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**Genes for Jadomycin B Biosynthesis and Regulation in  
*Streptomyces venezuelae* ISP5230**

**By**

**Liru Wang**

**Submitted in partial fulfillment of the requirements  
for the degree of Doctor of Philosophy**

**at**

**Dalhousie University  
Halifax, Nova Scotia  
March, 2002**

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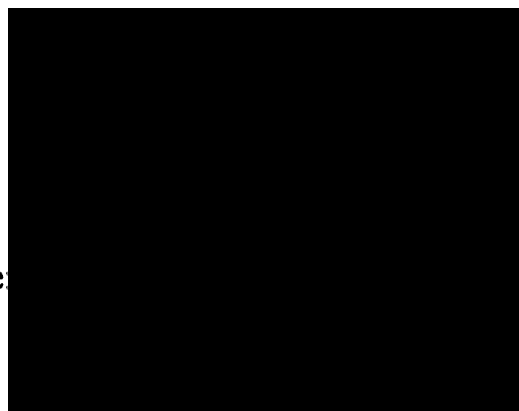
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## ABSTRACT

The sequenced region of the *Streptomyces venezuelae* ISP5230 chromosome, containing a gene cluster for biosynthesis of the antibiotic jadomycin B, was extended in both directions by chromosome walking. At the right-hand end, 13 new genes were added: these began with *jadM*, which encoded a phosphopantetheinyl transferase and partially overlapped *jadL*. Expression of *jadM* in *E. coli* and examination of the product by SDS-PAGE confirmed formation of a 29 KDa protein predicted from the *jadM* sequence. Northern hybridization indicated that biosynthesis of jadomycin B correlated with *jadM* expression. Since cultures of *S. venezuelae* disrupted in *jadM* were defective in jadomycin B production, but grew well and produced chloramphenicol normally, *jadM* was presumed to encode a holo-ACP synthase dedicated to jadomycin B biosynthesis. Downstream of *jadM* was a gene (*jadN*) encoding an acyl-CoA synthase/decarboxylase. This enzyme probably condenses acyl-coenzyme A precursors to synthesize the core linear polyketide. The adjacent genes *jadX*, *O*, *P*, *Q*, *S*, *T*, *U*, and *V* formed a sub-cluster involved in biosynthesis of the L-digitoxose moiety of jadomycin B. When the sub-cluster was cloned in *E. coli* and the genes were individually disrupted, transfer of the DNA into *S. venezuelae* by intergeneric conjugation furnished mutants altered in jadomycin B biosynthesis. HPLC and NMR analysis of intermediates accumulated in cultures of the insertionally inactivated mutants indicated that each gene mediates either formation of L-digitoxose or its attachment to jadomycin aglycone. Based on sequence similarities between the *jad* genes and genes in other species producing deoxysugar derivatives, a biosynthetic pathway generating L-digitoxose from D-glucose is proposed. *jadX* ensures complete conversion of the aglycone to the glycoside, but the mechanism is unclear. The gene (*jadR\**) following the sub-cluster encoded a TetR-type regulator. Since disrupting *jadR\** increased jadomycin B production, the protein functions as a repressor. *jadR\** is the last gene at the right-hand end of the *jad* cluster.

Chromosome walking to extend the left-hand end of the *jad* cluster added three new genes. Of these *jadW*<sub>1</sub> is a homologue of *barX* and *afsA*, which are associated with  $\gamma$ -butyrolactone autoregulators controlling morphogenesis and secondary metabolism in streptomycetes. *jadW*<sub>2</sub> is a homologue of 3- $\beta$ -keto steroid dehydrogenase, and *jadW*<sub>3</sub> is a homologue of 3- $\beta$ -keto ACP/CoA reductase. Disrupting *jadW*<sub>1</sub> not only stopped production of jadomycin B and chloramphenicol, but also prevented differentiation of the mycelium. Reintroducing *jadW*<sub>1</sub> into *jadW*<sub>1</sub>-disrupted mutants restored jadomycin B production above the wild-type titre, and allowed chloramphenicol production, implying that *jadW*<sub>1</sub> positively regulated synthesis of both antibiotics. Introducing *jadW*<sub>1</sub> into the wild type had a similar effect, and resulted in accumulation of acetyl-chloramphenicol. In contrast to the negative effect of inactivating *jadW*<sub>1</sub>, disrupting *jadW*<sub>2</sub> increased jadomycin B production 5-10 fold and allowed jadomycin B to be produced without the need for ethanol toxicity stress. Disrupting *jadW*<sub>2</sub> also interfered with spore pigmentation. Consistent with *jadW*<sub>2</sub>'s repressive role, introducing an extra copy into the wild type slowed growth, blocked production of jadomycin B and chloramphenicol, and prevented sporulation. Transforming the *jadW*<sub>2</sub>-disruptant with cloned *jadW*<sub>2</sub> restored wild-type growth and jadomycin B production. Disrupting *jadW*<sub>3</sub> caused no striking effects, but did reduce jadomycin production by 20-60%.

The structure for jadomycin B was re-examined and revised. Two additional antibiotics were detected in culture extracts of *S. venezuelae* and some *jadW* mutants.

## LIST OF ABBREVIATIONS

aa	amino acid
ACP	acyl carrier protein
ACPS	holo-acyl carrier protein synthase
<i>act</i>	gene cluster for actinorhodin biosynthesis
A-factor	$\gamma$ -butyrolactone effector in <i>S. griseus</i>
amp <sup>r</sup>	ampicillin resistance gene
Ap <sup>R</sup>	ampicillin resistance
Am <sup>R</sup>	apramycin resistance
apr <sup>r</sup>	apramycin resistance gene
ArCP	aryl carrier protein
ATP	adenosine-5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CDA	calcium-dependent antibiotic
<i>cml</i>	gene for chloramphenicol biosynthesis
Cm	chloramphenicol
CoA	coenzyme A
dATP	deoxyadenosine-5'-triphosphate
dCTP	deoxycytidine-5'-triphosphate
dGTP	deoxyguanosine-5'-triphosphate
dTTP	deoxythymidine-5'-triphosphate
DNA	deoxyribonucleic acid

<b>EDTA</b>	<b>ethylenediamine tetraacetic acid</b>
<i>fren</i>	<b>gene cluster for frenolicin biosynthesis</b>
<b>FAS</b>	<b>fatty acid synthase</b>
<i>gra</i>	<b>gene cluster for granolicin</b>
<b>HPLC</b>	<b>high performance liquid chromatography</b>
<b>HSD</b>	<b>hydroxysteroid dehydrogenase/isomerase</b>
<b>IPTG</b>	<b>isopropyl-<math>\beta</math>-thiogalactopyranoside</b>
<i>jad</i>	<b>gene cluster for jadomycin biosynthesis</b>
<b>JdB</b>	<b>jadomycin B</b>
<b>kb</b>	<b>kilobase</b>
<b>KS</b>	<b>ketosynthase</b>
<b>mer</b>	<b>oligomer</b>
<b>MOPS</b>	<b>morpholinopropanesulfonate</b>
<i>mmy</i>	<b>gene cluster for methylenomycin biosynthesis</b>
<b>NOE</b>	<b>nuclear overhauser enhancement</b>
<b>NRPS</b>	<b>non-ribosomal peptide synthetase</b>
<b>nt</b>	<b>nucleotide</b>
<b>ORF(s)</b>	<b>open reading frame(s)</b>
<b>PAGE</b>	<b>polyacrylamide gel electrophoresis</b>
<b>PCP</b>	<b>peptidyl carrier protein</b>
<b>PEG</b>	<b>polyethylene glycol</b>
<b>pfu</b>	<b>plaque forming unit</b>
<b>PKS</b>	<b>polyketide synthase</b>



<b>Ppan</b>	<b>phosphopantetheinyl</b>
<b>PPTase</b>	<b>phosphopantetheinyl transferase</b>
<b>RBS</b>	<b>ribosome binding site</b>
<i>red</i>	<b>gene cluster for undecylprodigiosin biosynthesis</b>
<b>RNA</b>	<b>ribonucleic acid</b>
<b>RNase</b>	<b>ribonuclease</b>
<b>rRNA</b>	<b>ribosomal RNA</b>
<b>rpm</b>	<b>revolutions per minute</b>
<b>SARP</b>	<i>Streptomyces</i> antibiotic regulatory protein
<b>SCB1</b>	$\gamma$ -butyrolactone effector in <i>S. coelicolor</i> A3(2)
<b>SDS</b>	<b>sodium dodecyl sulfate</b>
<b>TEMED</b>	<b>N,N,N',N'-tetramethylenediamine</b>
<b>TES</b>	<b>N-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid</b>
<b>TPNS</b>	<b>sodium-triisopropyl-naphthalene sulphonate</b>
<b>Tris</b>	<b>tris-(hydroxymethylamino)methane</b>
<b>tsr</b>	<b>thiostrepton resistance gene</b>
<b>Ts<sup>R</sup></b>	<b>thiostrepton resistance</b>
<b>VB</b>	<b>virginiae butanolide</b>
<b>w/v</b>	<b>weight per volume</b>
<b>X-gal</b>	<b>5-bromo-4-chloro-3-indoyl-<math>\beta</math>-D-galactopyranoside</b>

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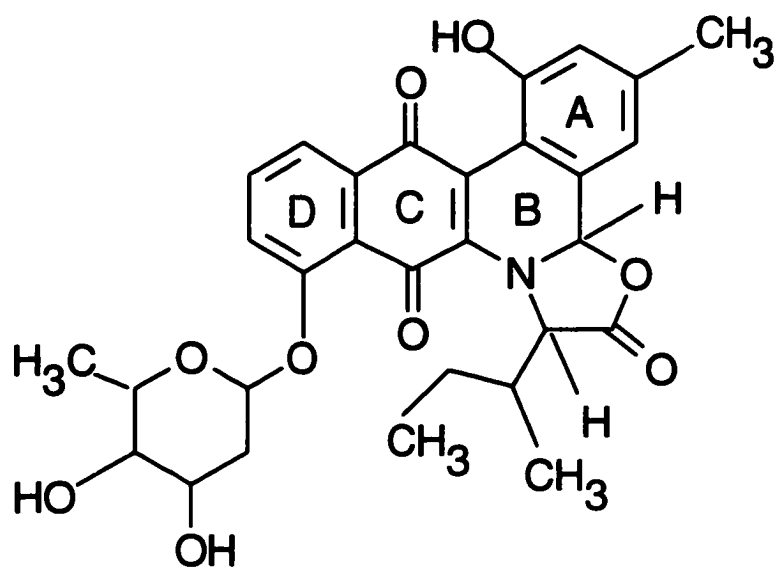
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## INTRODUCTION

The polyketide family of secondary metabolites shares with the fatty acids of primary metabolism, a general biosynthetic mechanism that forms the carbon skeletons of molecules by sequential condensation of activated low-molecular-weight acids such as acetate, propionate and butyrate. The condensation reaction generating polyketides is catalyzed by enzymes similar to the fatty acid synthases (FASs) found in all organisms where fatty acids are required for the biosynthesis of lipids (Hopwood, 1997). Polyketide synthases (PKSs) direct head-to-tail assembly of acyl precursors to generate polyketide intermediates that are then processed into a broad range of final products. The PKSs fall into three organizational groups: type I are multifunctional proteins, whereas type II are multienzyme complexes. Type III PKSs are characteristically found in plants, and are distinctly different in organization from type I and type II enzymes (Jez *et al.*, 2001). Genes for PKSs have been cloned and characterized from numerous bacteria, fungi and plants (Hopwood and Sherman, 1990; Hopwood, 1997).

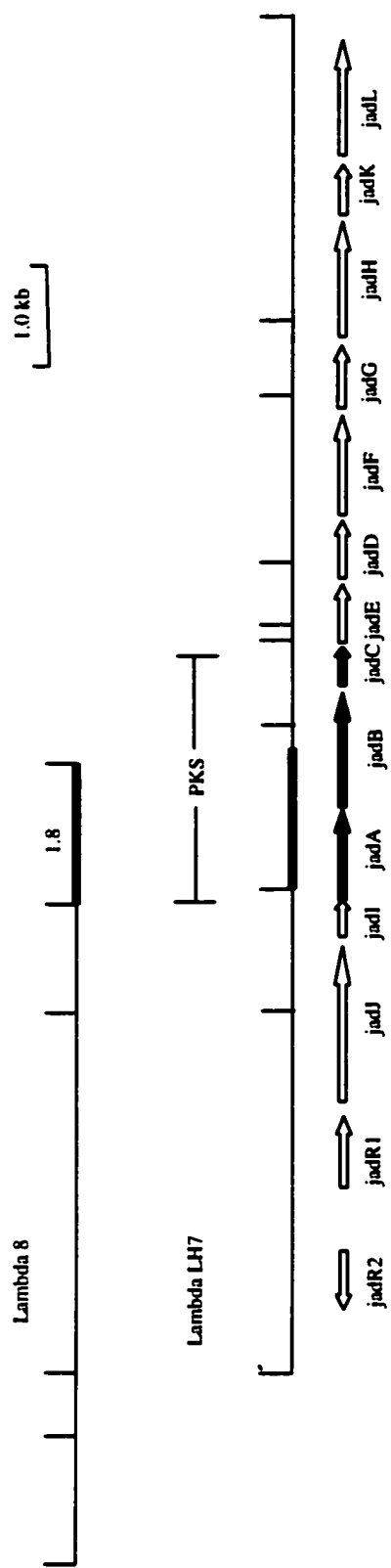
*Streptomyces venezuelae* ISP5230 produces, in addition to chloramphenicol (Cm), a second antibiotic, jadomycin B (JdB; Fig. 1; Doull, *et al.*, 1993; Doull, *et al.*, 1994), deduced from its chemical structure and the pattern of its labeling by [<sup>13</sup>C] acetate (Crowell, 1993) to be a member of the polyketide family. In the initial approach to cloning the PKS genes for JdB, a 1.8-kb fragment of *S. venezuelae* DNA that hybridized with *act* probes from *S. coelicolor* A 3(2) able to detect type-II PKS genes was cloned in a recombinant lambda phage vector 8 (Ramalingam, 1989). By using the 1.8-kb fragment



**Figure 1.** The chemical structure reported for JdB (Doull *et al.*, 1993; 1994).

to probe a genomic library of *S. venezuelae* DNA, Han *et al* (1994) isolated three additional lambda clones. From these clones, ORFs encoding JdB biosynthesis enzymes were isolated and identified (Fig. 2). Three of the ORFs encoded components of a PKS: *jadA* and *jadB* encoded ketoacyl synthases ( $KS_{\alpha}$  and  $KS_{\beta}$ , respectively), and *jadC* encoded an acyl carrier protein (ACP). Additional molecular genetic evidence established the biosynthetic origin of the jadomycin aglycone, and showed that it is derived from a decapolyketide generated by an iterative type-II PKS complex encoded by a chromosomal gene cluster (Han, *et al.*, 1994; Yang *et al.*, 1995; Yang *et al.*, 1996; Meurer *et al.*, 1997; Kulowski *et al.*, 1999).

Immediately downstream of the PKS gene cluster are *jadE* encoding a ketoreductase, and *jadD* encoding a bifunctional cyclase/dehydrase, two genes associated with cyclization and aromatization. These are followed by three genes *jadF*, *jadG* and *jadH*, with deduced amino acid sequences showing similarity to oxidoreductases. The *jadG* product resembles anthrone oxidases, and is postulated to generate the quinone keto groups of JdB and rabelomycin, a metabolite biosynthetically related to jadomycin (Yang *et al.*, 1996). The *jadH* product is a monooxygenase that probably acts during cyclization of the polyketide to generate an angucycline intermediate (McVey, 1998). The *jadF* product is involved in opening ring B of an angucycline precursor to allow insertion of the isoleucine component of jadomycin aglycone (Yang *et al.*, 1996). Because the deduced amino acid sequence of *jadK* did not resemble any protein in the GenBank database, the function of *jadK* in JdB biosynthesis is not yet known. Its disruption reduced, but did not



**Figure 2.** *SacI* restriction enzyme maps of Lambda 8 and Lambda LH7. Arrows below the LH7 map show ORFs detected. Filled arrows indicate the core PKS cluster. The 1.8-kb fragment used by Han *et al* (1994) to probe the *S. venezuelae* ISP5230 genomic library is shown as black bars in both maps.

block jadomycin production (J. He, personal communication). The product of *jadL* has characteristics of a transmembrane transporter protein (McVey, 1998), and the partial *jadM* immediately downstream of *jadL*, and not shown in Fig. 2, encodes an amino acid sequence with similarity to HetI, a phosphopantetheinyl transferase involved in producing a secondary metabolite that regulates heterocyst spacing in *Anabaena* strain PCC7120 (Black & Wolk, 1994; McVey, 1998). The region upstream of the PKS cluster contained four ORFs (Yang *et al.*, 1995). The product of *jadI* was identified as a four-ring cyclase associated with angucycline formation (Kulowski *et al.*, 1999), and the adjacent *jadJ* encoded the biotin carboxylase and biotin carboxylase carrier protein domains of an acetyl-coenzyme A carboxylase generating malonyl-coenzyme A destined for use in polyketide biosynthesis (Han *et al.*, 2000). Upstream of *jadJ* are two ORFs involved in regulating production of JdB. One (*jadR*<sub>1</sub>) encodes a polypeptide resembling in amino acid sequence the response-regulator protein of two-component signaling systems (Yang *et al.*, 2001). The other ORF (*jadR*<sub>2</sub>) encodes a polypeptide similar in amino acid sequence to a group of repressor proteins (Yang, *et al.*, 1995).

The present research was designed to establish the size and organization of the *jad* cluster. By chromosomal walking, the sequenced regions at both ends of the cluster were extended. In the downstream direction 13 new genes were added from a 12-kb fragment. By aligning their deduced protein sequences with functional homologues in other organisms, as well as by determining the effects of gene disruptions, the functions of 11 of them were assigned. Since the products of the last two genes at the downstream end of the sequenced region exhibited primary metabolic functions, they were presumed to lie

outside the *jad* cluster. Chromosome walking to extend the *jad* cluster in the upstream direction added three new genes, *jadW*<sub>1</sub>, *jadW*<sub>2</sub> and *jadW*<sub>3</sub>, on a 3.0-kb genomic fragment. One of these genes is implicated in a regulatory mechanism involving  $\gamma$ -butyrolactone effectors.



