptomyces coelicolor A3(2). The jadX product also showed 24 % sequence identity to the product of ORF5 (GenBank AF127374) from Streptomyces lavendulae, but the probability of chance identity is high and the function of ORF5 is uncertain.

jadO. In BLASTX searches with its deduced amino acid sequence, jadO showed similarity to genes for many nucleotide diphosphate (NDP)-hexose 2,3-dehydratases. The closest match (72 % identity) was with UrdS, involved in forming the d-olivose and l-rhodinose moieties of urdamycin, but JadO also resembled dTDP-4-keto-6-deoxy-l-hexose 2,3-dehydratase encoded by aveBVI of Streptomyces avermitilis (59 % identical aa), d-olivose, d-oliose and d-mycarose 2,3-dehydratases (MtmV) of Streptomyces argillaceus (47 % identity), Ty1C2V in Streptomyces fradiae (46 % identity) and eryBVI in Saccharopolyspora erythraea (44 % identity). Alignment of 2,3-dehydratase...
The deduced amino acid sequence of *jadP* showed appreciable similarity to oxidoreductases in GenBank. The closest match (51% identical aa) was with an enzyme used in landomyacin biosynthesis by *Streptomyces cyanogenus*; other similar oxidoreductases were Gra-orf26, used by *Streptomyces violaceoruber* to synthesize the deoxysugar in granaticin, and a gene product of *Amycolatopsis mediterranei* (45% identical aa). Related gene products included the 3-ketoreductase AknQ (43% identical aa) used during glycosylation of aclarcibin by *Streptomyces galilaeus*, and dTDP-3,4-diketo-2,6-dideoxyglucose 3-ketoreductase (38% identity) used by *Streptomyces antibioticus* for C-2 deoxy- genation during dTDP-1-oaleandroside biosynthesis. Participation of such an oxidoreductase in the biosynthesis of the 2,6-dideoxyhexose in jadomycin B would be consistent with the biosynthetic pathway for other dideoxy sugars proposed by Thorson et al. (1993) and Draeger et al. (1999). This requires a 2,6-dehydratase (e.g. *JadO*) and an oxidoreductase (e.g. *JadP*) acting in sequence on NDP-4-keto-6-deoxy-o-glucose to produce 4-keto-2,6-dideoxy-o-glucose as a key intermediate.

### Table 2. Features of ORFs downstream of *jadM* in the *jad* cluster of *Streptomyces venezuelae*

<table>
<thead>
<tr>
<th>ORF†</th>
<th>Size of product (aa)‡</th>
<th>Distance from preceding ORFs (bp)</th>
<th>G + C content (mol%)</th>
<th>Putative function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>jadN</em></td>
<td>609</td>
<td>4 bp overlap with <em>jadM</em></td>
<td>70/0</td>
<td>Acyl-coenzyme A decarboxylase</td>
</tr>
<tr>
<td><em>jadX</em></td>
<td>172</td>
<td>22</td>
<td>72/3</td>
<td>Not known</td>
</tr>
<tr>
<td><em>jadO</em></td>
<td>475</td>
<td>1 bp overlap with <em>jadX</em></td>
<td>75/9</td>
<td>NDP-hexose 2,3-dehydratase</td>
</tr>
<tr>
<td><em>jadP</em></td>
<td>376</td>
<td>26</td>
<td>76/8</td>
<td>NDP-hexose 3-ketoreductase</td>
</tr>
<tr>
<td><em>jadQ</em></td>
<td>269</td>
<td>68</td>
<td>75/1</td>
<td>NDP-glucose phosphate nucleotidyltransferase</td>
</tr>
<tr>
<td><em>jadS</em></td>
<td>395</td>
<td>47</td>
<td>75/7</td>
<td>Glycosyltransferase</td>
</tr>
<tr>
<td><em>jadT</em></td>
<td>332</td>
<td>32</td>
<td>69/1</td>
<td>NDP-hexose 4,6-dehydratase</td>
</tr>
<tr>
<td><em>jadU</em></td>
<td>196</td>
<td>8</td>
<td>68/6</td>
<td>NDP-4-keto-6-deoxyhexose 3,5-epimerase</td>
</tr>
<tr>
<td><em>jadV</em></td>
<td>341</td>
<td>41</td>
<td>76/5</td>
<td>NDP-4-keto-6-deoxyhexose 4-ketoreductase</td>
</tr>
<tr>
<td><em>jadR</em></td>
<td>204</td>
<td>160</td>
<td>73/8</td>
<td>tetR homologue</td>
</tr>
</tbody>
</table>

† Genes are listed in the order that the ORFs occur in the *jad* cluster. ‡ The putative start codon for *jadP* and *jadV* is GTG; for other ORFs it is ATG. § *jadR* is transcribed towards *jadV*; the distance between the *jadR* and *jadV* stop codons is 16 bp.

