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

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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.

Fig. 1. Structure of jadomycin B.

deduced chemical structure (Ayer *et al.*, 1991; Doull *et al.*, 1993) and the pattern of labelling by [^{13}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 antibiotic 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 deoxysugar biosynthesis genes expand opportunities to genetically engineer new microbial metabolites with medical applications (Olano *et al.*, 1999; Gaisser *et al.*, 2000).

Deoxysugars 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 deoxysugar 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 (Quiros *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 deoxysugars 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 ascarylose (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 *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, *jadXOPQSTUV*, occupying approximately 9 kb of the chromosome downstream of the *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 *jadXOPQSTUV* 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. *Escherichia coli* strains used for plasmid isolation and λ 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 *E. coli* DH5 α strains were grown in LB medium containing appropriate antibiotics (Sambrook *et al.*, 1989). Strains of *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 *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 λ DNA was purified as described previously (Wang *et al.*, 2001). Plasmid DNA was isolated from *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 *E. coli* cells were prepared and transformed by the procedures of Sambrook *et al.* (1989). In transformations of *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

Strain, plasmid or phage	Genotype/phenotype	Source/reference
<i>Streptomyces venezuelae</i>		
ISP5230	Wild-type, jadomycin B producer	Stuttard (1982)
VS1079/1080	ISP5230 with <i>jadO</i> disrupted by Am ^R gene in opposite orientation	This study
VS1081/1082	ISP5230 with <i>jadP</i> disrupted by Am ^R gene in opposite orientation	This study
VS1083/1084	ISP5230 with <i>jadQ</i> disrupted by Am ^R gene in opposite orientation	This study
VS1085/1086	ISP5230 with <i>jadX</i> disrupted by Am ^R gene in opposite orientation	This study
VS1087/1088	ISP5230 with <i>jadS</i> disrupted by Am ^R gene in opposite orientation	This study
VS1089/1090	ISP5230 with <i>jadT</i> disrupted by Am ^R gene in opposite orientation	This study
VS1091/1092	ISP5230 with <i>jadU</i> disrupted by Am ^R gene in opposite orientation	This study
VS1093/1094	ISP5230 with <i>jadV</i> disrupted by Am ^R gene in opposite orientation	This study
<i>Escherichia coli</i>		
DH5 α F'IQ	F'80dlacZ Δ (lacZYA-argF) U169 <i>deoR recA1 endA1 hsdR17</i> (r _k ⁻ , m _k ⁻) <i>phoA supE11</i> λ^- <i>thi-1 gyrA96 relA1/F' proAB⁺ lacI^q ZΔM15 zsf::Tn5</i> [Km ^R]	Gibco-BRL
ET 12567	<i>dam⁻ dcm⁻ hsdM⁻</i>	MacNeil <i>et al.</i> (1992)
ET 12567(pUZ8002)	ET12567 containing pUZ8002	M. Paget, John Innes
Plasmid		
pBluescript II	Phagemid derivative of pUC18, SK(+) and KS(+), f1 <i>ori</i> , and primer-binding sites for T3 and T7	Stratagene
pHJL400	<i>tsr amp lacZ</i> , bifunctional	Larson & Hersherberger (1986)
pUC18	<i>ori lacZ amp</i>	
pJV225	pBluescript II SK(+) containing Am ^R gene flanked by multiple cloning sites	Chang <i>et al.</i> (2001)
pJV326	pHJL400 with 0.76 kb <i>Pst</i> I insert containing <i>oriT</i> , bifunctional	He <i>et al.</i> (2001)
pJV401	pBluescript II SK(+) with 6.6 kb <i>Xho</i> I insert from phage LW3	Wang <i>et al.</i> (2001)