## **LITERATURE REVIEW**

### **I. Streptomyces**

Streptomyces are Gram-positive soil bacteria with two characteristic features not found in the well studied bacterium *Escherichia coli*: (1) during their life cycle they undergo a morphological change in which the filamentous hyphae formed for vegetative growth develop into spore-producing structures; (2) the streptomyces undergo physiological development to produce diverse secondary metabolites that include such pharmaceutically useful compounds as anti-tumour agents, immunosuppressants, and about 70% of current commercially available antibiotics.

#### **A. Secondary metabolites from streptomyces**

Primary metabolites are essential for the survival and well-being of all organisms, whereas secondary metabolites have no such “housekeeping” role. Although as much a product of the genetic make-up of the organism as are the primary metabolite pathways, secondary metabolism is activated only during a particular stage of growth, or during a period of stress caused by nutritional limitation or other environmental challenge (Vining 1990). Many secondary metabolites specifically inhibit bacteria or fungi by interfering with their metabolic processes. They typically appear in the transition phase, rather than during vegetative growth of the producer, as might be expected if their role is to suppress nutritional competition. Primary and secondary metabolism are interconnected, since

primary metabolism provides the precursor molecules employed as starting materials for secondary metabolic pathways. Mann (1987) considered the three principal starting materials to be: (1) shikimic acid, supplying many aromatic compounds; (2) amino acids, leading to alkaloids and peptide-derived metabolites; (3) acetate, a precursor of polyketide-derived macrocyclic and aromatic compounds.

The industrial and clinical importance of antibiotics produced by streptomycetes has fostered research into the molecular genetics of antibiotic biosynthesis. Sequencing of the *Streptomyces coelicolor* A3(2) genome, recently completed at the Sanger Institute ([http://www.sanger.ac.uk/Projects/S\\_coelicolor/](http://www.sanger.ac.uk/Projects/S_coelicolor/)), has stimulated further interest and provides an invaluable resource for exploiting the genetics of secondary metabolism.

## **B. Genes for antibiotic biosynthesis**

*Streptomyces coelicolor* A3(2), which has been investigated as a model streptomycete, produces four identified antibiotics, actinorhodin, undecylprodigiosin, methylenomycin, and the calcium-dependent lipopeptide CDA (Hopwood *et al.*, 1995). The genes for actinorhodin (*act*) and undecylprodigiosin (*red*) have been identified in clusters within the chromosome; those for methylenomycin lie on the large linear SCP1 plasmid, which can integrate into the chromosome. The recently finished genome sequence of *Streptomyces avermitilis* has 25 kinds of secondary metabolite gene clusters in its genome (Omura *et al.*, 2001), including that for the antiparasitic agent avermectin, a commercially important product used in human and veterinary medicine. Other such clusters are involved in the

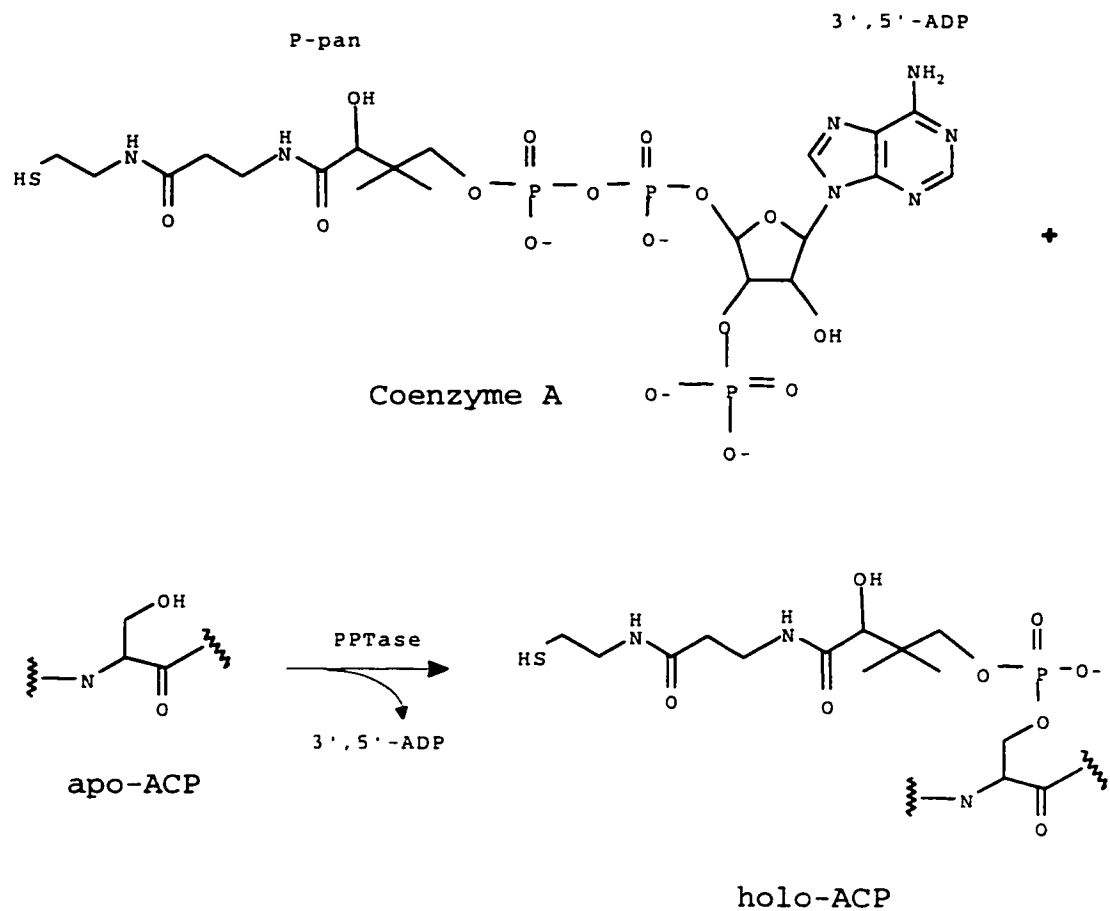
biosynthesis of melanoid, carotenoid, siderophore, polyketide, and peptide compounds. Eight of the gene clusters encode various type-I polyketide synthases, two clusters are involved in the biosyntheses of type-II polyketide-derived compounds, and eight clusters are associated with the biosyntheses of compounds by nonribosomal peptide synthetases (NRPSs). The genes for synthesis of a polyketide-derived metabolite are usually linked in a single cluster (Sherman *et al.*, 1989; Butler *et al.*, 1989).

## **II. Assembly of Polyketide Intermediates**

Among the many types of naturally occurring metabolites of polyketide origin are aromatic compounds (which are generated by type-II PKSs) and complex cyclic ester (macrolide) structures (generated by type-I PKSs). Polyketide-derived substances show biological and pharmaceutically important activities. Some notable examples are the erythromycins and tetracyclines (antibiotics), doxorubicin (an anticancer drug), avermectin (an antiparasitic drug), FK506 and rapamycin (immunosuppressant agents), and lovastatin (a cholesterol-lowering agent). The reaction mechanism of polyketide assembly is similar in principle to that used in the biosynthesis of fatty acids, but the  $\beta$ -carbonyl groups of intermediates formed by the PKSs remain largely unreduced and may cyclize to produce aromatic compounds. In the biosynthesis of fatty acids the intermediates formed by multifunctional FASs are processed through reduction, reduction and dehydration, or reduction, dehydration and further reduction to produce less reactive compounds.

The first condensation reaction in the two biosynthesis pathways uses a ketoacyl synthase, and is followed by a series of reactions catalyzed by enzymes such as ketoreductase, dehydrase and enoyl reductase that generate a variety of extended carbon chains. Both PKS and FAS enzyme complexes require post-translational modification of their constituent acyl carrier proteins to become catalytically active (Christopher *et al.*, 1997). The inactive apo-proteins (Fig. 3) are converted to active holo-enzymes by esterification of a specific serine hydroxyl with the 4'-phosphopantetheine prosthetic group of coenzyme-A (Lambalot *et al.*, 1996). The gene for the phosphopantetheinyl transferase of fatty acid synthase (ACPS) has been identified in *Escherichia coli*, and similar genes have been described for the phosphopantetheinyl transferases activating enterobactin synthetase (EntD) and surfactin synthetase (Sfp), responsible for biosynthesis of the secondary metabolites enterobactin in *E. coli* and surfactin in *Bacillus subtilis*, respectively (Lambalot *et al.*, 1996; Quadri *et al.*, 1998).

For the biosynthesis of polyketide-derived antibiotics by type-II PKSs in streptomycetes, each PKS complex has a dedicated *holo*-ACP, which in turn requires a specific *holo*-ACP synthase. The latter enzymes function as integral components of the antibiotic biosynthesis pathway, and differ from those that participate in fatty acid biosynthesis (Hopwood & Sherman, 1990; Hutchinson, 1995). However, *fren* and *gra* apo-ACPs could be phosphopantetheinylated *in vitro* by purified *E. coli* ACPS. When combined with ACP-, KS $\alpha$ - and KS $\beta$ -deficient *act* ketosynthase isolated from *S. coelicolor* A3(2), the holo-ACPs formed *in vitro* were fully functional in polyketide synthesis (Carreras *et al.*, 1997). Moreover, co-expression of actinorhodin and griseusin ACPs with ACPS in *E.*



**Figure 3.** Schematic for conversion of an inactive apo-protein to an active holo-enzyme. P-pan, phosphopantetheine; PPTase, phosphopantetheinyl transferase.

*coli* gave high titres of active *holo*-ACPS (Cox *et al.*, 1997), and *E. coli* ACPS efficiently modified post-translationally the *apo*-ACPs involved in biosynthesis of granaticin, frenolicin, oxytetracycline, and tetracenomycin (Gehring *et al.*, 1996). These results imply that *E. coli* ACPS has broad substrate specificity. However, it will not recognize the *apo*-forms of several PCP and ArCP domains, including the *apo*-PCP domain of *E. coli* EntF and the *apo*-ArCP domain of *E. coli* EntB (Lambalot *et al.*, 1996; Gehring *et al.*, 1997; Quadri *et al.*, 1998).

### **III. Genetics of Polyketide Biosynthesis**

#### **A. Organization of genes for type-II PKSs**

The genes for actinorhodin biosynthesis are clustered on a 21-kb fragment of chromosomal DNA containing 23 ORFs (Hopwood *et al.*, 1995). In the downstream region (right-hand end) of the *act* cluster, three *actI*-ORFs (*actI*-ORF1, -ORF2, and -ORF3), adjacent to genes *actIII*, IV and VB, are involved in the earliest stages of actinorhodin carbon chain assembly, catalyzed by actinorhodin PKS. The *act* cluster also contains a pathway-specific positive activator gene (*actII*-ORF4), genes involved in export of actinorhodin (*actII*-ORF2 and -ORF3), and a repressor gene *actII*-ORF1. Similar gene organizations have been found during studies of antibiotic biosynthesis in *Streptomyces* species producing granaticin (Sherman *et al.* 1989), tetracenomycin (Bibb *et al.*, 1989), and oxytetracycline (Kim *et al.*, 1994). The use of *actI* and *actIII* as probes has allowed the cloning of PKS gene clusters from *S. halstedii* (Blanco *et al.*, 1992), *S.*

*curacoi* (Bergh & Uhlen, 1992), *S. roseofulvus* (Bibb *et al.*, 1994), *S. griseus* (Yu *et al.*, 1994), and *S. venezuelae* ISP5230 (Han *et al.*, 1994). All of these clusters encoded type-II PKSs involved in the biosynthesis of aromatic antibiotics, and containing a minimal set of core subunits required for *in vivo* polyketide biosynthesis. This set consists of the two keto-synthase components ( $KS_{\alpha}$  and  $KS_{\beta}$ ), an acyl carrier protein (ACP), a malonyl transferase (MT), a holo-ACP synthase (HS), and a keto-reductase (KR). Additional subunits that may be present include ketoreductases and aromatases responsible for forming specific polycyclic aromatic products from the nascent chain. The diversity of products derived from polyketides is increased by tailoring enzymes that catalyze post-assembly processes.

## **B. Regulation of polyketide biosynthesis**

In the model streptomycete *Streptomyces coelicolor* A3(2), where various genes with pleiotropic regulatory effects have been identified, genes in the pathways for biosynthesis of actinorhodin (*act*) and undecylprodigiosin (*red*) are activated by expression of the pathway-specific regulatory genes *actII-ORF4* and *redD*, respectively. Various other pleiotropic regulatory genes (*afs*, *aba*, *abs*, *ptp*, *rel*, *bld*) as well as some autoregulator genes (Hara *et al.*, 1983; Horinouchi *et al.*, 1983; Ishizuka *et al.*, 1992; Umeyama *et al.*, 1996; Chater and Bibb, 1997) influence biosynthesis of the two antibiotics, and Hopwood (1988) suggested that these function via *actII-ORF4* and *redD*, and thus act at a higher level. Some of these “global” regulatory genes control both antibiotic production and

other cellular processes. Overall, the results have suggested that control of antibiotic production might be operating on several levels.

#### **a. First-level control**

This would be exerted (Hopwood, 1988) by genes that act pleiotropically on both secondary metabolism (e.g., antibiotic production) and morphological differentiation (e.g. the formation of aerial mycelium). Examples include the *bld* genes (Champness, 1988; Bibb, *et al.*, 2000; Gehring *et al.*, 2001; Kelemen *et al.*, 2001; Elliot *et al.*, 2001), and those such as *relC* (Ochi, 1990) and *relA* (Martinez-Costa *et al.*, 1996; Chakraborty *et al.*, 1996; Chakraborty and Bibb, 1997) that modulate the stringent response. Genetic studies with *S. coelicolor* A3(2) revealed several classes of bald (*bld*) mutants unable to form aerial mycelium (Merrick, 1976). Those also blocked in antibiotic production may have defective global regulators that switch on both activities. Only for *bldA* is the mechanism of action even partially understood. In *S. coelicolor* A3(2) *bldA* codes for a tRNA-like gene product that recognizes the UUA (leucine) mRNA codon. This codon is rare in *Streptomyces* due to their high G+C content (Fernandez-Moreno *et al.*, 1991) and in *S. coelicolor* and *S. lividans* *bldA* is the only gene encoding the tRNA translating UUA. Leskiw *et al.* (1991) suggested that TTA codons are absent from genes that function during vegetative growth, and from the structural genes for actinorhodin production, but are present in genes regulating secondary and morphological development. Consequently, *bldA* is not essential for growth, but controls antibiotic biosynthesis and sporulation through the limited availability of tRNA<sup>leu</sup>.



Because a *bldD* mutant elicited formation of aerial mycelium in all other *bld* mutants tested, Willey *et al.* (1993) placed *bldD* at the top of a regulatory hierarchy. Phenotypically, *bldD* and *bldA* mutants are very similar: neither class of mutants produces actinorhodin, undecylprodigiosin, methylenomycin A or CDA, the four antibiotics found in wild-type *S. coelicolor* A3(2), nor does either class form aerial mycelium. Recent studies (Elliot *et al.*, 1996; 2001) showing that *bldD* encodes a small, highly charged protein able to negatively regulate its own transcription, suggest that the *bldD* product is a transcriptional regulator. The *bldD* mutation acts *in vivo* to repress premature expression of *whiG*, *bldN* and *bdtA* during vegetative growth. Other *bld* mutants act in different ways: *bldK* encodes subunits of the membrane-spanning oligopeptide-permease family of ATP-binding cassette (ABC) transporters (Nodwell *et al.*, 1996). *bldB* encodes a small protein with a high negative charge and a DNA-binding sequence implicated in the regulation of catabolite control (Pope *et al.*, 1996 & 1998).

The nucleotide (p)ppGpp plays a role in triggering the onset of antibiotic production (Chakraburty *et al.*, 1996). Mutation of *relA*, which encodes ppGpp synthetase in *S. coelicolor* A3(2), resulted in loss of ppGpp synthesis, and failure to produce undecylprodigiosin and actinorhodin under conditions of nitrogen limitation (Chakraburty and Bibb, 1997). This implied that transcription of the respective pathway-specific regulatory genes *redD* and *actII-ORF4* had been diminished. The *relA* mutant also showed a marked delay in the onset and extent of morphological differentiation, resulting in conspicuously altered colony morphology.

### **b. Second-level control**

Second-level control is exerted by genes with pleiotropic effects on one or more antibiotic biosynthetic pathways, but not on morphological development. Examples are *absA*, *absB*, *afsB*, *afsR*, *afsQ1* and *afsQ2*, and *ptpA*; also in this group is *afsS* (Matsumoto *et al.*, 1995). Strains of *S. coelicolor* A3(2) mutated in *absA* or *absB* are defective in the biosynthesis of all four antibiotics, but express methylenomycin resistance (Adamidis *et al.*, 1990; Brian *et al.*, 1996); *afsB* mutants are defective in actinorhodin, undecylprodigiosin and A-factor production, but produce methylenomycin and CDA normally (Hara *et al.*, 1983); disruption of chromosomal *afsR* in *S. coelicolor* A3(2) delays and reduces actinorhodin production, and extra copies of DNA containing *afsR* cause overproduction of actinorhodin, undecylprodigiosin and  $\gamma$ -butyrolactones in *S. lividans* (Horinouchi *et al.*, 1983); cloned copies of *afsQ1* and *afsQ2* restore actinorhodin and undecylprodigiosin production in *absA* mutants of *S. coelicolor* (Ishizuka *et al.*, 1992); transformants of *S. lividans* containing *ptpA* on a high copy number vector overproduce undecylprodigiosin and A-factor, but transformation with *ptpA* on a low copy number vector, and disruption of the chromosomal *ptpA* of *S. coelicolor*, do not affect production of the pigmented antibiotics or A-factor (Umeyama *et al.*, 1996).