**Fig. 3.** Alignment of the amino acid sequences of *JadO* with NDP-hexose 2,3-dehydratases by CLUSTAL W. Highly conserved amino acids are in bold letters and are marked below the alignment with an asterisk (all aa identical), a colon (all aa similar) or a period (several aa identical). Highly conserved aligned sequences are designated regions 1-4. Sources of the sequences: *LanS, lanS* in *Streptomyces cyanogenus*; *UrdS, urdS* in *Streptomyces fradiae*; *AveBVI, aveBVI* in *Streptomyces avermitilis*; *EryBVI, eryBVI* in *Saccharopolyspora erythraea*; *TylCVI, tyl-orf6* in *Streptomyces fradiae*; *MtmV, mtmV* in *Streptomyces argillaceus*; *Orf10*, *orf10* in *Streptomyces violaceoruber* Tu22; *JadO, jadO* in *Streptomyces venezuelae*.

Amino acid sequences omitting *JadO* showed four highly conserved areas (regions 1–4; bold letters in Fig. 3). When *JadO* was included in the alignment, sequence conservation was limited to only two of these areas – region 3 (HSEEGGRG at aa 459–468; 87% identity) and region 4 (HGHXNVQARTLLA at aa 514–527). The narrower consensus with *JadO* present presumably reflects divergence of the more substrate-specific *JadO* enzyme activity. Some 2,3-dehydratases listed in Fig. 3 can participate in the synthesis of two or three different deoxysugars, or of a trideoxyhexose and an aminosugar (Hoffmeister et al., 2000).

**jadP.** The deduced amino acid sequence of *jadP* showed appreciable similarity to oxidoreductases in GenBank. The closest match (51% identical aa) was with an enzyme used in landomyacin biosynthesis by *Streptomyces cyanogenus*; other similar oxidoreductases were Gra-orf26, used by *Streptomyces violaceoruber* to synthesize the deoxysugar in granaticin, and a gene product of *Amycolatopsis mediterranei* (45% identical aa). Related gene products included the 3-ketoreductase AknQ (43% identical aa) used during glycosylation of aclarcibin by *Streptomyces galilaeus*, and dTDP-3,4-diketo-2,6-dideoxyglucose 3-ketoreductase (38% identity) used by *Streptomyces antibioticus* for C-2 deoxy- genation during dTDP-1-oaleandroside biosynthesis. Participation of such an oxidoreductase in the biosynthesis of the 2,6-dideoxyhexose in jadomycin B would be consistent with the biosynthetic pathway for other dideoxy sugars proposed by Thorson et al. (1993) and Draeger et al. (1999). This requires a 2,6-dehydratase (e.g. *JadO*) and an oxidoreductase (e.g. *JadP*) acting in sequence on NDP-4-keto-6-deoxy-o-glucose to produce 4-keto-2,6-dideoxy-o-glucose as a key intermediate.