pJV410	pUC18 with 6.0 kb <i>Pst</i> I– <i>Bam</i> HI insert from pJV401 containing <i>jadMNXO</i> and downstream genes	This study
pJV411	pBluescript II SK(+) with 3.5 kb <i>Kpn</i> I– <i>Xho</i> I fragment from pJV401 containing 5' region of <i>jadO</i> and downstream genes	This study
pJV413A/B	pUC18 with 6.0 kb <i>Xho</i> I– <i>Bam</i> HI fragment containing 1.6 kb Am ^R gene in <i>Eco</i> RV site; pJV413A/B in opposite orientation	This study
pJV414A/B	pUC18 with 6.0 kb <i>Xho</i> I– <i>Bam</i> HI fragment containing 1.6 kb Am ^R gene in <i>Kpn</i> I site; pJV414A/B in opposite orientation	This study
pJV415A/B	pJV326 with 7.6 kb <i>Eco</i> RI– <i>Xba</i> I fragment subcloned from pJV414A/B	This study
pJV416A/B	pBluescript II SK(+) with 3.5 kb <i>Kpn</i> I– <i>Xho</i> I fragment containing 1.6 kb Am ^R gene in blunted <i>Bst</i> EII site; pJV416A/B in opposite orientation	This study
pJV417A/B	pBluescript II SK(+) with 2.2 kb <i>Not</i> I– <i>Xho</i> I fragment containing 1.6 kb Am ^R gene in blunted <i>Mae</i> II site; pJV417A/B in opposite orientation	This study
pJV418A/B	pUC18 with 2.0 kb <i>Bam</i> HI fragment containing 1.6 kb Am ^R gene in <i>Nru</i> I site; pJV418A/B in opposite orientation	This study
pJV419A/B	pUC18 with 2.3 kb <i>Stu</i> I– <i>Kpn</i> I fragment containing 1.6 kb Am ^R gene in blunted <i>Bam</i> HI site; pJV419A/B in opposite orientation	This study
pJV420A/B	pUC18 with 2.9 kb <i>Nru</i> I fragment containing 1.6 kb Am ^R gene in blunted <i>Bst</i> EII site; pJV420A/B in opposite orientation	This study
pJV421A/B	pUC18 with 3.8 kb <i>Bam</i> HI fragment containing 1.6 kb Am ^R gene in <i>Nru</i> I site; pJV417A/B in opposite orientation	This study
pJV423	pBluescript II SK(+) with 2.0 kb <i>Bam</i> HI fragment from LW12	This study
pJV424	pBluescript II SK(+) with 0.56 kb <i>Nru</i> I– <i>Bam</i> HI fragment from pJV423	This study
pJV425	pUC18 carrying in its <i>Sma</i> I site a 5.6 kb <i>Stu</i> I fragment containing <i>jadS</i> and downstream genes subcloned from λ LW18	This study
pJV426	pBluescript II SK(+) with 4.2 kb <i>Nru</i> I– <i>Bam</i> HI fragment containing genes downstream of <i>jadS</i>	This study
pJV427	pJV326 with 4.2 kb <i>Nru</i> I– <i>Bam</i> HI fragment containing genes downstream of <i>jadS</i>	This study
Phage		
λ LW3	λ GEM-11 with 11.5 kb insert of <i>Streptomyces venezuelae</i> DNA	Wang <i>et al.</i> (2001)
λ LW12	λ GEM-11 with 9.5 kb insert of <i>Streptomyces venezuelae</i> DNA	This study
λ LW18	λ GEM-11 with 15.5 kb insert of <i>Streptomyces venezuelae</i> DNA	This study
λ gene bank	λ GEM-11 with 9.0–23.0 kb inserts of <i>Streptomyces venezuelae</i> genomic DNA	Facey (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 Am^R 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, *Bam*HI; BII, *Bst*EII; Ev, *Eco*RV; K, *Kpn*I; Ma, *Mae*II; MI, *Mlu*I; Nc, *Nco*I; Nr, *Nru*I; Nt, *Not*I; St, *Stu*I; X, *Xho*I.

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 thio-strepton 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 *Kpn*I–*Xho*I 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 *Exo*III/SI deletion kit (MBI Fermentas) and the cloned DNA was sequenced by the dideoxynucleotide chain-termination method. A 0.6 kb *Bam*HI–*Xho*I fragment from the *Xho*I end of pJV411 (see Fig. 2) was subcloned as pJV422 and used to probe a genomic library prepared in λ GEM-11 from a partial *Sau*3AI 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 *Nru*I–*Bam*HI fragment of pJV423, subcloned in pJV424, was labelled with [^{32}P] 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 *Stu*I 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).