### **c. Third-level control**

This level of control is exerted on individual biosynthetic processes by pathway-specific regulators. In *S. coelicolor* A3(2), extra copies of *redD* and *actII-ORF4* elicit over-

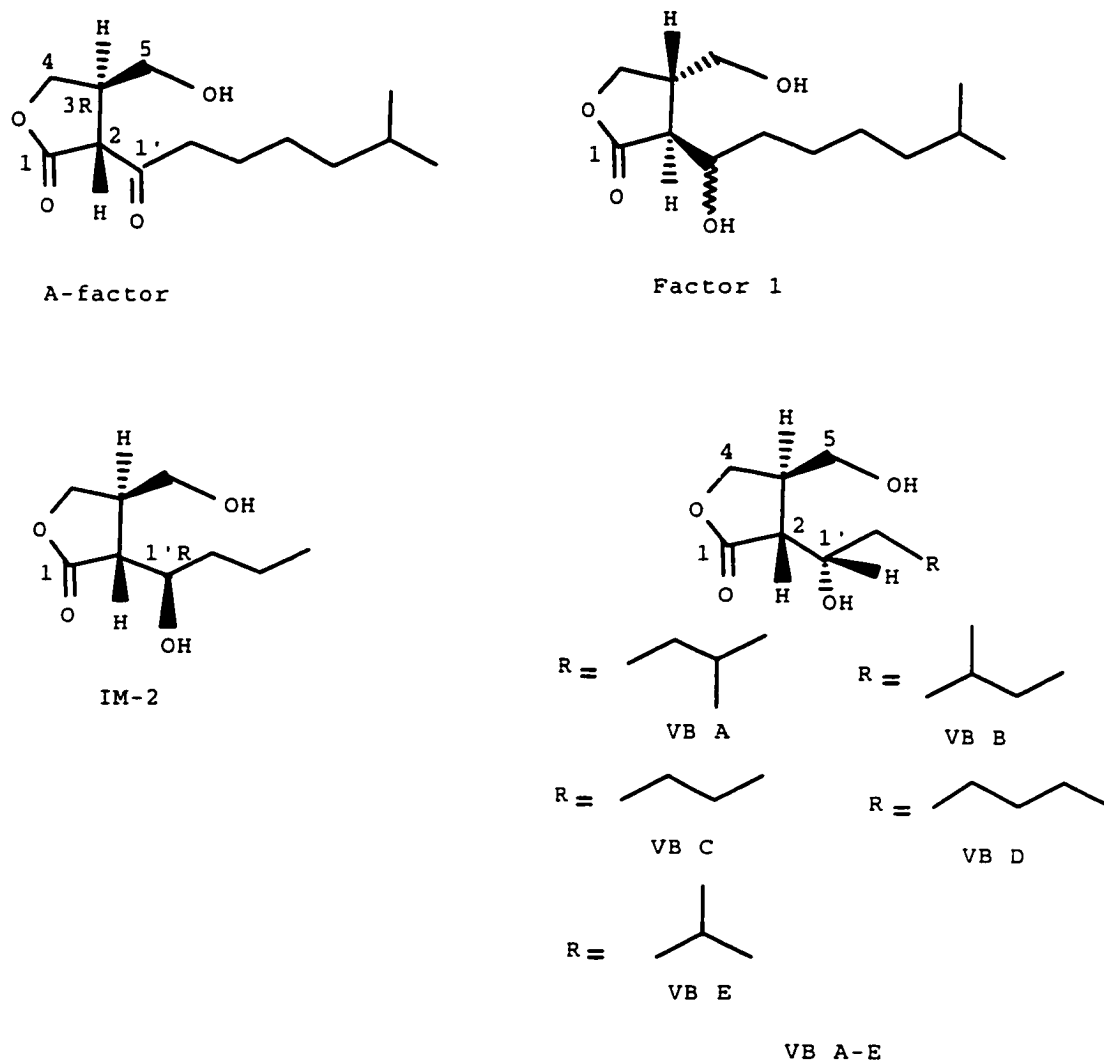
production of undecylprodigiosin and actinorhodin, respectively (Malpartida and Hopwood, 1986; Narve and Feitelson, 1990). The failure of *redD* and *actII-ORF4* mutants to co-synthesize the antibiotics in mixed cultures with any other *red* or *act* mutant classes, considered with the lack of expression of *red* and *act* biosynthetic structural genes in *redD* and *actII-ORF4* mutants, suggest that *redD* and *actII-ORF4* are pathway-specific activator genes. Similar pathway specific transcriptional activators, designated *Streptomyces* antibiotic regulatory proteins (SARP), have been found in other streptomycetes (Wietzorrek and Bibb, 1997). These include species producing *cdaR* (Chong, 1998), *dnrI* (Stutzman-Engwall *et al.*, 1992), *ccaR* (Perez-Llarena *et al.*, 1997), and the N terminal region of the AfsR protein (Horinouchi *et al.*, 1990). Members in the SARP family usually regulate only one antibiotic pathway, and appear to have similar mechanisms of transcriptional activation through DNA binding to specific nucleotide sequences (Arias *et al.*, 1999).

#### **d. Regulation by $\gamma$ -butyrolactones**

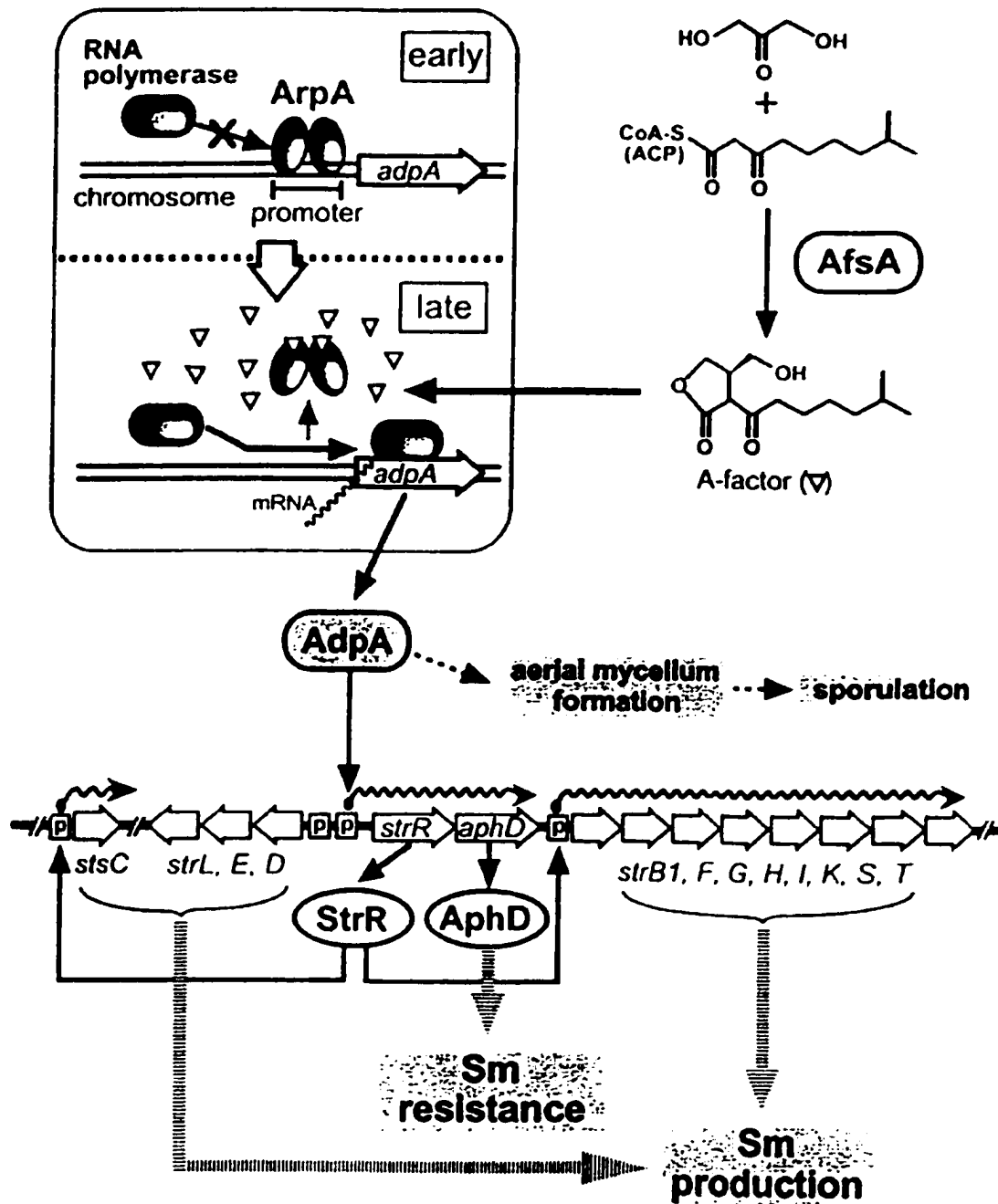
In several streptomycetes,  $\gamma$ -butyrolactones – often called autoregulators (Yamada and Nihira, 1998) – play important roles as extracellular factors triggering both morphological differentiation and secondary metabolism. It has been estimated (Horinouchi & Beppu, 1992; Yamada, 1999) that this type of regulatory molecule is present in at least 60% of streptomycetes. A-factor (2-isocaprolyl-3R-hydroxymethyl- $\gamma$ -butyrolactone) identified from *Streptomyces griseus* is the most intensively studied representative so far. The structure of A-factor contains a 1'-keto group that is varied in

some  $\gamma$ -butyrolactone autoregulators by stereospecific reduction (Fig. 4). The *R* ( $\alpha$ ) and *S* ( $\beta$ ) series of 1'-hydroxy- $\gamma$ -butyrolactones have been identified in, respectively, the virginiae butanolide (VB) autoregulators of *Streptomyces virginiae* (Kawachi *et al.*, 2000), and the IM-2 group from *Streptomyces* sp. FRI-5 (Hashimoto *et al.*, 1992). Six different  $\gamma$ -butyrolactones varying in structure were isolated from *S. coelicolor* A 3(2) (Efremenkova *et al.*, 1985) and more recently a  $\gamma$ -butyrolactone (SCB1) with IM-2 stereochemistry has been characterized from this species (Takano *et al.*, 2000). Among several other naturally occurring  $\gamma$ -butyrolactones cited by Takano *et al.* (2000), Factor I in *Streptomyces viridochromogenes* influences anthracycline formation (Graefe *et al.*, 1983).

Regulatory mechanisms involving  $\gamma$ -butyrolactones act pleiotropically, and appear to be engaged at more than one level of control. In *S. griseus*, where A-factor has been shown to function at extremely low concentration as a signaling molecule for both antibiotic production and cell differentiation, a regulatory cascade leading to activation of streptomycin biosynthesis has been proposed (Fig. 5; Ohnishi *et al.*, 1999). In this model, A-factor is gradually accumulated in a growth-dependent manner by the action of AfsA, which probably condenses a 3-carbon intermediate from glycolysis with a  $\beta$ -keto acyl intermediate from fatty acid biosynthesis (Ando *et al.*, 1997). When the concentration of A-factor reaches a certain critical level, it binds to a cytoplasmic receptor protein (ArpA). In the absence of A-factor, ArpA is firmly bound to the promoter region of *adpA*, preventing transcription. When A-factor binds to ArpA, the receptor dissociates from the *adpA* promoter, allowing transcription and translation of *adpA*, which is a pleiotrophic



**Figure 4.**  $\gamma$ -Butyrolactones isolated from streptomycetes. A-factor from *S. griseus* controls streptomycin production and sporulation; IM-2 from *S. lavendulae* controls showdomycin and minimycin production; VB from *S. virginiae* controls virginiamycin production; Factor 1 from *S. viridochromogenes* controls anthracycline production and sporulation.



**Figure 5.** The A-factor regulatory cascade leading to the onset of streptomycin biosynthesis in *S. griseus* (Ohnishi *et al.*, 1999).

regulatory gene controlling both streptomycin production and cell differentiation (Miyake *et al.*, 1989, 1990). Its product exerts a positive effect on the promoter of the regulatory gene *strR*, which initiates streptomycin production by allowing transcription of most of the streptomycin biosynthesis and resistance genes (see Fig. 5; Ohnishi *et al.*, 1999).

BarX is a regulatory protein controlling the biosynthesis of VB and virginiamycins, as well as resistance to virginiamycin M1 in *S. virginiae* (Kawachi *et al.*, 2000). Disruption of *barX* caused no apparent changes in morphology of *S. virginiae*. Even though *afsA* and *barX* exhibit very high sequence similarity, and receptor proteins for A-factor, and VB share certain characteristics, there is evidence (Kawachi *et al.*, 2000) that the two genes encode proteins with different functions in the parent strains. Although BarX is involved in regulating the VB biosynthetic pathway, it differs from A-factor in not functioning as an enzyme in  $\gamma$ -butyrolactone synthesis. The timing of A-factor production in *S. griseus* cultures is different from that of VB production in *S. virginiae*. VB is produced just before the onset of virginiamycin biosynthesis, and acts only on the regulatory cascade for secondary metabolism, whereas A-factor is produced in a growth-dependent manner and controls a very early stage common to both morphological and physiological differentiation (Ohnishi *et al.*, 1999). Genes for the VB-type regulatory system may lie within the gene cluster for biosynthesis of the secondary metabolite they control, so that production of VB and virginiamycin is synchronized. In the VB system, *barA* which encodes a receptor homologous to the A-factor receptor ArpA, is located close to *barX* (Kinoshita *et al.*, 1997). In contrast, *afsA*, encoding the key enzyme for A-factor

biosynthesis (Ando *et al.*, 1997), is near one end of the linear chromosome (Lezhava *et al.*, 1997), and is distant from *arpA* (Ohnishi *et al.*, 1999).

Streptomycetes appear to contain redundant  $\gamma$ -butyrolactone regulatory systems, some of which control both physical and morphological development while others control only one of these processes. There is evidence that *S. coelicolor* A3(2) contains seven different  $\gamma$ -butyrolactones (Anisova *et al.*, 1984; Takano *et al.*, 2000) and at least three *arpA*-like genes (Onaka *et al.*, 1998). More information about their distribution, and detailed knowledge of the regulatory cascades in which they participate are needed to determine whether they function as all-or-none switches or can fine-tune levels of metabolic activity.

#### **IV. Post-assembly Modification of Polyketide Metabolites by Glycosylation**

Cloning and characterization of genes for the biosynthesis of polyketides have shown that clusters for aromatic polyketide-derived antibiotics are usually 20-50 kb long (Bechthold *et al.*, 1995; Westrich *et al.*, 1999). Clusters for other, more complex polyketide-derived products can be 70-110 kb in length (Xue *et al.*, 1998; Ikeda *et al.*, 1999). Analysis of the clusters indicated that genes for assembly of the polyketide are centrally located; a set of regulatory genes, usually present adjacent to and upstream of the PKS genes, controls transcription of the clusters. Genes for post-assembly modification of the polyketide may be at one end, or scattered at both ends of the PKS genes. Glycosylation is the most common modification, and the genes supporting this activity are generally found at both



ends of the core PKS cluster. This kind of organization makes it difficult to distinguish genes for sugar biosynthesis from the genes coding for regulatory proteins and for other polyketide modification enzymes. Consequently, information on the pathways for biosynthesis of the glycosidic components of polyketide antibiotics is relatively limited.

In contrast, a great deal has been learned about the aglycone components since Hopwood and Sherman (1990) reviewed the molecular genetics of polyketide synthesis with a focus on the genes encoding their PKSs. Progress has also been made on the recombination of PKS genes to construct novel metabolites (Hutchinson and Fuji, 1995; Hutchinson, 1999; McDaniel *et al.*, 1995 & 1999; Shen *et al.*, 1999). However, the polyketide skeleton made by PKSs is never the final bioactive product of a pathway. Post-assembly modifications are as important as the reactions that create the initial polyketide skeleton. The increased knowledge of the genes for polyketide aglycone biosynthesis has been supplemented by research on biosynthesis of the other substituents. Various chemical and biochemical aspects of sugar biosynthesis pathways have become active fields of interest.