**jadQ.** The product of *jadQ* was similar to NDP-glucose synthases in many streptomycetes producing glycosicid metabolites; the strongest resemblance was to TDP-glucose synthase of *Streptomyces griseus* (75% identical aa). Other relevant gene products were glucose-1-phosphate thymidyltransferase of *Streptomyces avermitilis* (71% identical aa), dTDP-1-glucose synthase of *Streptomyces galilaeus* (70% identical aa), α-d-glucose-
1-phosphate thimidylyltransferase of Streptomyces venezuelae ATCC 15439 (67% identical aa) and glucose-1-phosphate thimidylyltransferase of Streptomyces fradiae. The NDP-hexose phosphate transferases consist of four identical subunits, each with a molecular mass of 29 kDa (Thorson et al., 1994); analysis of their sequence shows two conserved regions (Katsube et al., 1991; Liu & Thorson, 1994; Kirschning et al., 1997). In an alignment of JadQ with NDP-hexose phosphate transferases, these regions could be recognized at two locations. One near the N terminus was present in all sequences and is associated with activator binding. A more centrally located consensus sequence, present in all transferases except AscA and LanZ2, is believed to bind the substrate. In the AscA sequence, the substrate binds further downstream (Katsube et al., 1991; Thorson et al., 1994) and the difference in binding site is consistent with the different role of AscA in catalysing biosynthesis of a 3,6- rather than a 2,6-dideoxy sugar. That pathways to dideoxy sugars may be initiated with specific sugar phosphates is indicated by the presence in ‘Streptomyces cyanogenus’ of the two genes, lanG and lanZ2, encoding NDP-hexose synthases for the d-olivose and l-rhodinose moieties, respectively, of landomycin (Westrich et al., 1999).

jadS. The deduced amino acid sequence of jadS matched those of many glycosyltransferases. The closest resemblance was to a sugar-flexible elloramycin glycosyltransferase encoded by oleGI (44% identical aa) and used by Streptomyces olivaceus for biosynthesis of the polyketide-derived antitumour agent elloramycin. The sequence also resembled (35-42% identical aa) those of glycosyltransferases used in the biosynthesis of nogalamycin by Streptomyces nogalater, the angucycline antibiotics landomycin and urdamycin by Streptomyces fradiae, daunorubicin by Streptomyces peuceticus and for transfer of forosamine to the aglycone by Saccharopolyspora spinosa. Genes for glycosyltransferases have been identified in a number of gene clusters for polyketide antibiotic biosynthesis (Baltr & Seno, 1988; Krugel et al., 1993; Bechthold et al., 1995; Westrich et al., 1999; Trefzer et al., 1999; Kunzel et al., 1999). Where examined, the gene products catalysed specific reactions; thus LanGT1–4 transferred d-olivose and l-rhodinose at different stages during landomycin biosynthesis and UrdGT2 transferred d-olivose, but not l-rhodinose in the urdamycin pathway. In a comparison of the functions and structural features of NDP-sugar glycosyltransferases based on the amino acid sequences of 555 cloned genes, all but two could be assigned to 26 families (Campbell et al., 1997). Using the stereochemistries of substrates and reaction products as criteria, the glycosyltransferases of streptomycete genes such as the urdT2, dnrS and oleGI were all placed in family 1, representing a group of enzymes that invert configuration at the reaction site. Since JadS closely resembles UrdGT2, DnrS and OleGI, it probably is a family 1 glycosyltransferase, acting similarly.

jadT. The jadT product showed 68% sequence identity to the TDP-glucose 4,6-dehydratase used in streptomycin biosynthesis by Streptomyces griseus and 58-69% identity to NDP-hexose 4,6-dehydratases involved in the biosynthesis of glycosidic components of polyketide-derived secondary metabolites (granaticin, landomycin, urdamycin A, erythromycin, avermectin) by ‘Streptomyces cyanogenus’, Streptomyces fradiae, Streptomyces galilaeus, Streptomyces violaceoruber, Saccharopolyspora erythraea and ‘Streptomyces avermitilis’. In actinomycetes, genes for 4,6-dehydratases are more highly conserved than those for glycosyltransferases (Liu & Thorson, 1994), and fragments of strD, E, L and M from Streptomyces griseus N2-3-11 have served as effective probes for locating 6-deoxy- or 4,6-dideoxy-sugar biosynthesis genes (Decker et al., 1996). The phylogenetic tree for protein sequences deduced from the cloned genes or obtained from purified NDP-dehydratases indicates that actinomycete 4,6-dehydratase genes are more closely related to each other than to dehydratase genes from species of other orders. The relationship between dehydratases from strains producing natural compounds with similar dideoxy sugar moieties is even closer, and NDP-glucose 4,6-dehydratase gene probes have proven advantageous in cloning novobiocin and coumermycin A(1) biosynthesis gene clusters (Steffensky et al., 2000; Wang et al., 2000).