Construction of pXE4::Am^R. The Am^R gene was excised from pJV225 (Chang *et al.*, 2001) as a *Hind*III fragment and ligated into the *Hind*III site of pXE4 (Ingram *et al.*, 1989) with the same transcriptional orientation as *xylE*. The pXE4::Am^R 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 *Kpn*I. The linear fragment was blunt-ended by treatment with SI nuclease and ligated with the 1.6 kb *Eco*RV cassette containing *apr* retrieved 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 passed 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 Flett *et al.* (1997) and Mazodier *et al.* (1989). Single colonies of the transconjugant strains VS1079/1080, each resistant to apramycin but sensitive to thiostrepton, 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 Am^R cassette in alternative directions at the *Eco*RV, *Bst*EII, *Mae*II, *Nru*I, *Bam*HI, *Bst*EII and *Nru*I 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 Am^R 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 (Am^R gene in opposite orientation to the disrupted gene).

Construction of merodiploids for gene complementation.

The 3.6 kb *Bam*HI fragment containing *jadT*, *U*, *V* and *R** was ligated to the *Bam*HI end of the fragment cloned in pBluescript II as pJV424 to give pJV426. The 4.2 kb *Nru*I–*Bam*HI insert in pJV426 was retrieved by *Eco*RI–*Hind*III digestion, end-filled by incubation with the Klenow fragment of DNA polymerase I and inserted into *Eco*RI-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 8.4 min, contained the aglycone. They were evaporated *in vacuo* and the residues (1.0–1.1 mg), taken up in CDCl₃, were examined with 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 controls, in shaken cultures. Enzyme activity in sonicated cell extracts was measured colorimetrically by the methods of Ingram *et al.* (1989) and Kieser *et al.* (2000). Protein in cell extracts was measured with the bicinchoninic acid reagent (Pierce).

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 a 7.0 kb *Xho*I fragment in pJV401. Sequencing a 3.4 kb *Xho*I–*Kpn*I 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 acyl-coenzyme 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 *jaxX* 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 *jaxX* reported previously (Wang *et al.*, 2001) is now considered incorrect. The remainder of *jadO* was obtained by subcloning (as pJV411) the 3.6 kb *Kpn*I–*Xho*I 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 *Bam*HI–*Xho*I 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 *Bam*HI fragment in pJV423 and the LW18 insert as a *Stu*I fragment in pJV425. Sequencing and analysis of the inserts in pJV411, pJV423 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-oliase and D-mycarose 2,3-dehydratases (MtmV) of '*Streptomyces argillaceus*' (51 % identical aa) and the products of *lanS* in '*Streptomyces cyanogenus*' (68 % identity), ORF10 in *Streptomyces violaceoruber* (47 % identity), TylCVI in *Streptomyces fradiae* (46 % identity) and *eryBVI* in *Saccharopolyspora erythraea* (44 % identity). Alignment of 2,3-dehydratase

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

ORF†	Size of product (aa)‡	Distance from preceding ORFs (bp)	G + C content (mol %)	Putative function
<i>jadN</i>	609	4 bp overlap with <i>jadM</i>	70.0	Acyl-coenzyme A decarboxylase
<i>jadX</i>	172	22	72.3	Not known
<i>jadO</i>	475	1 bp overlap with <i>jadX</i>	75.9	NDP-hexose 2,3-dehydratase
<i>jadP</i>	376	26	76.8	NDP-hexose 3-ketoreductase
<i>jadQ</i>	269	68	75.1	NDP-glucose phosphate nucleotidyltransferase
<i>jadS</i>	395	47	75.7	Glycosyltransferase
<i>jadT</i>	332	32	69.1	NDP-hexose 4,6-dehydratase
<i>jadU</i>	196	8	68.6	NDP-4-keto-6-deoxyhexose 3,5-epimerase
<i>jadV</i>	341	41	76.5	NDP-4-keto-6-deoxyhexose 4-ketoreductase
<i>jadR*</i>	204	16§	73.8	<i>tetR</i> homologue

† 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.