#### **A. Naturally occurring deoxysugars**

Our understanding of the formation of the unusual sugars in polyketide antibiotics is still limited (Liu & Thorson, 1994; Kirschning *et al.*, 1997; Trefzer *et al.*, 1999). Many glycosylated secondary metabolites contain deoxy sugars found elsewhere in nature as constituents of glycoproteins, bacterial cell walls, and plant glycosides. In particular, the 2,6- and 4,6-dideoxyhexoses are found in a broad range of bioactive compounds, and

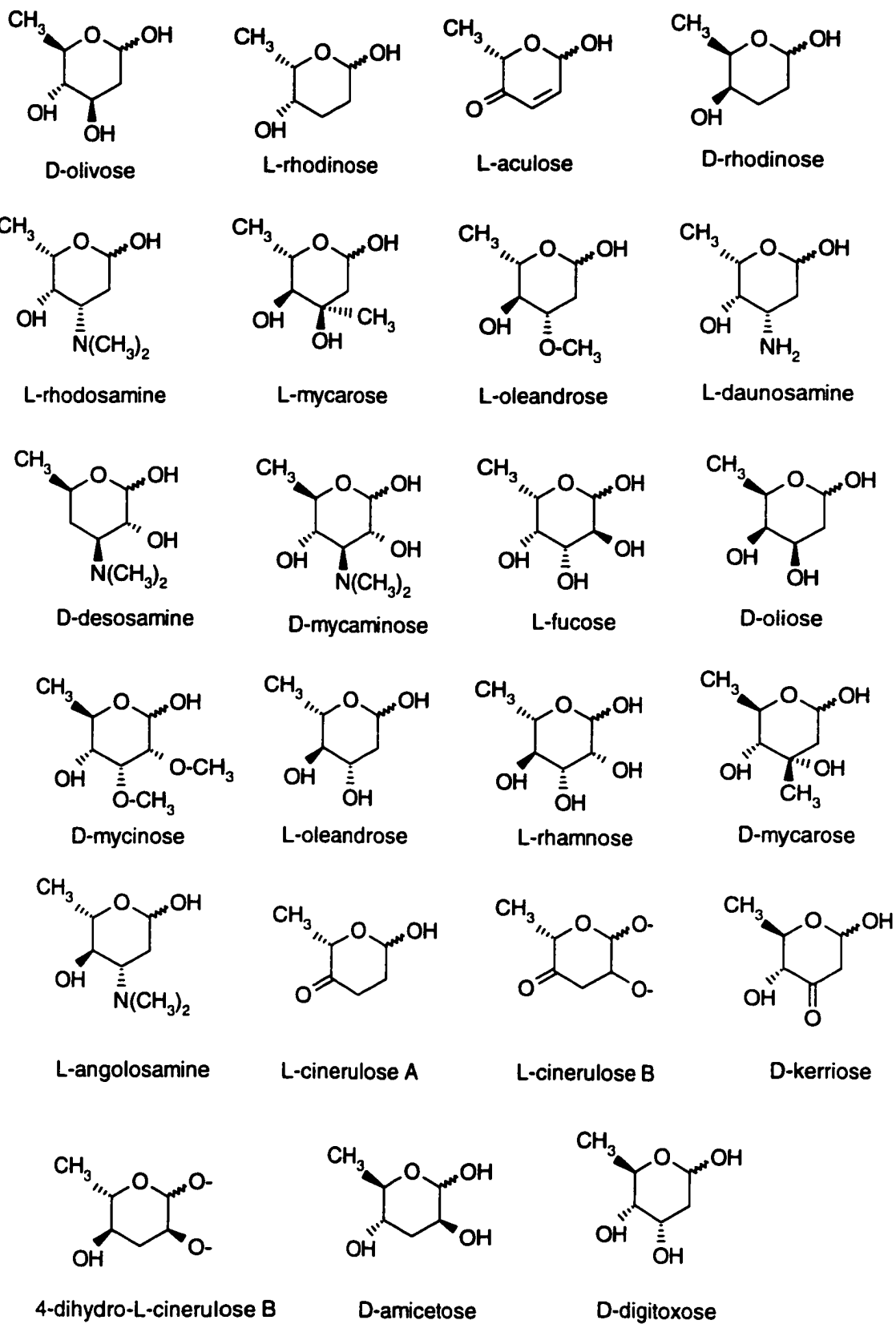
are formed biosynthetically from common monosaccharides. Modifications of the biosynthetic pathways generate deoxyaminosugars. Although the precise function of the carbohydrate moiety in most glycosylated molecules has not yet been fully elucidated, incorporation of a deoxysugar as a structural component seems to alter the way in which a compound or organism interfaces with its surroundings. Examples include the requirement of daunosamine for the antineoplastic activity of doxorubicin and daunorubicin (Krugel *et al.*, 1993; Otten *et al.*, 1995), the inactivation by glycosyl transfer of the antibiotic activity of the macrolide during oleandomycin biosynthesis (Hernandez *et al.*, 1993; Quiros *et al.*, 1998), and the role of the oligosaccharide moieties in binding antibiotics at the minor groove of DNA to form stable antibiotic-DNA complexes (Walker *et al.*, 1990). The structural variety of deoxysugars suggests diversity in their biosynthesis. However, their contribution to the mechanism of action of the final products is still poorly understood.

Perhaps the best known deoxysugar is 2-deoxy-D-ribose, which provides the scaffold on which the DNA helix ascends. In microorganisms deoxygenated sugars other than 2-deoxy-D-ribose, can be found as elements of lipopolysaccharides (Bochkov *et al.*, 1991), extracellular polysaccharides (Andrianopoulos *et al.*, 1998), and antibiotics (Weber *et al.*, 1990; Westrich *et al.*, 1999). Lipopolysaccharides are constituents of the Gram-negative bacterial cell wall, and form the endotoxins of these organisms. Extracellular polysaccharides can be found as capsular compounds and in colanic acid. Polyketide antibiotics very often contain sugars deoxygenated at C-2, C-3, C-4 and C-6 (Fig. 6). Among them are D-olivose (2,6-dideoxy-D-arabino-hexopyranose, Hutchinson and Fuji,

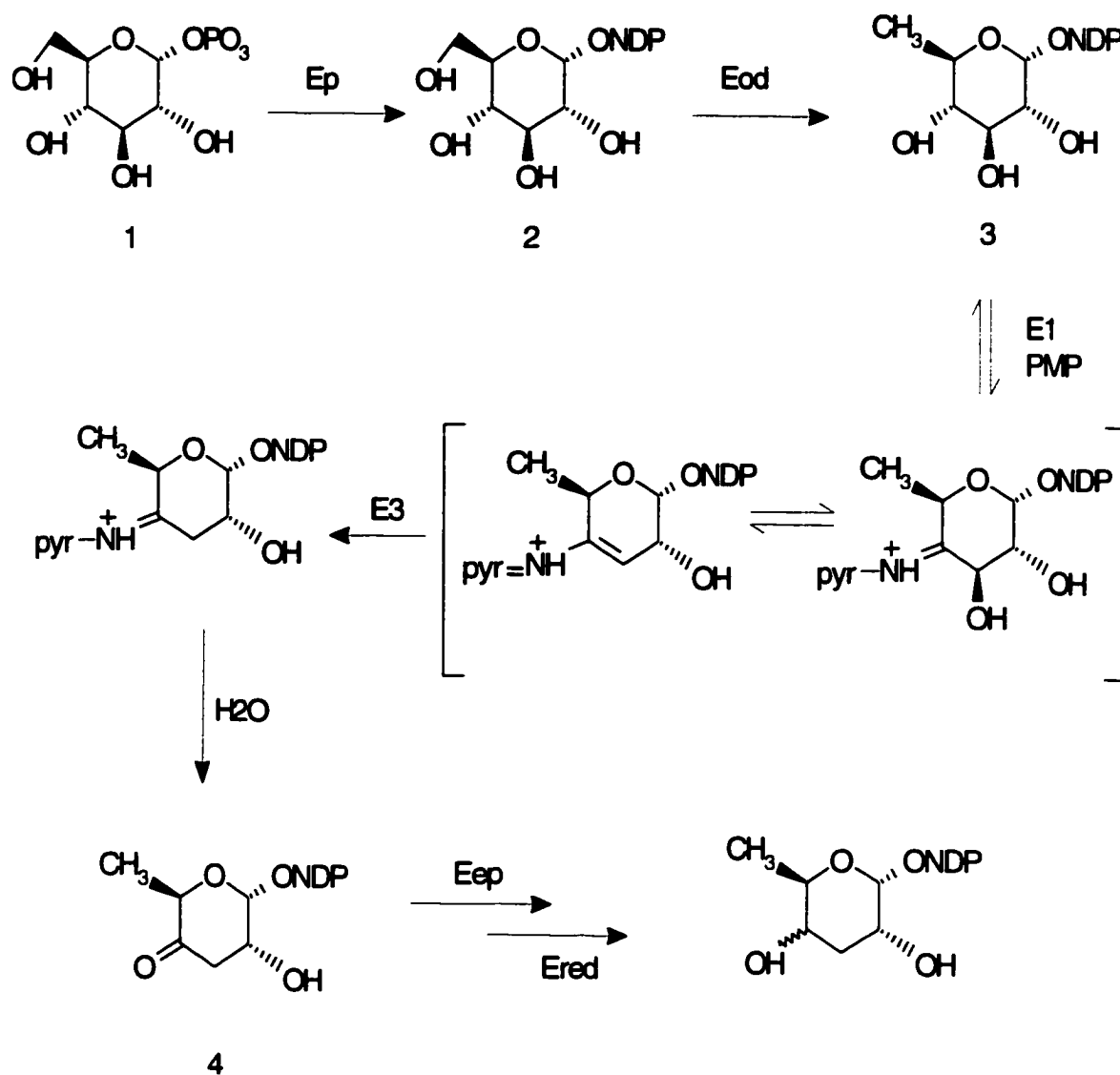
1995), D-digitoxose (2,6-dideoxy-L-ribohexopyranose, Murakami *et al.*, 2001), L-rhodinose (2,3,6-trideoxy-L-*threo*-hexopyranose, Stevens *et al.*, 1964), L-aculose (2,3,6-trideoxy-L-hex-2-enopyran-4-ulose, Yashimoto *et al.*, 1979), L-cinerulose A (2,3,6-trideoxy-L-hexopyran-4-ulose) and L-cinerulose B (Trefzer *et al.*, 1999), the 2,6-dideoxysugars L-oleandrose (Hernandez *et al.*, 1993), L-rhodinose (Westrich *et al.*, 1999; Kunzel *et al.*, 1999), L-fucose (Torkkell *et al.*, 1997), and D- or L-mycarose (Merson-Davies and Cundliffe, 1994), the 2,4,6-trideoxysugar L-daunosamine, the 3,4,6-trideoxysugar D-desosamine (Hutchinson, 1995), and the 4,6-dideoxysugar D-mycaminose (Merson-Davies and Cundliffe, 1994).

### **B. 3,6-Dideoxygenation and possible 2,6-dideoxygenation mechanisms**

The most prevalent glycosidic components in polyketide antibiotics are 2,6- and 4,6-dideoxysugars. However, mechanistic studies on their biosynthesis are scarce; the only pathway in which biochemical mechanisms have yet been advanced is that for formation of the 3,6-dideoxyhexose ascarylose in lipopolysaccharides of *Yersinia* (Thorson *et al.*, 1993; Liu and Thorson, 1994; Kirschning *et al.*, 1997; Fig. 7). The precursor for this pathway is  $\alpha$ -D-glucose-1-phosphate (**1**), which is first activated to CDP-D-glucose (**2**) in a cytidylyl-transferase (Ep)-catalyzed reaction. The ascarylose pathway is initiated by the CDP-D-glucose 4,6-dehydratase (Eod)-catalyzed conversion of CDP-D-glucose to 6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**3**). Compound **3** is then converted to CDP-3,6-dideoxy-D-*glycero*-4-hexulose (**4**) in two consecutive enzymatic reactions catalyzed by CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (E1) and CDP-6-deoxy- $\Delta^{3,4}$ -

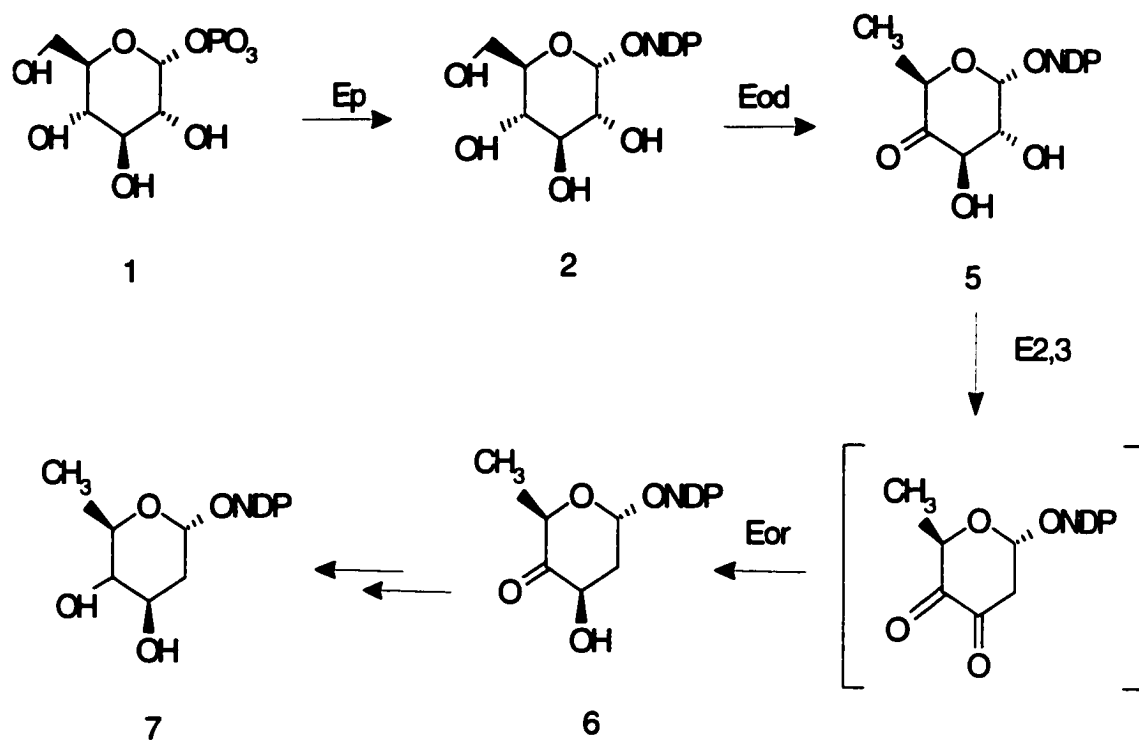


**Figure 6.** Deoxysugars moieties of polyketide antibiotics.



**Figure 7.** Biosynthesis of 3,6-dideoxyhexose from NDP-D-glucose. Ep, cytidyltransferase; Eod, CDP-D-glucose 4,6-dehydratase; E1, CDP-6-deoxy-L-threo-D-glycero-4-hexulose-3-dehydrase; E3, CDP-6-deoxy- $\Delta^{3,4}$ -glucoseen reductase; Eep, C-5 epimerase; Ered: C-4 reductase.

glucoseen reductase (E3). The final steps from **4** to the various 3,6-dideoxyhexoses are carried out by a C-5 epimerase (Eep), and a C-4 reductase (Ered). Cell-free extracts of *Streptomyces peucetius* and *Saccharopolyspora erythraea* (Thorson *et al.*, 1993) lack a homolog of E3. Since E3 is an indispensable enzyme for C-3 deoxygenation in the 3,6-dideoxyhexose pathway, its absence from these cell extracts indicates that the 2,6- and 4,6- dideoxylation mechanisms for biosynthesis of the 2,6-dideoxyhexose (daunosamine) in daunorubicin, and of the 2,6- and 4,6-dideoxyhexoses (cladinose and desosamine, respectively) in erythromycin differ from the 3,6-dideoxylation mechanism in Gram-negative bacteria. A comparison of the genes for ascarylose biosynthesis with those for biosynthesis of the 2,6-dideoxysugars in polyketide antibiotics, and the results of a feeding experiment with isotope-labeled precursors, suggested the pathway shown in Fig. 8 for forming 2,6- dideoxysugars (Liu and Thorson, 1994; Draeger *et al.*, 1999; Trefzer *et al.*, 1999).  $\alpha$ -D-Glucose-1-phosphate is activated to an NDP-D-glucose (**2**) by NDP-hexose pyrophosphorylase (Ep), then converted to NDP-4-keto-6-deoxy-D-glucose (**5**) by an NDP-D-glucose 4,6-dehydratase (Eod). Subsequent C-2 deoxygenation catalyzed by a 2,3-dehydratase (E2,3) gives an unstable intermediate that is reduced by an oxidoreductase (Eor) to NDP-4-keto-2,6-dideoxy-D-glucose (**6**). Compound **6** has been detected by NMR (Snipes *et al.*, 1978) in *S. violaceoruber* after reactions catalyzed by E2,3 and Eor. From the 2,6- dideoxysugar (**7**; see Fig. 8) various known glycosidic components could then be formed by epimerization, methylation, and further reduction (Westrich *et al.*, 1999; Liu and Thorson, 1994).



**Figure 8.** The possible pathway for the biosynthesis of 2,6-dideoxyhexose. Ep: NDP-hexose pyrophosphorylase; Eod: NDP-glucose 4,6-dehydratase; E2,3: 2,3-dehydratase; Eor: oxidoreductase.

### **C. Enzymes for the biosynthesis of deoxysugars**

Enzymes for biosynthesis of ascarylose have been characterized biochemically. However, most of the information on biosynthesis of other deoxysugars has come only from molecular biological techniques. Functions deduced from the DNA sequences of genes have been verified, where possible, by gene disruption to create blocked mutants that accumulate biosynthetic intermediates. Identification of the intermediates usually offers supporting evidence for the role of putative biosynthetic enzymes. The information not only casts light on biosynthetic pathways, but can lead to the design of hybrid natural compounds with potentially useful biological activity. To achieve the latter goal, the enzymes need to be characterized so that their biosynthetic role can be assigned unambiguously. Detecting the activity of enzymes directly is difficult because the correct substrates are often unstable and cannot be prepared by fermentation. Molecular cloning has allowed the genes and gene clusters involved in biosynthesis of the sugar moieties of antibiotics to be isolated. Over-expressing specific cloned genes in *E. coli* or other *Streptomyces* can provide enzymically active proteins for deoxysugar biosynthesis in amounts that permit biochemical study. The putative biosynthetic pathway for 2,6-dideoxysugars (see Fig. 8) furnishes the following enzymes:

#### **a. NDP-hexose phosphate (Ep)**

Genes for NDP-hexose phosphate synthesis have been cloned from *Yersinia* and many streptomycetes (Liu and Thorson, 1994; Kirschning *et al.*, 1997). Thorson *et al.* (1994)



have shown that hexose-1-phosphate nucleotidyl transferases consist of four identical 29 kDa subunits, two conserved regions in the amino acid sequences have been identified. The one in the N-terminal region is postulated to form part of the activator binding site; the second domain, located in the center of the transferases, is involved in binding the substrate.

#### **b. NDP-hexose 4,6-dehydratase (Eod)**

Genes encoding this enzyme are highly conserved in actinomycetes. Decker *et al.* (1996) used DNA probes derived from *strDELM* in *Streptomyces griseus* N2-3-11 to successfully detect genes encoding enzymes responsible for the formation of 6-deoxysugars in various actinomycete strains, including the dehydratase genes involved in biosynthesis of urdamycin A and landomycin. A phylogenetic tree constructed with protein sequences deduced from the cloned genes and already known NDP-dehydratases indicates that these enzymes in actinomycetes are more closely related to each other than to dehydratases from other orders. An even closer relationship exists between dehydratases from strains producing natural compounds with similar deoxysugar moieties.