jadU. In a BLASTX search, the product of jadU most closely resembled (61% identical aa) AknL (dTDP-4-keto-6-deoxyhexose 3,5-epimerase,) used in aclacinomycin biosynthesis by Streptomyces galilaeus. It also resembled the putative epimerase (58% identical aa) used by Streptomyces peuceticus to form the daunorubicin precursor thymidine diphospho-L-daunosamine, the NDP-4-keto-6-deoxy-epimerase (58% identical aa) used by ‘Streptomyces avermitilis’ for avermectin biosynthesis and the TDP-4-keto-6-deoxyhexose 3,5-epimerase (56% identical aa) used by ‘Micromonospora megalomica subsp. nigra’ for biosynthesis of the antiparasitic agent megalomicin.

jadV. The product of jadV showed strong similarity (52% identical aa) to TDP-4-keto-6-deoxyhexose 4-ketoreductase in ‘Micromonospora megalomica subsp. nigra’, the putative deoxyhexose reductase (49% identical aa) in Streptomyces griseus, dTDP-4-keto-6-deoxy-L-hexose 4-reductase (46% identical aa) in ‘Streptomyces avermitilis’, the NDP-hexose 4-ketoreductase TylCIV (46% identical aa) in Streptomyces fradiae and EryBIV (47% identical aa) involved in making L-mycarose and D-desosamine in Saccharopolyspora erythraea.

jadR*. The deduced amino acid sequence of the 612 bp ORF (jadR*) downstream of jadV, but transcribed in the opposite direction, showed similarity to TetR-family transcriptional regulators in Streptomyces coelicolor A3(2) (29% identical aa) and Mycobacterium tuberculosis (27% identical aa), the probable transcription regulator PA1226 in Pseudomonas aeruginosa (29% identical aa) and the putative TetR regulatory proteins in Prauserella rugosa (31% identical aa) and Mycobacterium leprae (29% identical aa).
Disruption of jadX, O, P, Q, S, T, U and V

Plasmids containing genes for disruption were linearized at a restriction site near the centre of the insert and ligated with a cassette containing the AmR gene from E. coli (Chang et al., 2001). Fragments of DNA containing the disrupted gene were recloned in the promiscuous vector pJV326 (He et al., 2001) and the plasmid constructs were transferred from E. coli to Streptomyces venezuelae by intergeneric conjugation (Mazodier et al., 1989). Single colonies of the members of each transconjugant pair, VS1079/1080, VS1081/1082, VS1083/1084, VS1085/1086, VS1087/1088, VS1089/1090, VS1091/1092 and VS1093/1094, were isolated. In the first member of each transconjugant pair the AmR gene had been inserted in the opposite orientation to the gene disrupted (A series). In the second member of each pair, the two genes were in the same orientation (B series). Probing genomic DNA digests from VS1079, VS1081, VS1083 and VS1085 with the 4·0 kb Ncol–BamHI fragment from λ LW3 gave a hybridizing band at 4·0 kb from Streptomyces venezuelae ISP5230 and at 5·6 kb from the jadXOQP mutants (Fig. 4a). When genomic DNA from Streptomyces venezuelae ISP5230, VS1087 and VS1089 was probed with the 2·9 kb NruI fragment from λ LW18 (see Fig. 2), a hybridizing band was located at 2·9 kb from Streptomyces venezuelae ISP5230 and at 4·5 kb from strains disrupted in jadS or jadT (Fig. 4b). When the genomic DNA from Streptomyces venezuelae ISP5230, VS1091 and VS1093 was probed with the 2·15 kb MluI–BamHI fragment (see Fig. 2), a hybridizing band at 2·15 kb was detected from Streptomyces venezuelae ISP5230 and at 3·75 kb from strains carrying disrupted jadU or jadV (Fig. 4c). These are the results expected from double cross-overs disrupting jadX, O, P, Q, S, T, U and V. Cultures of each of the jad-disrupted transconjugant strains grew normally on MYM agar and in jadomycin-production medium. Analysis of culture extracts by HPLC, and comparison with jadomycin B and jadomycin aglycone standards, showed in all pairs (both A and B series) of insertionally inactivated strains, except those from jadX, a single major peak corresponding to jadomycin aglycone. Extracts showing only the aglycone peak were pooled and the product, purified by flash chromatography on silica gel H, was examined by 1H and 13C NMR spectroscopy. The major signals corresponded in chemical shift values to the resonances in 13C and 1H NMR spectra published for jadomycin aglycone (Ayer et al., 1991). Cultures of jadX-disrupted mutants with the AmR gene in each orientation accumulated both jadomycin B and the aglycone; the amounts of each product were similar, but each represented only 10–20% of the jadomycin B yield in wild-type controls. The results suggest that the jadX product facilitates accumulation of the glycosylated antibiotic, but is not required for its formation.