region 1			region 2		
LanS	81	HRSGRFFSVEGLHV	146	QLSPTVQATRSN	
UrdS	77	HRSGRFFTVGLHV	147	QLSPTVQATRSN	
AveBVI	80	HRSGRFFSVEGLHV	145	QLSPTVQATRSN	
EryBVI	94	HESGRFFSIEGLHV	159	QLSPTLQATRSN	
TylCVI	43	HESGRFFSIEGLRV	108	QLSPTVQATRSN	
Orf10	63	HTSGRFFSIEGLRV	128	QLSPTVQATRSN	
MtmV	82	HDSGRFFTVGLRA	147	QLSPTVQATRSN	
JadO	75	PPQRPLLHRRGPAR	141	EPAPAVADPRGH	
		. : : *		: * : :	
region 3			region 4		
LanS	408	YEA VHSEEGGR -FLNAESRYLLIE	454	LVR HGHYVNVQARTLL ACLNA	
UrdS	407	YEA VHSEEGGR -FLNAESRYLLIVD	453	LVR HGHYVNVQARTLL ACLNA	
AveBVI	403	YAA VHSEEGGR -FLHAQARYLFVE	449	LLRYGRYVNVQARTLLSLLT	
EryBVI	443	YDA VHSEEGGR -FYRNENRYMLIE	488	LLGHSHYVNIQLRSIIACASA	
TylCVI	389	YDT VQSEEGGR -FYHARNRYAIVE	440	LLAHGNYVNVVELRTLIACAH	
Orf10	409	YDT LQSEEGGR -FHHAENRYVVVE	455	LLHHSNYVNVVEARSLVACIQ	
MtmV	430	YDA VQSEEGGR -FYRALSRYLVVE	474	LLQHSNYCNIQARSLIACLS	
JadO	455	YEA VHSEEGGRGFLQTPRAATFLG	511	TTR HGHYLVNVQARTLL ACVHA	
		* : : ***** *		: . . * * : : * : : :	

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 (HSEEGGRGF at aa 459–468; 87% identity) and region 4 (HGHYxNVQARTLLA 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 landomycin 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 aclarubicin by *Streptomyces galilaeus*, and dTDP-3,4-diketo-2,6-dideoxyglucose 3-ketoreductase (38% identity) used by *Streptomyces antibioticus* for C-2 deoxygenation during dTDP-L-oleandroside 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-D-glucose to produce 4-keto-2,6-dideoxy-D-glucose as a key intermediate.

***jadQ*.** The product of *jadQ* was similar to NDP-glucose synthases in many streptomycetes producing glycosidic 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 thymidyltransferase of *Streptomyces venezuelae* ATCC 15439 (67% identical aa) and glucose-1-phosphate thymidyltransferase 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-dideoxysugar. That pathways to deoxysugars 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 *oleG1* (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 peuceitius* 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 (Baltz & 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 *urdGT2*, *dnrS* and *oleG1* 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 OleG1, 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-dideoxysugar 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 deoxysugar 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) AklN (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 peuceitius* 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 megalomicea* 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 megalomicea* 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).

Fig. 4. Southern hybridization of *Streptomyces venezuelae* genomic DNA. (a) *Streptomyces venezuelae* genomic DNA digested with *NcoI*/*Bam*HI and probed with the 4.0 kb *NcoI*–*Bam*HI fragment from λ LW3 (see Fig. 2). Lanes: 1, *Streptomyces venezuelae* ISP5230; 2, VS1079; 3, VS1081; 4, VS1083; 5, VS1085; M, DNA size markers. (b) *Streptomyces venezuelae* genomic DNA digested with *NruI* and probed with the 2.9 kb *NruI* fragment from λ LW18 (see Fig. 2). Lanes: 1, *Streptomyces venezuelae* ISP5230; 2, VS1087; 3, VS1089. (c) *Streptomyces venezuelae* genomic DNA digested with *MluI*/*Bam*HI and probed with the 2.15 kb *MluI*–*Bam*HI fragment from λ LW18. Lanes: 1, *Streptomyces venezuelae* ISP5230; 2, VS1091; 3, VS1093. The fragment at 5.6 kb in lane 1 is ascribed to incomplete digestion of the 5.6 kb *Bam*HI fragment by *MluI* (see Fig. 2).

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 *Am^R* 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 *Am^R* 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 *NcoI*–*Bam*HI fragment from λ LW3 gave a hybridizing band at 4.0 kb from *Streptomyces venezuelae* ISP5230 and at 5.6 kb from the *jadXOPQ* 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*–*Bam*HI 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 ^{13}C NMR spectroscopy. The major signals corresponded in chemical shift values to the resonances in ^{13}C and ^1H NMR spectra published for jadomycin aglycone (Ayer *et al.*, 1991). Cultures of *jadX*-disrupted mutants with the *Am^R* 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 *Am^R* gene promoter