#### **c. NDP-2,3-dehydratase (E2,3) and oxidoreductase (Eor)**

In the 2,6-dideoxysugar biosynthesis pathway (see Fig. 8) the formation of 4-keto-2,6-dideoxy-D-glucose needs both an NDP-2,3-dehydratase and an oxidoreductase.

Compound **5** is believed to be first rearranged by E2,3 to an intermediate that can be reduced by Eor. In the 3,6-dideoxysugar biosynthetic pathway, this step involves reactions catalyzed by E1 and E3 (Thorson and Liu, 1993; see Fig. 7). The polyketide antibiotic sugars are commonly modified following 6-deoxygenation by loss of the oxygen function from C-2. Very little is known about the enzymes catalyzing this process (Draeger *et al.*, 1999), but alignment of the deduced amino acid sequences of NDP-2,3 dehydratases (Fig. 9) is informative. The comparison of this enzyme in four polyketide biosynthetic pathways shows close similarity. Several regions (asterisks in Fig. 9) are highly conserved, and the sequence QLSPTVQATRSNYT (amino acids 127-140 of LanS, see bold letters in Fig. 9) shows 100% identity. The degree of similarity among these 2,3 dehydratases is just as high as for the NDP-hexose 4,6-dehydratases of actinomycetes.

#### **d. Glycosyltransferase**

Genes coding for glycosyltransferases with various substrate specificities have been identified in clusters for polyketide antibiotic biosynthesis (Baltz & Seno, 1988; Krugel *et al.*, 1993; Bechthold *et al.*, 1995; Trefzer *et al.*, 1999). For example, in the landomycin biosynthesis pathway there are four glycosyltransferases (*lanGT1-4*) for the specific transfer of D-olivose and L-rhodinose; in urdamycin biosynthesis *urdGT2* catalyzes the transfer of activated D-olivose to an angucyclinone precursor. Other sugar transferases are needed to attach L-rhodinose, and perhaps also D-olivose. The degree of identity between glycosyltransferases in different pathways is not as high as for the Eod and E2,3 enzymes of sugar biosynthesis. A classification of nucleotide-diphospho-sugar glycosyl-

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lanSxx0      ---MLSSLVRTGGTGRRLRPRHDPVSAERIAASAAAVTGASLRTEDFPQWLEGRRAHRFT
aveBVI      MSVRADADHTEPSTAHRAARRRPARVPRPLRRRGRHR-RRTSLDAFTGWWTRRSAGHRFR
mtmVxx2     ---MTQAIMSRKHPAVPPDGLDAPDLRRIAESAAADS-GLLSLGGVHDWFEERRKAIKRD
orf10(ole)  -MIWGIPAMSEAMGSVPTAG-----SEVS--STCAL-----LSWLDARRRANRLT
            :                               * * * * :

lanSxx0      VDRI PFDALDGWSFDDATGNLVHRSGRFFSVEGLHVTRDEIGHREWYQPI IKQPEVGILG
aveBVI      VERI PFHGMDAWSFHPGTGNLAHRSGRFFSVEGLHVRGGEQPFPEWQQPI IHQPEIGILG
mtmVxx2     VRRIPFAELQRWGFHPVTGNLAHDSGRFFTV EGLRARSEGDPVSWSQPIMHQPEVGFLG
orf10(ole)  VEHVPFRELSGWQFDENTGNLRHTSGRFFSIEGLRVTRDHCWFGSWTQPI IVQPEIGILG
            * ::* *  :.* * .  **** * *****:;***:.  .* ***: ***:;***

lanSxx0      ILVKEFDGVLHFLMQAKMEPGNRNLLQLSPTVQATRSNYTKVHKGADVKEYIEYFTQP-GR
aveBVI      ILAKKFDGVLHFLMQAKMEPGNINLVQLSPTVQATRSNYTKVHGGAADVKEYLEYFTQP-RR
mtmVxx2     ILVKEFDGVLHCLMQAKAEPGNAEGLQLSPTVQATRSNYTGVEGSAVRYLEYFTDP-GK
orf10(ole)  LLVKRFDGILHVLVQAKNEPGNIGGLQLSPTVQATRSNYTRVHRGGGVRYLEYFASPRGR
            :*.*.***:* * :*** **** :***** * * * . * :;***:.* :

lanSxx0      GRFIADVLQSEHGSWFFHKSNRNMIVEAVGDVPLDDDFCWLTGQIGQLLHRDNVNMDS
aveBVI      ATVVVDVLQSEHGAWFHRKFNRNIVVETDEDEVPLDDDFRWLTGQIGELMHRDNLVNMDA
mtmVxx2     GRPLVDVLQSEHGSWFFRKRNRNLLVEAVGDVPEHEDYRWLTGQVRRLLGVPDLVNMDT
orf10(ole)  GRVLADVLQSEQGSWFLHKRNRNMEVEALDDVPLDDDFHWISLGLRKLKLLRPHLVNMDT
            : .:*****:*:* * * * : * * : * * * * : * * : * * * * : * * :

lanSxx0      RTVLACAPFP-----DDGADALLSDTELVSWFTAERSRH
aveBVI      RTVLACLPTP-----FDEPAALHSDAELLSWYAAERSRH
mtmVxx2     RTVLACLVPVAPPDGSLEGVTTDGSRGAAVAALTASLSAQAPAAHTLPELLRWFNGLRARS
orf10(ole)  RTVLSCLPPDP---APDGRQPSAPAAPFAAAVTRSLTRGATALHTMGEILGWLTDESR
            ***** * *          . * : * : * * * :

lanSxx0      DVRAERVPLHD--LPGWVRGTSTIDHELGRYFRVVAVSVEAGSREVTGWTQPLFEPLGLG
aveBVI      SVHARRVPLAG--IPGWTTGAESIAHHDARYFRVAVRVEASNREVAAWTQPLIEPCGHG
mtmVxx2     EVAAQLVPLTS--VDGWVRDTDEIRHTSGRHFAIVAVSVEAGNREVKGWTQPLLSPRGTG
orf10(ole)  ELVQQRVPLEETAFCGWRDDHAIAHKDGDYFRVIGVSVRASSREVSSWSQPLLPVPGG
            .: . *** . ** . * * . : * : * * * . * : * * * : * * *

lanSxx0      VTAFLTRRIGGVPHVLVHARVEGGFLDTVELGPTVQYTPDNYGHLTGEDRPPFLDLVLD
aveBVI      ITAFLTRRIGGVPHLLAHRVEGGFLDTIELGPTVQYTPRNYAHLTGPARPRFLDLVLEA
mtmVxx2     VVAFLAKRIDGVLHVLARAHPEPGYVDLIELAPTVQCVDDHAHLPPPEQRPRFLDQVLA
orf10(ole)  LAAFVTRRIRIGVLHVLHARTEAGLLNGPEMAPTVQCRPLNRYAVPAEYRPAVLDYVLSA
            :.***:;* * * * : * * : * : * : * : * : * * * * * : * : * * * *

lanSxx0      DPARIQYEAVHSEEGGRFLNAESRYLLIEADEEQAPLDPPAGFQWVTPAQLTSLVRHG
aveBVI      APDRIRYAAVHSEEGGRFLHAQARYLFEADESQAPNDPPPGYRWCTPGQLTQLLRYGRY
mtmVxx2     RPEQILYDAVQSEEGGRFYRALSRVYLVVETDEDPVVPD---DYRWMTVAQLVALLQHSNY
orf10(ole)  DPGRIRYDTLQSEEGGRFHAENRYVVEA-EDDFPVEVPRDFRWLTLLHQILALLHHSNY
            * * * * :;***** . * * * : * : * : * : * * * * * : * : * * *