Expression from the AmR gene promoter

Since construction of the AmR cassette in pJV225 (Chang et al., 2001) from the E. coli plasmid pKC203 (Rao et al., 1983; Kaster et al., 1983) retained the AmR gene promoter and omitted a terminator sequence, use of the cassette for insertional inactivation could potentially maintain expression of polycistronically transcribed genes downstream of the insertion site. The promoter activity of the cassette was verified by inserting it upstream of xylE in the bifunctional promoter probe plasmid pXE4 designed by Ingram et al. (1989). On MYM agar, colonies of Streptomyces venezuelae ISP5230 transformed with pXE4::AmR (but not controls transformed with pXE4) became bright yellow when sprayed with 0·5 M catechol. Mycelial extracts from shaken cultures of the pXE4 transformants grown in MYM medium averaged 0·7 (± 0·6) mU catechol 2,3-dioxygenase activity (mg protein)−1, comparable to the 1–6 mU (mg protein)−1 reported in Streptomyces lividans and Streptomyces coelicolor A3(2) mycelium carrying pXE4 (Ingram et al., 1989), and contrasting with the 19·0 (± 1·2) mU (mg protein)−1 in extracts from
cultures of pXE4::AmR transformants. Extracts from cultures of the pXE4::AmR transformants in Gal2I medium gave 28·6 mU (mg protein)−1. This level of catechol dioxygenase activity implied expression of xylE from the AmR gene promoter.

**Complementation of dysfunctional genes by merodiploid construction**

To determine whether failure to produce jadomycin B after insertional inactivation of jadS was due to the mutation in jadS or to a polar effect preventing expression of downstream genes, a 3·6 kb BamHI fragment of DNA from pJV425, containing jadT, U, V and R, was ligated to the BamHI end of the 0·6 kb DNA fragment cloned in pJV424. The 4·2 kb NrdI–BamHI insert in the resulting plasmid (pJV426) was retrieved by EcoRI/HindIII digestion and recloned in the oriT-containing bifunctional vector pJV326 to give pJV427. This promiscuous plasmid was transferred conjunctively into Streptomyces venezuelae strain VS1087, in which jadS had been inactivated by insertional plasmid (Fig. 5) for biosynthesis of the jadomycin B sugar. First, α-D-glucose 1-phosphate is activated to an NDP-glucose by the jadQ product (NDP-glucose synthase); the activated glucose is then converted to NDP-4-keto-6-deoxy-d-glucose by the dehydratase JadT. This irreversible step commits the sugar nucleotide to a pathway in which NDP-4-keto-6-deoxy-d-glucose is a key precursor for 2-deoxysugars and other glycosidic metabolites (Liu & Thorson, 1994). The subsequent C-2 deoxyxygenation is catalysed by a pair of enzymes, JadO (a 2,3-dehydratase giving the labile intermediate NDP-3,4-diketo-6-deoxyglucose or its 2,3-enol; Draeger et al., 1999) and JadP (an oxidoreductase that reduces the intermediate to NDP-4-keto-2,6-dideoxy-d-glucose). The required epimer of the 2,6-dideoxy sugar is probably formed by JadU (NDP-4-keto-2,6-dideoxyhexose epimerase) via an enediol intermediate. Eventually JadV (NDP-4-keto-2,6-dideoxyhexose 4-keto-reductase) reduces the NDP-4-keto-2,6-dideoxyhexose to NDP-l-digitoxylose, and a glycosyltransferase encoded by jadS attaches the activated sugar to jadomycin aglycone.