Since construction of the *Am^R* cassette in pJV225 (Chang *et al.*, 2001) from the *E. coli* plasmid pKC203 (Rao *et al.*, 1983; Kaster *et al.*, 1983) retained the *Am^R* 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::*Am^R* (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::Am^R transformants. Extracts from cultures of the pXE4::Am^R transformants in Gal2I medium gave 28.6 mU (mg protein)⁻¹. This level of catechol dioxygenase activity implied expression of *xylE* from the Am^R 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 *Bam*HI fragment of DNA from pJV425, containing *jadT*, *U*, *V* and *R*^{*}, was ligated to the *Bam*HI end of the 0.6 kb DNA fragment cloned in pJV424. The 4.2 kb *Nru*I–*Bam*HI insert in the resulting plasmid (pJV426) was retrieved by *Eco*RI/*Hind*III digestion and recloned in the *oriT*-containing bifunctional vector pJV326 to give pJV427. This promiscuous plasmid was transferred conjugally into *Streptomyces venezuelae* strain VS1087, in which *jadS* had been inactivated by insertion of the oppositely oriented Am^R gene. A transconjugant selected for thiostrepton resistance (indicating the presence of free or integrated pJV427) was grown in Gal2I medium under conditions favourable for jadomycin B production, but produced only jadomycin aglycone. The results indicated that introducing the genes downstream of *jadS* behind the functional promoter in pJV326 did not restore jadomycin B biosynthesis. This was consistent with the results obtained from disruption of *Streptomyces venezuelae* with the Am^R gene cassette in suggesting that inactivation of *jadS* *per se*, rather than a polar effect on expression of downstream genes, was responsible for accumulation of jadomycin aglycone. To confirm that the downstream genes in pJV427 are indeed expressed in thiostrepton-resistant transconjugants, the plasmid was conjugally transferred into strains VS1089, VS1091 and VS1093, in which *jadS* is intact but *jadT*, *jadU* and *jadV*, respectively, have been inactivated by insertion of the oppositely oriented Am^R gene. Selection of thiostrepton-resistant transconjugants and HPLC analysis of extracts from cultures grown in Gal2I medium showed that all three transconjugants produced jadomycin B exclusively, implying complementation of the disrupted genes.

DISCUSSION

The pathway for biosynthesis of 3,6-dideoxysugars has been firmly established (Liu & Thorson, 1994) and the enzymes involved have been investigated (Trefzer *et al.*, 1999), but much less is known about the 2,6- and 4,6-dideoxygenation pathways that generate the glycosidic components of many secondary metabolites. As in the present investigation, knowledge of biosynthetic reactions is limited to prediction of enzyme functions based on gene sequences, their relationship to the existing database and the phenotypic changes that result from specific gene disruptions. Biochemical characterization of intermediates accumulated after gene

inactivation provides supporting evidence for enzyme function and has not only cast light on several sugar biosynthetic pathways, but also helped in the design of hybrid natural compounds (Zhao *et al.*, 1998; Kunzel *et al.*, 1999; Olano *et al.*, 1999).

The absence of the dideoxysugar component from the substance produced when *jadO*, *P*, *Q*, *S*, *T*, *U* and *V* were disrupted implicates the products of these genes in biosynthesis or attachment of the glycosidic moiety. The conclusion is strengthened by the results from BLAST searches showing marked similarities between the deduced amino acid sequences of the genes disrupted and enzymes for deoxysugar biosynthesis in other species. Based on their sequence homologies, *jadO*, *P*, *Q*, *S*, *T*, *U* and *V* are assigned to seven biochemical reactions required in *Streptomyces venezuelae*. The combined evidence from all sources supports a plausible pathway (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 deoxygenation is catalysed by a pair of enzymes, *JadO* (a 2,3-dehydratase giving the labile intermediate NDP-3,4-diketo-2,6-dideoxyglucose 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-dideoxysugar 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-digitoxose, 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-epimerases 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-dideoxysugar biosynthesis in other

Fig. 5. Proposed pathway for biosynthesis of the L-digitoxose moiety in jadomycin B by *Streptomyces venezuelae* ISP5230.

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 poly-

ketides, the enzymes for glycosylation do not exhibit absolute specificity (Katz & Donadio, 1993; Trefzer *et al.*, 1999). Relaxed substrate selection allows glycosyl-transferases 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 glycosyl-transferases used by producers of daunorubicin (Madduri *et al.*, 1998), erythromycin (Gaisser *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-Digitoxose 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-digitoxose 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 tetrocarcin group of antitumour antibiotics from *Micromonospora chalcona* (Tomita *et al.*, 1980) and in some antifungal polyene macrolides from streptomycetes (Zielinski *et al.*, 1979).

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