lanSxx0      VNVQARTLLACLNATAVLSG
aveBVI      VNVQARTLLSLLTTRAVEL-
mtmVxx2     CNIQARSLIACLQSVW----
orf10(ole)  VNVEARSLVACIQALS----
            * : * * * * : : :

```

**Figure 9.** Alignment of NDP-2,3-dehydratases from various polyketide antibiotic producers. LanSxx0: *lanS* in *S. cyanogenus* S136; aveBVI: *aveBVI* in *S. avermitilis*; mtmVxx2: *mtmV* in *S. argillaceus*; orf10(ole): *orf10* in *S. violaceoruber* Tü22. Asterisks, colons and periods denote 4, 3, and 2 identical amino acids, respectively.

transferases based on amino acid sequence similarities has been proposed (Campbell *et al.*, 1997). A total of 555 sequences were analyzed, of which 553 were classified into 26 families that predict the function and intrinsic structural features of an enzyme. Interestingly, the glycosyltransferases of streptomycetes (such as *urdGT2*, *dnrS*, and *oleG1*) are all in family 1 of this classification. The stereochemistries of their substrates and products thus place streptomycete glycosyl transferases in the group of enzymes that invert configuration at the reaction site.

#### **D. Glycosylation**

Glycosylation is a post-PKS tailoring step. The products accumulated by various streptomycete mutants blocked in glycosylation indicate that glycosyl transfer establishing a C-glycosidic substituent precedes several other modification steps (Kirschning *et al.*, 1997; Rohr *et al.*, 1993). In the urdamycin A biosynthesis pathway, glycosyl transfer occurs before the final aglycone component is completed (Madduri *et al.*, 1998). For doxorubicin, glycosyltransfer of daunosamine is postulated to occur either at the aklavinone (basic aglycone) stage or after 11-hydroxylation to  $\epsilon$ -rhodomycinone, i.e., six to seven biosynthesis steps prior to completion of doxorubicin (Grimm *et al.*, 1994; Bartel *et al.*, 1990). For erythromycin A biosynthesis, L-mycarose is transferred first, generating 3-O-mycarosylerythronolide B, and then D-desosamine is transferred generating erythromycin D. Formation of erythromycin A requires a hydroxylation step mediated by the *eryK* product (a cytochrome P450 monooxygenase) and O-methylation of L-mycarose by the *eryG* product (an O-methyltransferase).

Many enzymes involved in post-polyketide modifications do not seem to have absolute specificity for a particular structure (Trefzer *et al.*, 1999). Glycosylation showing this relaxed substrate selection may indicate a role in resistance rather than in biosynthesis. In *Streptomyces antibioticus*, *oleI* is the glycosyltransferase gene located in the sugar biosynthesis region within the cluster; *oleD* is found in another region of the chromosome. Both gene products transfer a D-glucose from UDP-D-glucose to the 2'-hydroxy group of D-desosamine to inactivate oleandomycin. The *oleI* product is very specific for oleandomycin, but the *oleD* product can inactivate several other macrolides (Quiros *et al.*, 1998). The enzymes are intracellular and possibly confer resistance on the oleandomycin producer by glycosylating active antibiotic precursors as well as analogues within the cells. Flexibility for aglycone substrates has also been reported for the glycosyltransferases in producers of daunorubicin (Madduri *et al.*, 1998), erythromycin (Gaisser *et al.*, 1998), methymycin/neomethymycin (Zhao *et al.*, 1998) and urdamycin (Hoffmeister *et al.*, 2000).

## **V. Jadomycin B: Structure and Biosynthesis**

Southern analysis of genomic DNA from various actinomycetes using *actI* from *S. coelicolor* A3(2) as a probe for type-II PKS genes detected an *actI*-hybridizing DNA fragment in the Cm producer *S. venezuelae* UC2374 (Malpartida *et al.*, 1987). Independently, Ayer *et al.* (1991) found that cultures of *S. venezuelae* ISP5230, which secrete Cm when stressed by moderate nutrient limitation, produce a second antibiotic

containing an 8H-benz[b]oxazolophenanthridine moiety (jadomycin; Ayer *et al.*, 1991), when subjected to additional stresses, such as heat shock, phage infection, or exposure to toxic concentrations of ethanol (Doull *et al.*, 1993, 1994). The structure deduced for the active antibiotic JdB (see Fig. 1), included a dideoxyhexose as well as the angular tetracyclic aglycone. The aromatic structure of the aglycone suggested that JdB might be synthesized through a polyketide intermediate. To confirm this Han *et al.* (1994) used a 1.8-kb fragment of *S. venezuelae* that hybridized with the type-II PKS genes *actI* and *actIII* from *S. coelicolor* A3(2) (Ramalingam, 1989) to probe a genomic library of *S. venezuelae* ISP5230 DNA. Three lambda clones were identified and shown to contain a cluster of ORFs encoding JdB biosynthesis enzymes. The three core PKS genes were: *jadA* encoding a ketoacyl synthase, *jadB*, the closely related gene postulated to determine polyketide chain length, and *jadC*, encoding an acyl carrier protein (ACP). Molecular genetic evidence and an isotopic labelling experiment (Crowell 1993) clarified the biosynthetic origin of the jadomycin aglycone and established that it is derived from a decapolyketide generated by an iterative type-II PKS complex encoded by a chromosomal gene cluster (see Fig. 2; Han *et al.*, 1994; Yang *et al.*, 1995; Yang *et al.*, 1996; Meurer *et al.*, 1997; Kulowski *et al.*, 1999; Han *et al.*, 2000).

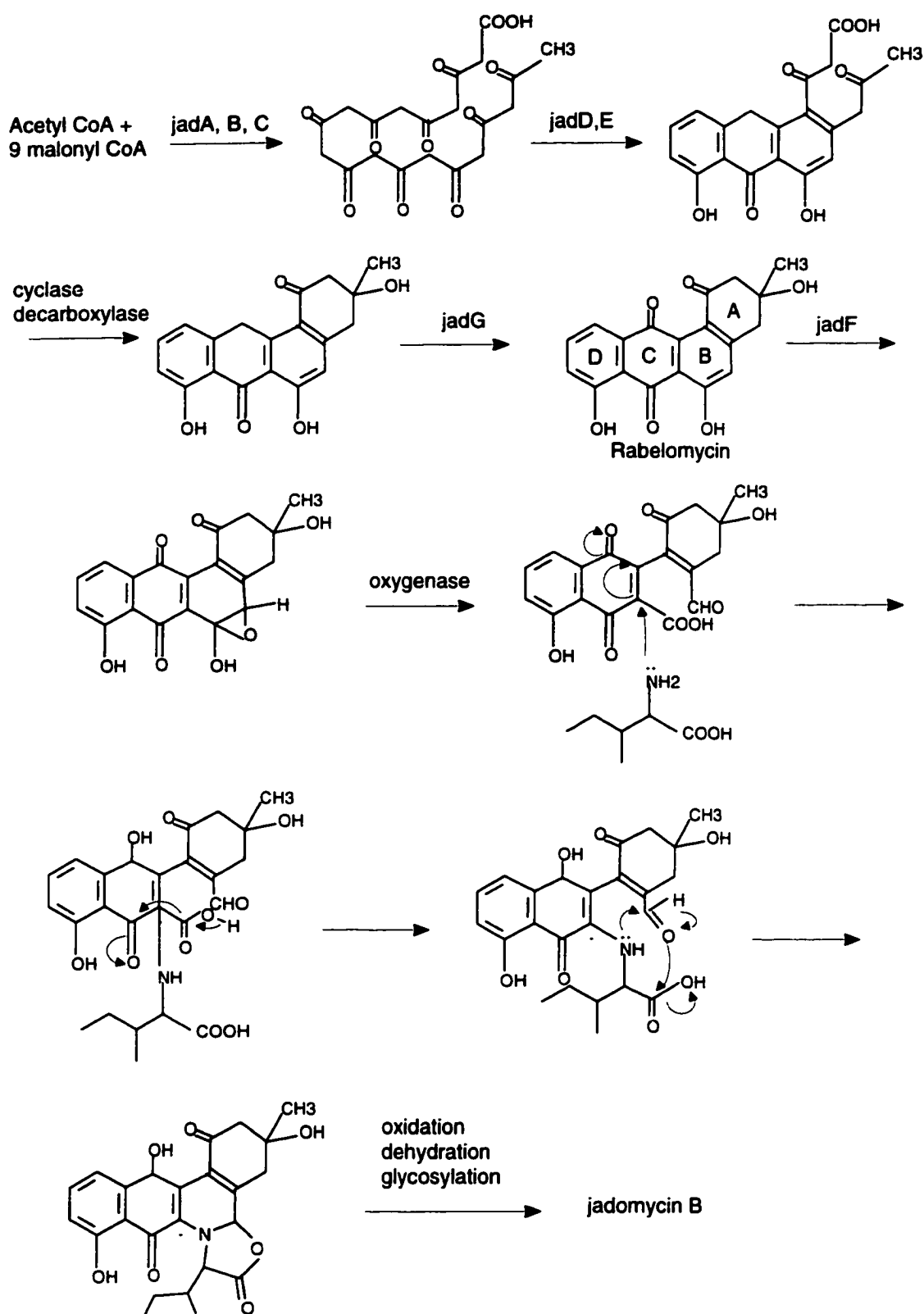
#### **A. The *jad* cluster in *S. venezuelae* ISP5230**

In the cluster of JdB biosynthesis (*jad*) genes in *S. venezuelae*, those involved in polyketide cyclization and aromatization, e.g., *jadD* (bifunctional cyclase/dehydrase) and *jadE* (ketoreductase), lie immediately downstream of the core PKS cluster. Further

downstream are genes encoding oxygenases (*jadF*, *jadG*, and *jadH*) (Yang *et al.*, 1995), and beyond them an unidentified gene (*jadK*) and one (*jadL*) for a transmembrane protein have been located (McVey, 1998). Upstream of the PKS genes are genes for a fourth-ring cyclase (*jadI*), a biotin carboxylase (*jadJ*; Han *et al.*, 2000), and genes regulating JdB production (*jadR*<sub>1</sub> and *jadR*<sub>2</sub>; Yang *et al.*, 1996; 2001). Based on these results and disruptions of the PKS cluster and *jadF*, a putative biosynthetic pathway for jedomycin was proposed (Fig. 10; Yang *et al.*, 1996). The products of *jadA-C* and *jadJ* were postulated to catalyze the synthesis of intermediates and their assembly into a linear polyketide chain. Cyclization was believed to be catalyzed and/or directed by the products of *jadD*, *E*, and *I*. These and the *jadG* product were expected to generate rabelomycin, the B ring of which would be opened by the oxygenase activity of the *jadF* product. Subsequent insertion of an isoleucine molecule at the site of ring opening, and further oxidation, dehydration, and then glycosylation would result in the formation of JdB. The location of genes for deoxysugar biosynthesis/attachment and for resistance and regulation was not determined.

## **B. Regulation of jedomycin B biosynthesis**

Since JdB production is associated with exposure of cultures to severe stress such as heat shock or ethanol toxicity, Doull *et al.* (1993; 1994) suggested that transcription of the *jad* cluster might be linked to the heat shock response. Singh (1992) showed that in either heat-shocked or ethanol-treated cultures JdB was produced 8-12 h after the stress was imposed, indicating a rapid transcriptional response by the *jad* genes. The requirement for



**Figure 10.** Tentative pathway for Jadomycin B biosynthesis (Yang *et al.*, 1996).



environmental stress superimposed on a nutritional imbalance to initiate JdB synthesis implies that *S. venezuelae* possesses a control mechanism different from streptomycetes that produce antibiotics in response to a physiological imbalance alone (Doull and Vining, 1995). Yang *et al.* (1995; 2001) identified a repressor-response regulator gene pair (*jadR*<sub>1</sub> and *jadR*<sub>2</sub>) controlling JdB production, and showed that *jadR*<sub>1</sub> encodes a polypeptide resembling in amino acid sequence the OmpR-PhoB subfamily of regulators. Introducing additional copies of the *jadR*<sub>1</sub> gene into the wild-type gave unstable strains; the transformants initially produced a larger amount of JdB, but the titre decreased as excess copies of *jadR*<sub>1</sub> were lost. In mutants where *jadR*<sub>1</sub> was deleted or disrupted, JdB was not produced (Yang *et al.*, 2001), indicating that *jadR*<sub>1</sub> encodes a positive regulator required for JdB production. In contrast, when *jadR*<sub>2</sub> was disrupted the mutant cultures produced JdB without needing the stress conditions required by wild-type strains, and overproduced the antibiotic when they were stressed with ethanol (Yang *et al.*, 1995). Transformants with an increased number of *jadR*<sub>2</sub> copies relative to *jadR*<sub>1</sub> failed to produce JdB, as predicted if *jadR*<sub>2</sub> encodes a repressor. The N-terminal region of *JadR*<sub>2</sub> resembled MtrR from *Neisseria gonorrhoeae*, as well as EnvR and AcrR from *E. coli*. These gene products, and also TetC in *E. coli* and TetR in *S. glaucescens*, control genes responsible for resistance to structurally diverse antimicrobial agents (Guilfoile and Hutchinson, 1992; Otten *et al.*, 1995), and the regulatory systems consist of repressor and resistance genes divergently transcribed. The sequence of *JadR*<sub>2</sub> closely resembles the deduced amino acid sequences of the repressor genes in these divergently transcribed systems, and the gene organization is similar in that oppositely oriented ORFs are present upstream of the *jadR*<sub>2</sub> repressor gene. However, the ORFs immediately upstream of *jadR*<sub>2</sub>

showed no similarity to known antibiotic resistance genes. Altering the proportions of *jadR*<sub>1</sub> and *jadR*<sub>2</sub> in the *S. venezuelae* chromosome by integration of vectors carrying intact or disrupted copies gave results suggesting that the two genes form an interactive regulatory pair different in function from the usual two-component regulatory systems in which a sensor-transmitter is associated with a response regulator. The *jadR*<sub>1</sub> and *jadR*<sub>2</sub> genes appear to link antibiotic synthesis to stress (Yang *et al.*, 2001), but the mechanism by which they sense or respond to stress signals (Kawachi *et al.*, 2000) is not known. The properties of *jadR*<sub>1</sub> and *jadR*<sub>2</sub> suggest that a complicated regulatory cascade controls antibiotic biosynthesis in *S. venezuelae* ISP5230.

## **Material and Methods**

### **I. Bacterial Strains, Plasmids and Phages**

Bacteria, plasmids and phages used in this study are listed in Table 1.

### **II. Chemicals and Biochemicals**

Reagent grade chemicals were used. Lysozyme, RNase-free DNase I and [ $\alpha$ - $^{32}$ P] dCTP were purchased from Amersham Pharmacia Biotech. Deoxyribonucleoside triphosphates (dNTPs), ethidium bromide, and RNase A were from Boehringer-Mannheim. RNA markers were from Gibco BRL, and the Klenow fragment was supplied by MBI Fermentas. Isopropyl- $\beta$ -thiogalactopyranoside (IPTG), sodium trichloroacetate (NaTCA) and PIPES disodium salt were from Aldrich Chemical Company; 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-gal) was from Diagnostic Chemicals Limited (Charlottetown, P.E.I.). Ultra-pure polyethylene glycol (PEG) 1000 used for transformations was from Koch-Light (Haverhill, UK); N-tris-(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES), tris-(hydroxymethyl)aminoethane (Tris), sodium dodecyl sulfate (SDS), deoxyribonucleic acid solution, and N,N,N',N'-tetramethylenediamine (TEMED) were from BioBasic Inc. (Scarborough, Ont.). Ammonium persulfate (AMPS), glucose, and glycerol were from BDH Chemicals, sodium-triisopropyl naphthalene sulphonate (TPNS) was from Eastman Kodak, ultrapure DNA-grade agarose, acrylamide, bis-acrylamide, ethylene diamine tetraacetic acid (EDTA), polyvinylpyrrolidone (PVP), Ficoll, mannitol,

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

<b>Strains</b>	<b>Genotype/phenotype</b>	<b>Source/reference</b>
<i>Streptomyces venezuelae</i>		
ISP5230	Wild type, JdB producer	Stuttard (1982)
VS1075/1076	ISP5230 with <i>jadM</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1079/1080	ISP5230 with <i>jadO</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1081/1082	ISP5230 with <i>jadP</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1083/1084	ISP5230 with <i>jadQ</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1085/1086	ISP5230 with <i>jadX</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1087/1088	ISP5230 with <i>jadS</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1089/1090	ISP5230 with <i>jadT</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1091/1092	ISP5230 with <i>jadU</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1093/1094	ISP5230 with <i>jadV</i> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1095/1096	ISP5230 with <i>jadW</i> <sub>1</sub> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1097	VS1095 (pJV435) transconjugant	This study
VS1098	ISP5230 (pJV435) transconjugant	This study

Table 1 to be continued on page 43.

Table 1. continued.

VS1099/1100	ISP5230 with <i>jadW</i> <sub>2</sub> replaced by Am <sup>R</sup> gene in opposite orientations	This study
VS1101/1102	ISP5230 with <i>jadW</i> <sub>3</sub> disrupted by Am <sup>R</sup> gene in opposite orientations	This study
VS1103/1104	ISP5230 with both <i>jadW</i> <sub>1</sub> and <i>jadW</i> <sub>2</sub> disrupted by Am <sup>R</sup> gene in opposite orientation	This study
VS1105	VS1099 (pJV463) transconjugant	This study
VS1106	ISP5230 (pJV463) transconjugant	This study
VS1107	ISP5230 with <i>jadR</i> * replaced by Am <sup>R</sup> gene	This study
<b><i>Streptomyces griseus</i></b>		
IFO13350	Wild type producing streptomycin and A-factor	S. Horinouchi
HH1	A-factor-deficient mutant	S. Horinouch
<b><i>Escherichia coli</i></b>		
BL 21 (DE3)	F <sup>-</sup> ompT <i>hsd</i> SB (rB <sup>-</sup> , mB <sup>-</sup> ,) <i>gal dcm</i>	Novagen
DH5αF'IQ	F' α80 <i>lacZ</i> Δ( <i>lacZYA-argF</i> ) U169 <i>deoR recA1 endA1 hsdR17</i> (r <sub>k</sub> <sup>-</sup> , m <sub>k</sub> <sup>-</sup> ) <i>phoA supE11 λ- thi-1 gyrA96 relA1/F' proAB<sup>+</sup> lacI<sup>q</sup>ZΔM15 zff::Tn5[Km<sup>r</sup>]</i>	Gibco BRL
ET 12567	<i>dam<sup>-</sup> dcm<sup>-</sup> hsdM</i>	McNeil <i>et al.</i> (1992)
ET 12567 (pUZ8002)	ET12567 containing pUZ8002	M. Paget, John Innes
LE 392	<i>del(lacIZY)6 galK2 galT22 metBI TrpR55 lambda-</i>	Maniatis <i>et al.</i> (1982)

Table 1 to be continued on page 44.

Table 1. continued.

**Plasmids**

pBluescript II	pUC18 phagemid with SK+ & KS+, f1 <i>ori</i> , T3 and T7 primer binding sites	Stratagene
pET 21+	pUC18 with <i>amp</i> , primer binding sites for T7 promoter, and terminator	Novagen
pHJL400	<i>tsr</i> , <i>amp</i> , <i>lacZ</i> , bifunctional	Larson and Hershberger (1986)
pXE4	containing promoterless <i>xylE</i> fragment from pTG402	J. Westpheling
pJV104	pBluescript II SK(+) containing 5'-region of <i>jadM</i> in a 0.55-kb <i>XhoI</i> - <i>SacI</i> insert	McVey (1998)
pJV105	pHJL400 with 4.0-kb <i>SacI</i> insert	McVey (1998)
pJV225	pBluescript II SK+ containing Am <sup>R</sup> gene flanked by multiple cloning sites	Chang <i>et al.</i> (2001)
pJV326	pHJL400 with 0.76-kb <i>PstI</i> insert containing <i>oriT</i> , bifunctional	He <i>et al.</i> (2001)
pJV401	pBluescript II SK(+) with 6.6-kb <i>XhoI</i> insert from phage LR3	This study
pJV402	pBluescript II SK(+) with 3.3-kb <i>XhoI</i> - <i>KpnI</i> insert containing 5'-region of <i>jadM</i> and downstream genes	This study
pJV403	pBluescript II SK(+) with 3.0-kb <i>EcoRV</i> - <i>XhoI</i> insert containing 5'-region of <i>jadM</i> and downstream genes	This study
pJV404A/B	pBluescript II SK(+) with 4.0-kb <i>SacI</i> fragment from pJV105 (two orientations)	This study
pJV405	pBluescript II SK(+) with 1.3-kb <i>NruI</i> - <i>XhoI</i> fragment from pJV404A	This study

Table 1 to be continued on page 45.

Table 1. continued.

pJV406	pBluescript II SK(+) with 4.7-kb <i>NruI-KpnI</i> fragment from pJV402	This study
pJV407A/B	pBluescript II SK(+) with 6.3-kb <i>NruI-KpnI</i> (blunted) fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in the <i>NruI-KpnI</i> site	This study
pJV408A/B	pJV326 with blunt-ended 5.6-kb <i>EcoRI-EcoRV</i> fragment inserted in the blunt-ended <i>BamHI</i> site	This study
pJV409	pET-21(+) with a blunt-ended 1.0-kb <i>XhoI-PvuII</i> fragment inserted in the blunt-ended <i>BamHI</i> site	This study
pJV410	pUC18 with 6.0-kb <i>PstI-BamHI</i> insert from pJV401 containing <i>jadMNXO</i> and downstream genes	This study
pJV411	pBluescript II SK(+) with 3.3-kb <i>KpnI-XhoI</i> fragment from pJV401	This study
pJV412A/B	pBluescript II SK(+) with 3.3-kb <i>XhoI-KpnI</i> fragment from pJV402 containing 1.6-kb $Am^R$ gene (alternative orientations) in the <i>NcoI</i> site	This study
pJV413A/B	pJV326 with 4.8-kb <i>XhoI-KpnI</i> fragment from pJV412 A/B	This study
pJV414A/B	pUC18 with 6.0-kb <i>XhoI-BamHI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in the <i>KpnI</i> site	This study
pJV415A/B	pJV326 with 7.6-kb <i>EcoRI-XbaI</i> fragment subcloned from pJV414A/B	This study
pJV416A/B	pBluescript II SK(+) with 3.5-kb <i>KpnI-XhoI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in blunted <i>BstEII</i> site	This study

Table 1 to be continued on page 46.

Table 1. continued.