In the mechanism forming 3,6-dideoxyhexoses in Gram-negative bacteria (Liu & Thorson, 1994), 5-epimerization (Eep) and 4-reduction (Ered) are involved in the last two steps. The lack of E3 activity (involving an ascD homologue) in synthesizing the 2,6-dideoxyhexose in daunorubicin and the 2,6- and 4,6-dideoxyhexoses in erythromycin implies that the mechanism generating 2,6- and 4,6-dideoxyhexoses in the Gram-positive streptomycetes differs from that giving 3,6-dideoxyhexoses in Gram-negative bacteria. Genes encoding the NDP-4-keto-6-deoxyhexose 3,5-epimerase and NDP-4-keto-6-deoxyhexose 4-ketoreductases have been cloned and sequenced, but the order in which the reactions occur is not known; thus it is uncertain whether NDP-2,6-dideoxy-d-threo-4-hexulose or NDP-2,6-dideoxy-l-erythro-4-hexulose is the natural substrate of the jadV gene product. In the absence of this information, the steps showing 5-epimerization preceding 4-keto-reduction in the route to NDP-l-digitoxose proposed in Fig. 5 are based only on the similarity of JadU and JadV sequences to those of enzymes believed to participate in the pathway for 2,6-dideoxy sugar biosynthesis in other...
bacteria (Thorson et al., 1993; Draeger et al., 1999; Trefzer et al., 1999; Aguirrezabalaga et al., 2000).

Products accumulated by various blocked Streptomyces mutants indicate that post-PKS tailoring by glycosyl transfer can precede other modification steps (Krohn & Rohr, 1997). Glycosyl transfer may also occur before synthesis of the sugar moiety is completed (Katz & Donadio, 1993; Madduri et al., 1998). In doxorubicin biosynthesis, the daunosamine moiety may be transferred either to ε-rhodomycinone or to the basic aglycone (aklavinone), six to seven biosynthesis steps before completion of doxorubicin (Bartel et al., 1990; Grimm et al., 1994). In erythromycin biosynthesis, L-mycarose and D-desosamine are transferred sequentially before hydroxylation and O-methylation of the L-mycarose moiety complete the formation of erythromycin A. However, accumulation of jadomycin aglycone as the sole product of strains disrupted at various steps in construction and transfer of the deoxysugar strongly suggest that specific glycosylation by JadS is a necessary final reaction in the jadomycin B biosynthesis pathway. In some other post-assembly reactions modifying polyketides, the enzymes for glycosylation do not exhibit absolute specificity (Katz & Donadio, 1993; Trefzer et al., 1999). Relaxed substrate selection allows glycosyltransferases encoded by the two genes oleI and oleD to play a role in self-resistance during oleandomycin biosynthesis (Quiros et al., 1998). Substrate flexibility for aglycones has also been reported for the glycosyltransferases used by producers of daunorubicin (Madduri et al., 1998), erythromycin (Gaiser et al., 1998) and urdamycin (Hoffmeister et al., 2000). In the methymycin/neomethymycin producer, Zhao et al. (1998) have demonstrated a potential application in genetically engineering hybrid antibiotics by replacing TDP-desosamine with other substrates to create two new macrolide glycosides.

Analysis of gene clusters for biosynthesis of polyketide-derived secondary metabolites has usually shown genes for constructing the aglycones to be centrally located and genes regulating transcription to be situated in the upstream region of the cluster. Genes for glycosylation and other post-polyketide modifications may be scattered on both flanks of the core PKS cluster. This
organization can complicate identification of individual biosynthesis pathways in antibiotics with more than one glycosidic substituent, but was not a factor in the jad cluster of Streptomyces venezuelae ISP5230. The single sugar in the jadomycin B structure and compact clustering of genes for its biosynthesis allow a probable sequence of biosynthetic reactions to be deduced. D-Digoxose is well known as a constituent of plant cardiac (Reichstein & Weiss, 1962) and other steroidal glycosides (Abe et al., 1994; Abe & Yamauchi, 2000; Huan et al., 2001; Warashina & Noro, 2000a, b), and is present in the microbial metabolite ammocidin (Murakami et al., 2001), but l-digoxose has been previously reported only in bioactive products from actinomycetes. It occurs in the glycosidic components of the macrocyclic antibiotic kijanimicin from Actinomadura kijaniata (Mallams et al., 1981), in the tetracarin group of antitumour antibiotics from Micro monospora chalcea (Tomita et al., 1980) and in some antifungal polyene macrolides from streptomycetes (Zielinski et al., 1979).

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