pJV417A/B	pBluescript II SK(+) with 2.2-kb <i>NotI-XhoI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in blunted <i>MaeII</i> site	This study
pJV418A/B	pUC18 with 2.0-kb <i>BamHI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in the <i>NruI</i> site	This study
pJV419A/B	pUC18 with 2.3-kb <i>StuI-KpnI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in blunted <i>BamHI</i> site	This study
pJV420A/B	pUC18 with 2.9-kb <i>NruI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in blunted <i>BstEII</i> site	This study
pJV421A/B	pUC18 with 3.8-kb <i>BamHI</i> fragment containing 1.6-kb $Am^R$ gene (alternative orientations) in the <i>NruI</i> site	This study
pJV423	pBluescript II SK(+) with 2.0-kb <i>BamHI</i> fragment from LW12	This study
pJV424	pBluescript II SK(+) with 0.56-kb <i>NruI-BamHI</i> fragment from pJV423	This study
pJV425	pUC18 carrying in its <i>SmaI</i> site a 5.6-kb <i>StuI</i> fragment in which <i>jadS</i> and downstream genes have been subcloned from Lambda LW18	This study
pJV426	pBluescript II SK(+) with 4.2-kb <i>NruI-BamHI</i> fragment containing <i>jadS</i> , <i>jadT</i> , <i>jadU</i> , <i>jadV</i> , and <i>jadR*</i> from pJV425	This study
pJV427	pJV326 with 4.2-kb <i>NruI-BamHI</i> fragment from pJV426	This study
pJV428	pXE4 with 1.6-kb $Am^R$ gene from pJV225	This study
pJV429	pBluescript II SK(+) with 4.0-kb <i>XhoI-BamHI</i> insert from lambda 8	This study

Table 1 to be continued on page 47.



Table 1. continued.

pJV430	pBluescript II SK(+) with 1.0-kb <i>Pst</i> I- <i>Bam</i> HI insert containing <i>jadW</i> <sub>1</sub> from pJV429	This study
pJV431	pUC18 with 1.0-kb <i>Xho</i> I( <i>Bam</i> HI)- <i>Sac</i> I fragment containing <i>jadW</i> <sub>1</sub> from pJV430	This study
pJV432A/B	pUC18 with 2.6-kb <i>Xho</i> I( <i>Bam</i> HI)- <i>Sac</i> I fragment containing 1.6-kb Am <sup>R</sup> gene (alternative orientations) in the <i>Kpn</i> I site;	This study
pJV433A/B	pUC326 with 2.6-kb <i>Xho</i> I- <i>Eco</i> RI fragment subcloned from pJV432A/B	This study
pJV434	pUC18 with 1.2-kb <i>Bsa</i> AI( <i>Sma</i> I) fragment containing <i>jadW</i> <sub>1</sub> from pJV429	This study
pJV435	pJV326 with 1.2-kb <i>Eco</i> RI- <i>Bam</i> HI containing <i>jadW</i> <sub>1</sub> fragment from pJV434	This study
pJV436	pBluescript II SK(+) with 0.3-kb <i>Sac</i> I- <i>Not</i> I fragment from pJV426	This study
pJV437	pUC18 with 1.75-kb <i>Bsa</i> AI ( <i>Sma</i> I) insert from pJV429 containing <i>jadW</i> <sub>2</sub> and <i>jadW</i> <sub>3</sub>	This study
pJV438	pUC18 with a 2.6-kb <i>Bsa</i> AI ( <i>Sma</i> I) fragment containing the 1.6-kb Am <sup>R</sup> gene inserted in its <i>Sac</i> I (blunted) site	This study
pJV439	pUC18 with a 3.6-kb <i>Xho</i> I ( <i>Bam</i> HI)- <i>Bsa</i> AI ( <i>Sma</i> I) fragment containing <i>jadW</i> <sub>1</sub> , <i>jadW</i> <sub>3</sub> and the Am <sup>R</sup> gene in its <i>Sac</i> I (blunted) site	This study
pJV440	pJV326 with 3.6-kb <i>Eco</i> RI- <i>Bam</i> HI fragment from pJV439	This study
pJV441	pUC18 with 2.3-kb <i>Sac</i> I- <i>Bam</i> HI fragment from pJV429 containing <i>jadW</i> <sub>3</sub>	This study
pJV442A/B	pUC18 with 3.9-kb <i>Sac</i> I- <i>Bam</i> HI fragment containing the Am <sup>R</sup> gene (alternative orientations) in its <i>Bsa</i> AI site	This study

Table 1 to be continued on page 48.

Table 1. continued.

pJV443A/B	pJV326 with 3.9-kb <i>EcoRI-BamHI</i> insert from pJV442A/B	This study
pJV444	pBluescript II SK(+) with 3.35-kb <i>EcoRI</i> fragment from pJV429	This study
pJV445	pBluescript II SK(+) with 4.4-kb <i>EcoRI</i> fragment containing Am <sup>R</sup>	This study
pJV446	pJV326 with 4.4-kb <i>EcoRI</i> fragment from pJV445	This study
pJV447	pBluescript II SK(+) with 1.0-kb <i>SacI</i> fragment from pJV429	This study
pJV448	pBluescript II SK(+) with 1.5-kb <i>SacI-NotI</i> fragment containing <i>jadW</i> <sub>2</sub>	This study
pJV449	pUC18 with 0.75-kb <i>BamHI-StuI</i> fragment containing <i>jadR</i> * from pJV425	This study
pJV450	pUC18 with 2.0-kb <i>BamHI</i> fragment containing <i>jadY</i> , <i>jadZ</i> from Lambda LW23	This study
pJV456	pBluescript II SK(+) with 3.3-kb <i>KpnI-StuI</i> fragment containing <i>jadU</i> , <i>jadV</i> , <i>jadR</i> *, and <i>jadY</i> from pJV425	This study
pJV457	pBluescript II SK(+) with 4.1-kb <i>KpnI-StuI</i> fragment containing Am <sup>R</sup> gene from pJV456	This study
pJV458	pJV326 with 4.1-kb <i>KpnI-StuI</i> fragment from pJV457	This study
pJV459	pUC18 with 0.75-kb <i>NruI-BamHI</i> fragment from pJV456 inserted in its <i>SmaI</i> and <i>BamHI</i> sites	This study
pJV460	pBluescript II SK(+) with 1.5-kb <i>NruI-StuI</i> fragment from pJV456 inserted in its <i>EcoRV</i> site	This study
pJV461	pXE4 with 0.75-kb <i>HindIII-EcoRI</i> (blunted)	This study

Table 1. to be continued on page 49.

Table 1. continued.

	fragment from pJV459 in its <i>Hind</i> III and <i>Bam</i> HI (Blunted) sites	
pJV462	pXE4 with 1.5-kb <i>Hind</i> III- <i>Bam</i> HI fragment from pJV460	This study
pJV463	pJV326 with 1.5-kb <i>Sac</i> I fragment containing <i>jadW</i> <sub>2</sub> from pJV447	This study
<b>Phages</b>		
Lambda 8	Lambda GEM-11 with an 9.5-kb insert of <i>S. venezuelae</i> DNA	Ramalingam (1989)
Lambda LW3	Lambda GEM-11 with an 11.5-kb insert of <i>S. venezuelae</i> DNA	This study
Lambda LW12	Lambda GEM-11 with a 13.5-kb insert of <i>S. venezuelae</i> DNA	This study
Lambda LW18	Lambda GEM-11 with a 15.5-kb insert of <i>S. venezuelae</i> DNA	This study
Lambda LW23	Lambda GEM-11 with a 14.5-kb insert of <i>S. venezuelae</i> DNA	This study
Lambda gene bank	Lambda GEM-11 with 9.0-23.0-kb inserts of <i>S. venezuelae</i> genomic DNA	Facey (1994)

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and sodium 4-aminosalicylate (PAS) were from Sigma Chemical Co. Bovine serum albumin fraction V was from Pierce, galactose was from Bioshop Canada Inc. (Burlington, Ont.), trypticase soy broth was from Becton Dickson and Company, Bacto-agar, Bacto-Peptone, nutrient broth, nutrient agar, yeast extract, malt extract and casamino acids were from Difco Laboratories. Samples of the  $\gamma$ -butyrolactone compounds A-factor and IM-2 were kindly provided by Dr. Takuya Nihira, Department of Biotechnology, Graduate School of Engineering, Osaka University.

### III. Media

Except where noted otherwise, media were sterilized in an autoclave at 120°C and 15 psi for 20 min. Heat-sensitive media, and some supplements (indicated) were sterilized by filtration through cellulose membranes with 0.2  $\mu$ m pores.

**LB broth** for *E. coli* cultures (Sambrook *et al.*, 1989)

Bacto-tryptone	10 g
Yeast extract	5 g
Sodium chloride	10 g
Distilled water to	1000 ml
pH	7.0

**LB agar** for routine maintenance of *E. coli* strains:

LB broth with 1.5% agar

**MYM agar** for maintaining streptomycetes and growing cultures for spore stocks.

Maltose	4.0 g
Yeast extract	4.0 g

Malt extract	10.0 g
Agar	15.0 g
Distilled water to	1000 ml

The medium was adjusted to pH 7.0 with NaOH before adding agar.

To select for thiostrepton- or apramycin-resistant *Streptomyces* colonies, MYM agar was supplemented with the antibiotic to a final concentration of 20 or 50 µg/ml, respectively.

**TO agar** for sporulation of *Streptomyces* (Chang, 1999)

Tomato paste	20 g
Pablum	20 g
Agar	20 g
Distilled water to	1000 ml
pH	6.8

**MYEME medium** (modified from Hopwood *et al.*, 1985) for *Streptomyces* cultures used

to prepare protoplasts:

Malt extract	3.0 g
Yeast extract	3.0 g
Bacto-peptone	5.0 g
Maltose	10.0 g
Sucrose	103 g
Distilled water to	1000 ml

The medium was adjusted to pH 7.5 with NaOH before autoclaving. To the sterilized solution 2.5 M MgCl<sub>2</sub>·6H<sub>2</sub>O and 10% (v/v) glycine were added to final concentrations of 5 mM and 1.0%, respectively.

**R2YE agar** (Kieser *et al.*, 2000) for regeneration of *Streptomyces* protoplasts:

Sucrose	103 g
K <sub>2</sub> SO <sub>4</sub>	0.25 g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	10.0 g
Glucose	10.0 g
Difco Casamino acids	0.1 g
Distilled water to	800 ml

Portions (80 ml) of the solution were added to 250 ml Erlenmeyer flasks, each containing 2.2 g Bacto-agar, and autoclaved. At the time of use, the medium was remelted and to each flask the following sterile solutions were added in the order listed:

$\text{KH}_2\text{PO}_4$ (0.5%)	1 ml
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (3.68%)	8 ml
L-proline (20%)	1.5 ml
TES buffer (5.37%, pH 7.2)	10 ml
10x trace element solution	0.2 ml
NaOH (1 N)	0.5 ml

The 10x trace element solution contained:

$\text{ZnCl}_2$	0.2 g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	2.0 g
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	0.1 g
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.1 g
$\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$	0.1 g
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$	0.1 g
Distilled water to	1000 ml

**Soft nutrient agar (Hopwood *et al.*, 1985):** used to overlay protoplasts during transformations, and to bioassay for streptomycin production.

Nutrient broth powder	8.0 g
Agar	3.0 g
Distilled water	1000 ml

**MS agar (Flett, *et al.*, 1997; Mazodier *et al.*, 1989):** used to culture streptomycetes for the conjugal transfer of plasmids from *E. coli*.

Mannitol	20 g
Soya-flour	20 g
Agar	16 g
Tap water to	1000 ml
pH	7.0

To the autoclaved medium before use, 2.5 M  $\text{MgCl}_2$  was added to a final concentration of 10 mM.

**GNY medium** (Malik and Vining, 1970) was used to grow *M. luteus* or *S. venezuelae* cultures; soft **GNY agar** (0.5%) seeded with *M. luteus* was used as an overlay for bioassaying Cm.

Glycerol	20.0 ml
Nutrient broth	8.0 g
Yeast extract	3.0 g
K <sub>2</sub> HPO <sub>4</sub>	5.0 g
Distilled water to	1000 ml

**TSBG medium** was used for growing *S. venezuelae* cultures.

Trypticase soy broth	30.0 g
Starch	10.0 g
Distilled water to	1000 ml

**Galactose-isoleucine medium (Gal2I)** (modified from Doull *et al.*, 1994) used to grow *S. venezuelae* and *S. venezuelae* mutant cultures for production of JdB or pathway intermediates.

Isoleucine	7.8 g
MgSO <sub>4</sub>	0.4 g
K <sub>2</sub> HPO <sub>4</sub>	1.16 g
KH <sub>2</sub> PO <sub>4</sub>	0.5 g
Salt solution	9.0 ml
Mineral solution	4.5 ml
FeSO <sub>4</sub> ·7H <sub>2</sub> O(0.2% w/v)	4.5 ml
Distilled water to	900 ml

The medium was dispensed as 21.5-ml aliquots into 125-ml Erlenmeyer flasks. After autoclaving, 2.5-ml of sterile 30% aqueous galactose and 1 ml 10% (w/v) malt extract were added to each flask. The salt solution contained 1% (w/v) NaCl and 1% CaCl<sub>2</sub>.

The mineral solution contained:

ZnSO <sub>4</sub> ·7H <sub>2</sub> O	880 mg
CuSO <sub>4</sub> ·5H <sub>2</sub> O	39 mg
MnSO <sub>4</sub> ·4H <sub>2</sub> O	6.1 mg
H <sub>3</sub> BO <sub>3</sub>	5.7 mg

$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	3.7 mg
Distilled water to	1000 ml

**GI medium** for Cm production:

Glucose	30 g (autoclaved separately)
$\text{MgSO}_4\cdot 7\text{H}_2\text{O}$	0.2 g
$\text{KH}_2\text{PO}_4$	4.5 g
$\text{K}_2\text{HPO}_4$	10.5 g
L-isoleucine	7.5 g
Salt solution (see above)	9.0 ml
$\text{FeSO}_4\cdot 7\text{H}_2\text{O}$	4.5 ml
Trace element solution A	4.5 ml
Distilled water to	1000 ml

Trace element solution A for GI medium (per 100 ml):

$\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$	400 mg
$\text{CuSO}_4\cdot 5\text{H}_2\text{O}$	18 mg
$\text{MnCl}_2\cdot 4\text{H}_2\text{O}$	2.4 mg
$(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\cdot 4\text{H}_2\text{O}$	1.7 mg
Boric acid	2.6 mg

#### IV. Gels, Buffers and Solutions

**Triton X-100:** 10% aqueous solution used for preparation of cell free extracts.

**X-gal:** 20 mg/ml in dimethylformide and **IPTG:** 200 mg/ml in water, sterilized by filtration. These two solutions were used for “blue-white” screening of *E. coli* transformants; those with recombinant plasmids gave white colonies. On a Petri plate containing LB agar with the appropriate filter-sterilized antibiotics, 40  $\mu\text{l}$  of the stock solution of X-gal and 4  $\mu\text{l}$  of the stock solution of IPTG were spread before use.

**Thiostrepton:** 50 mg/ml in dimethyl sulfoxide (DMSO). The final concentration was 50  $\mu\text{g/ml}$ .



**5X TBE buffer:** was used at 0.5 X in agarose gel electrophoresis for total RNA analysis.

Tris-base	54.0 g
Boric acid	27.5 g
0.5 M EDTA (pH 8.0)	20 ml
Distilled water	700 ml

**50X TAE buffer:** was used at 1X in agarose gel electrophoresis of DNA samples.

Tris-base	242 g
Glacial acetic acid	57.1 g
0.5 M EDTA (pH 8.0)	200 ml
Distilled water to	1000 ml

**TE buffer**

Tris-HCl	10 mM
EDTA	1 mM

The pH was adjusted to 8.0 with concentrated HCl.

**Lysozyme buffer** for DNA isolation from *Streptomyces*

Sucrose	0.3 M
Tris-HCl (pH 8.0)	25 mM
EDTA (pH 8.0)	25 mM

Immediately before use, lysozyme was added to a final concentration of 2 mg/ml.

**Acrylamide stock solution**

Acrylamide	29.2 %
Bis-acrylamide	0.8 %

**Spacer gel buffer (pH 6.8)**

Tris-HCl	0.5 M
SDS	0.4 %

**Separation gel buffer (pH 8.0)**

Tris-HCl	1.5 M
SDS	0.4 %

**Ammonium persulfate (AMPS)** for polymerization of polyacrylamide was dissolved at 0.24 % in water immediately before use.

**Polyacrylamide gel electrophoresis (PAGE)** required:

**a) 15 % PAGE separation gel consisting of:**

Acrylamide stock solution	7.5 ml
Separation gel buffer	5 ml
AMPS (0.24 %)	2.5 ml
Distilled water	2.5 ml
0.3 % TEMED	2.5 ml

**b) Spacer gel consisting of:**

Acrylamide stock solution	1 ml
Spacer gel buffer	2.4 ml
AMPS (0.24%)	1.25 ml
Distilled water	4 ml
2.0 % TEMED	1.25 ml

**c) Sample buffer consisting of:**

Distilled water	4.0 ml
0.5 M Tris-HCl (pH 6.8)	1.0 ml
Glycerol	0.8 ml
10 % SDS	1.6 ml
$\beta$ -Mercaptoethanol	0.2 ml
0.05% (w/v) bromophenol blue	0.2 ml

**d) 5x Electrode (running) buffer for PAGE**

Tris-base	45.0 g
Glycine	216 g
SDS	15.0 g

Distilled water to 3 L, stored at 4°C and warmed to room temperature before use.

**RNase solution** for DNA purification: RNase I "A" was dissolved at a concentration of 5 mg/ml in water or in a solution containing 10 mM Tris-HCl and 15 mM NaCl. The solution was heated in a boiling water bath for 15 min and cooled slowly to room temperature. RNase solution was dispensed as 1-ml aliquots and stored at -20°C.

**P-buffer** (Kieser *et al.*, 2000)

Sucrose	103 g
K <sub>2</sub> SO <sub>4</sub>	0.25 g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	2.02 g
Trace element solution C	2 ml
Distilled water to	800 ml

The solution was dispensed in 80-ml aliquots and autoclaved. Before use, the following sterile solutions were added:

KH <sub>2</sub> PO <sub>4</sub> (0.5%)	1.0 ml
CaCl <sub>2</sub> ·7H <sub>2</sub> O (0.25 M)	10.0 ml
TES buffer (5.73%, pH 7.2)	10.0 ml

Trace element solution C consisted of:

ZnCl <sub>2</sub>	40 mg
FeCl <sub>3</sub> ·6H <sub>2</sub> O	200 mg
CuCl <sub>2</sub> ·2H <sub>2</sub> O	10 mg
MnCl <sub>2</sub> ·4H <sub>2</sub> O	10 mg
Na <sub>2</sub> B <sub>4</sub> O <sub>7</sub> ·10H <sub>2</sub> O	10 mg
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	10 mg
Distilled water to	1000 ml

**SM buffer** for phage elution (Sambrook *et al.*, 1989)

Tris-HCl (1 M, pH 7.5)	20 ml
MgSO <sub>4</sub> (1 M)	1 ml
NaCl (5 M)	20 ml
Gelatine	1 g (melted in 10 ml water)
Distilled water to	1000 ml

**Phenol mixture:**

Phenol	500 g
8-hydroxyquinoline	0.5 g

The solution was equilibrated with 50 mM Tris-HCl (pH 8.3).

**Phenol/chloroform:** phenol mixture: chloroform: isoamyl alcohol, 50:50:1

**Modified Kirby mixture** for extraction of RNA (Kieser *et al.*, 2000):

TPNS	1% (v/v)
Sodium 4-aminosalicylate	6% (v/v)

Phenol mixture (above)	6% (v/v)
Tris-HCl (pH 8.3)	50 mM

**10x DNase I buffer:**

Tris-HCl (pH 7.8)	0.5 M
MgCl <sub>2</sub>	0.05 M

**Buffers for catechol 2,3-dioxygenase assay (Kieser *et al.*, 2000):****a) Sample buffer**

Phosphate buffer (pH 7.5)	100 mM
EDTA-Na (pH 8.0)	20 mM
Acetone	10 % (v/v)

**b) Assay buffer**

Phosphate buffer (pH 7.5)	10 mM
Catechol	0.2 mM

Catechol stock solution (20 mM) was prepared in ethanol and stored at -20°C.

**Solutions used for hybridizations:** To prepare 20x SSC, 175.3 g NaCl and 88.2 g trisodium citrate in water at pH 7.0 were made to 1 liter. Denaturing solution contained 1.5 M NaCl and 0.5 M NaOH. Neutralizing solution contained 1.5 M NaCl, 0.5 M Tris-HCl (pH 7.2), and 1 mM EDTA. Denhardt's reagent (100x) contained 2% (w/v) BSA, 2% (w/v) Ficoll and 2% (w/v) polyvinylpyrrolidone. Pre-hybridization solution for Southern blots contained 5x SSC, 5x Denhardt's reagent, 0.5% (w/v) SDS and 100 µg/ml of denatured, sheared herring sperm DNA. The pre-hybridization solution for Northern blots contained 0.1% SDS, 50% formamide, 5x SSC, 50 mM NaPO<sub>4</sub> (pH 6.7), 0.1% sodium pyrophosphate, 5x Denhardt's solution, and 20 mg/ml DNA solution. Hybridization solutions were pre-hybridization solutions supplemented with an [ $\alpha^{32}$ P] dCTP-labeled probe.

## **V. Maintenance of Stock Cultures**

### **A. Bacteria:**

*E. coli* stocks were prepared as overnight cultures on LB agar supplemented with 100 mg/ml ampicillin for strains harboring plasmids. Cells were collected, suspended in 20% glycerol, and kept at -20°C for short-term storage or -70°C for long-term storage.

### **B. Streptomycetes:**

Streptomycetes were maintained temporarily as plate cultures on agar. For *S. venezuelae*, MYM agar (with appropriate antibiotics for strains with plasmids) was used. TO agar was used for strains that sporulated poorly on MYM agar. For long-term maintenance, spores were harvested, washed with sterile water, re-suspended in 20% (v/v) glycerol and stored at -70°C. Cultures of *S. griseus* IFO13350 and HH1 were maintained on nutrient agar (Horinouchi *et al.*, 1984), or in 20% (v/v) glycerol and stored at -20°C.

### **C. Phage:**

Lambda phage from single plaques were eluted into 1 ml SM buffer with a drop of chloroform added, and stored at 4°C. For long term maintenance, 7.0% DMSO was used instead of a drop of chloroform, and storage was at -70°C.

## **VI. Culture Conditions**

### **A. Cultures for plasmid isolation:**

A single colony was used to inoculate 1 ml of LB broth containing appropriate antibiotics in a 1.5 ml tube. The culture was incubated at 37°C overnight on a shaker at 220 rpm. Cells were harvested by centrifugation. For large scale plasmid isolation, *E. coli* strains were cultured in 4 ml of LB broth supplemented with appropriate antibiotics. Cells were harvested after incubation as above.

### **B. Cultures for phage DNA isolation:**

A 10 ml culture of *E. coli* strain LE392 was grown overnight in LB broth at 37°C with shaking. A portion (0.5 ml) was used to inoculate 10 ml of LB broth containing 10 mM MgSO<sub>4</sub>, 0.2% maltose, and 20 µl of stock lambda phage. The infected cells were shaken overnight at 37°C before isolation of phage DNA.

### **C. Streptomyces**

#### **a. Cultures for genomic DNA isolation:**

Erlenmeyer flasks (125 ml) containing 25 ml of MYM medium supplemented with 2.5 M of  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  to a final concentration of 5 mM were inoculated with the streptomycete and incubated at 30°C for 24–48 h with shaking at 220 rpm.

**b. Cultures for isolating of total RNA from *S. venezuelae*:**

The culture conditions were the same as those used for JdB production (see below).

**c. Cultures for jadomycin B production:**

MYM medium (25 ml) dispensed in 125-ml Erlenmeyer flasks was normally inoculated with 20  $\mu\text{l}$  of spore suspension and incubated for 24 h at 30°C with shaking to prepare vegetative inocula. However, to initiate vegetative inocula of VS1095/1096 or VS1106, a 2-cm<sup>2</sup> piece of agar containing mycelium was used instead of a spore suspension, and the MYM culture was incubated for 72 h at 30°C with shaking. Portions (1 ml) of vegetative inocula were used to inoculate 25 ml of Gal2I medium in 125-ml Erlenmeyer flasks. The Gal2I cultures were grown at 30°C with shaking (220 rpm) for 6.5 h, at which time they were supplemented with 0.75 ml of absolute ethanol. Incubation was then resumed under the same conditions for an additional 48 h. In some experiments the ethanol supplement was omitted or the period of post-supplement incubation varied. In another experiment testing the effect of higher pH on production of JdB, MOPS buffer (0.1 M final concentration; Shapiro and Vining, 1983) was added to Gal2I medium and the pH was adjusted to 7.3 with NaOH before inoculation.

**d. Cultures for chloramphenicol production:**

A 250-ml Erlenmeyer flask containing TSGB medium (25 ml) was normally inoculated with 20  $\mu$ l of spore suspension and shaken (220 rpm) for 24 h at 30°C to prepare vegetative inocula. For the bald strains VS1095/1096 and VS1106, a 2-cm<sup>2</sup> piece of agar containing mycelium was used to initiate vegetative inocula. Portions (1 ml) of a vegetative inoculum culture were used to inoculate GI medium (25 ml) in 250-ml Erlenmeyer flasks. Appropriate antibiotic supplements were included if needed. Cultures in GI medium were shaken (220 rpm) at 30°C at for 7 days.

**e. Cultures of *S. griseus*:**

Nutrient broth (50 ml) in a 250 ml Erlenmeyer flask was inoculated with *S. griseus* spores or mycelium and shaken (220 rpm) for 3-5 days at 30°C.

**f. Cultures for measuring catechol 2,3-dioxygenase activity**

*S. venezuelae* transformed with pXE4 or pXE4::*apr*<sup>r</sup> was shaken (220 rpm) at 30°C in MYM medium for one day, or in Gal2I medium for 48 h after ethanol treatment as described for JdB production. In one experiment, ethanol treatment was omitted.



## **VII. DNA and RNA Manipulation**

### **A. Plasmid DNA preparation:**

Plasmid DNA was isolated from *E. coli* cells by the alkaline lysis method (Sambrook *et al.*, 1989).

### **B. Phage DNA purification:**

Bacteriophage  $\lambda$  DNA was purified essentially as reported by Yamamoto *et al.* (1970) and modified by Sambrook *et al.* (1989).

### **C. Genomic DNA extraction:**

The procedures of Hopwood *et al.* (1985) were used. Mycelium was collected from a 10-ml culture sample by centrifugation, washed twice with 10.3% sucrose and resuspended in 2 ml of lysozyme solution. After incubation at 37°C for 30 min with periodic gentle shaking, EDTA (0.5 M, 0.24 ml) and 26  $\mu$ l of 2 mg/ml pronase K were carefully mixed in and 0.14 ml of 10% (w/v) SDS was added. The mixture was incubated at 37°C for 2 h, then extracted with 4 ml of neutral phenol/chloroform by inverting the tube several times, and centrifuging at 10,000 x g for 10 min. The aqueous layer was transferred with a wide-mouth pipette to a new tube, and the extraction was repeated until no protein collected between the two phases. To the aqueous solution, 0.1 volume of 3 M sodium

acetate was added; one volume of ethanol was then layered on the top. Genomic DNA was spooled on a glass hook by gentle stirring at the interface of the two layers.

**D. Total RNA isolation (Kieser *et al.*, 2000):**

Streptomycete mycelium was harvested by centrifugation (5000 rpm) and immediately transferred to a 50-ml plastic tube (pre-cooled on ice) containing 5 ml modified Kirby mixture and about 14 g of 4.5-5.5-mm glass beads. After the suspension had been vigorously vortexed for 3-4 min, 5 ml phenol/chloroform (room temperature) was added and vortexing was continued for a further 2-3 min before the mixture was centrifuged at 7,000 rpm for 10 min at 4°C. The upper (aqueous) layer was added to 3 ml phenol/chloroform mixture, vortexed for 1 min, and the phases were separated as above. These steps were repeated until no significant amount of material was present at the phase interface. To the upper layer 0.1 volume of 3 M sodium acetate (pH 6.0) and one volume of isopropanol were added to precipitate the total RNA. The pellet was redissolved in 1 ml distilled water and reprecipitated with sodium acetate and isopropanol. After rinsing with 5 ml 80% ethanol, it was air dried and dissolved in 1 ml distilled water. The solution was incubated with 0.1 volume of DNase I buffer and 7.5 U RNase-free DNase I at 37°C for 1 h, and phenol/chloroform was added with vortexing. The phases were separated, RNA was precipitated as before, and the pellet was washed with 80% ethanol. Finally the RNA was redissolved in 0.2-0.5 ml of distilled water, divided into 50-100 µl aliquots and stored at -70°C.

## **E. Gel electrophoresis**

### **a. Agarose gel electrophoresis for DNA and RNA analysis:**

For routine DNA gel electrophoresis 0.5-1.5 % agarose gels in 1x TAE buffer were used. For RNA analysis a 1.0 % agarose gel in 1x TBE buffer was used. To avoid degradation of RNA during gel electrophoresis, the agarose, 1x TBE buffer and loading dye were each separately autoclaved and kept sterile before use. The gel tank, the plate on which the gel was poured, and the comb used to form the wells were immersed in 100 mM NaOH for more than two hours, then rinsed in sterile water before use. Electrophoresis at 80-100 V was usually carried out at room temperature for 1 h.

### **b. Polyacrylamide gel electrophoresis:**

The polyacrylamide gel (15%) was prepared in separation gel buffer and electrophoresis was carried out at 175 V and room temperature for 1-3 h. Polypeptides separated by SDS-PAGE were simultaneously fixed and stained with methanol:water:glacial acetic acid (45:45:1) containing 0.25% (w/v) of Coomassie Blue R250 for 4-12 h at room temperature. The gel was destained by agitation in methanol/acetic acid/water alone at room temperature; the destaining solution was replaced with fresh solution until clear bands were visible. The gel was stored in 20% glycerol at 4°C.

**F. Elution of DNA from agarose gels:**

Bands containing DNA fragments were excised and extracted from agarose gels by using the UltraClean15 DNA Purification Kit (MO BIO Laboratories, Inc.) and procedures recommended by the supplier.

**G. Generating nested, overlapping deletions, subcloning and sequencing:**

To sequence the DNA insert retrieved from Lambda LW3 and recloned in pBluescript SK(+) as pJV401, a set of overlapping deletions covering the entire length of the fragment was created with an ExoIII/S1 nuclease deletion kit supplied by MBI Fermentas. Procedures were based on the supplier's protocol. Restriction mapping of pJV401 allowed overlapping regions of the insert to be chosen for further subcloning. The regions of *S. venezuelae* DNA isolated from lambda phages were mapped and subcloned as pJV423, pJV425, pJV429, and pJV450 in pBluescript II SK(+) and pUC18 sequencing vectors, as for pJV401. To sequence *S. venezuelae* DNA upstream of the core PKS cluster, pJV429 was mapped and subcloned as above. All of the constructs were sequenced at the Dalhousie University/National Research Council Joint Sequencing Laboratory by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977).

## **H. Computer-assisted DNA sequence analysis:**

The DNA sequence data obtained from the Joint Sequencing Laboratory was analyzed with Gene runner version 3.05 (Hastings Software, Inc., Hastings-on-Hudson, NY) to detect sequence overlaps, and examined with ORF Finder (NCBI) and FramePlot 2.3 (Ishikawa and Hotta, 1999) to locate ORFs by biased usage of G and C nucleotides in the third letter of codons (Wright and Bibb, 1992). BlastX was used to query GenBank with individual ORF sequences, and BlastP was used for searching the protein database with the translated ORF sequences. To locate functional domains, an NCBI CDD search, which compared protein sequences with the conserved domain database, was used. Sequences were aligned and their relatedness was assessed with ClustalW (Thompson *et al.*, 1994).

## **I. GenBank accession numbers**

The DNA sequences reported in this research have been submitted to GenBank. For the sequence of *jadM* the accession number is AF222693; for *jadN*, *X*, *O*, *P*, *Q*, *S*, *T*, *U*, *V*, *R\**, *Y*, and *Z* the accession number is AY026363; for *jadW<sub>1</sub>*, *jadW<sub>2</sub>*, and *jadW<sub>3</sub>* the accession number is AU24659.

## **VIII. Transformation**

### **A. *E. coli***

#### **a. Preparation of *E. coli* competent cells (Hopwood *et al.*, 1985):**

A 2-ml inoculum culture of the *E. coli* strain was grown overnight at 37°C with shaking. Two 25-ml portions of LB broth in 150-ml Erlenmeyer flasks were each supplemented with 0.2 ml of 2.5 M MgCl<sub>2</sub>, and 1 ml of the overnight inoculum culture. After 3-4 h growth (OD ca 1.3 at 622 nm), the cultures were cooled on ice for 10 min and centrifuged (10 min, 4000 rpm, 4°C). The cell pellet was re-suspended in 10 ml of cold 0.1 M CaCl<sub>2</sub>, centrifuged again (10 min), and re-suspended in 10 ml of 0.1 M CaCl<sub>2</sub>. After cooling on ice for 20 min, the cells were re-suspended in 5 ml of 0.1 M CaCl<sub>2</sub> and 1 ml of glycerol. The competent cells were then stored at -70°C as 100 µl aliquots in pre-cooled 1.5 ml microcentrifuge tubes.

**b. Transformation of *E. coli*:**

To 100 µl of stored competent cell suspension, 1-2 µl (0.1-0.2 µg) of plasmid DNA or approximately 6 µl of ligation mixture was added. The mixture was kept on ice for 20-30 min, heat-shocked for 90 s at 42°C, and cooled on ice for 2 min before adding 0.9 ml of LB medium, and incubating the mixture at 37°C for 60-90 min. Portions (100-500 µl) of the cell suspension were spread on LB-agar supplemented with appropriate antibiotics and/or IPTG and X-gal, and incubated overnight at 37°C. Colonies on the plates were used to screen for the desired clones.

**B. *Streptomyces***

**a. Transformation of protoplasts:**

To prepare *S. venezuelae* protoplasts, procedures described by Kieser *et al* (2000) were used. MYEME broth (25 ml) and 0.1 ml of spore suspension were incubated at 30°C on a shaker for 24-36 h. Mycelium was harvested by centrifugation and washed twice with 10 ml of 7.32% (w/v) mannitol. The pellet was re-suspended in 4 ml of sterile P buffer supplemented with 2 mg/ml of lysozyme; after incubating at 30°C for 30-60 min, protoplast formation was complete. The suspension was drawn into a 10 ml pipette three times, and mixed with 5 ml of P buffer, then filtered aseptically through cotton wool and centrifuged gently at 3000 rpm for 10 min to sediment the protoplasts. These were washed twice with P buffer and resuspended in 1 ml of P buffer. For each transformation, 100 µl of the protoplast suspension was mixed with 5-20 µl of DNA in TE buffer. Immediately, 200 µl of 25% PEG 1000 in P buffer was mixed in by pipetting up and down once. As soon as possible (no longer than 2 min after adding the PEG) 100-200 µl of the mixture was gently spread on R2YE medium pre-dried more than 4 h in a laminar-flow hood. The plates were incubated at 30°C for 14-16 h, then overlaid with 3 ml of soft agar containing appropriate antibiotics for selection. Transformants were usually observed after 4-6 days incubation.

**b. Conjugal plasmid transfer from *E. coli* to *Streptomyces*:**

A modification of the methods of Mazodier *et al.* (1989) and Flett *et al.* (1997) was used. Competent cells of *E. coli* ET12567 (pUZ8002) grown under Cm (25 µg/ml) and Km (50

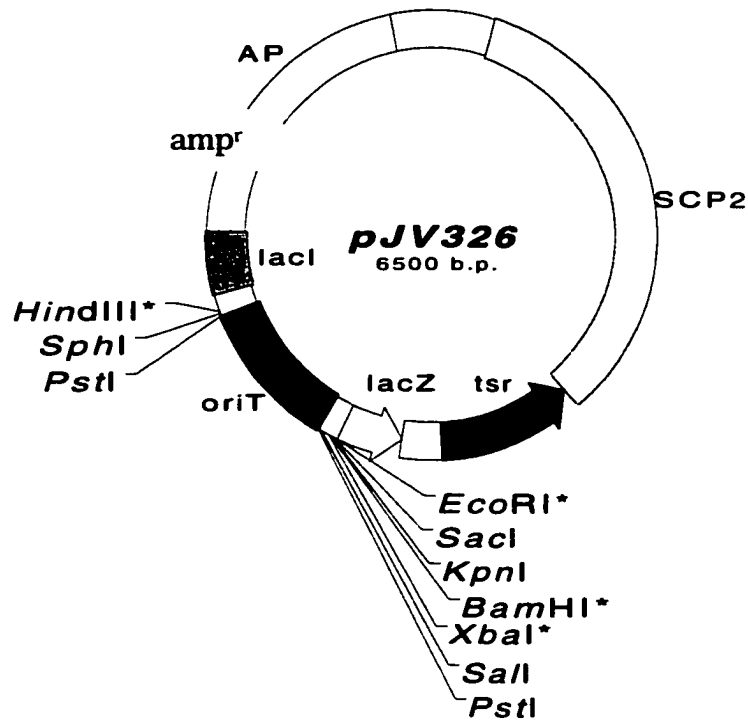
$\mu\text{g/ml}$ ) selection were transformed with the *oriT*-containing plasmid pJV326 (Fig. 11, He *et al.*, 2001) under conditions selecting for the incoming vector only. LB broth (10 ml) containing Cm, Km, and other appropriate antibiotics selecting for the *oriT*-containing plasmid was inoculated with the transformed *E. coli* ET12567 (pUZ8002) and grown overnight at 37 °C. The overnight culture was diluted 1/100 in fresh LB broth supplemented with antibiotics for appropriate selection, and grown at 37 °C to an  $\text{OD}_{600}$  of 0.4-0.6. The cells were washed twice with equal volumes of LB broth, then resuspended in 0.1 volume of LB broth. To 500  $\mu\text{l}$  of washed *E. coli* suspension, approximately  $10^8$  streptomycete spores that had been heat-shocked at 50°C for 10 min and allowed to cool to room temperature were added. The mixture was centrifuged briefly and most of the supernatant was decanted; the pellet was resuspended in the remaining liquid (200-300  $\mu\text{l}$ ), plated on MS agar, and incubated at 30°C for 5-7 h. The agar surface was overlaid with 1 ml water containing 0.5 mg nalidixic acid and the appropriate antibiotic for plasmid selection; incubation was then continued at 30°C for colony development. Potential transconjugants were picked to selective media containing nalidixic acid for further examination.

## **IX. Hybridizations**

### **A. Plaque hybridization:**

Procedures described by Sambrook *et al.* (1989) and Kieser *et al.* (2000) were used to screen the *S. venezuelae* ISP5230 genomic DNA library in Lambda GEM-11 prepared by





**Figure 11.** Restriction map of pJV326. The plasmid was constructed from pHJL400 by inserting a 0.76-kb *Pst*I fragment containing *oriT* from pKC1218 in the polylinker *Pst*I site. The SCP2 and *tsr* regions are from the *S. coelicolor* A3(2) plasmid SCP2\*. The regions of DNA containing *lacZ*, *lacI*, and *amp<sup>r</sup>* are from *E. coli* plasmid pUC19. Enzymes labeled with asterisks are unique sites in the vector (He *et al.*, 2001).

Facey (1994). Phage propagated in *E. coli* LE392 were suspended in 2.5 ml of top agar and spread on LB-agar in 9-cm Petri plates. Plaques formed during overnight incubation at 37°C. Phage DNA in the plaques was adsorbed on a nylon membrane by carefully placing the membrane on the agarose surface. Lines drawn on the surface of the membrane were marked by drawing lines on the back of the plate. The membrane was removed after 2 min and placed, plaque-side-up, on filter paper saturated with 10% (w/w) SDS for 5 min. It was transferred sequentially for 7 min at room temperature to filter papers saturated with denaturing and neutralizing solutions. Finally the membrane was transferred to a fresh filter paper and air-dried. DNA was bound to the nylon by baking (80°C for 1-2 h) before the membrane was immersed overnight at 65°C in hybridization solution containing a <sup>32</sup>P-labeled probe. After hybridization the membrane was washed twice at room temperature by agitation for 10-15 min with 1x SSC/0.1% SDS, and once with 0.1x SSC/0.1% SDS at 65°C for 1 h. It was then exposed to a *Bio-Rad* CS phosphor-imaging screen for approximately 24 h. Hybridization signals on the screen were detected using the GS-525 Molecular Imaging system (*Bio-Rad* Laboratories, Mississauga, Ont).

The probe was labeled in a solution containing the following:

Linearized denatured DNA probe (>50 ng)	1-45 µl
[α- <sup>32</sup> P]dCTP (3000 Ci/mmol)	5 µl
Ready to Go Reaction Mix labelling bead	one bead
Distilled water to	50 µl

These were mixed by pipetting and incubated at 37°C for 30 min, then heated at 95-100°C for 4 min to ensure complete denaturation of the DNA, and cooled on ice for 2 min before addition to the pre-hybridization solution.

**B. Southern hybridization:**

Plasmid DNA digested with restriction enzymes was fractionated by agarose gel electrophoresis, and the gel was stained and photographed. It was soaked in 0.25 N HCl for 15 min to depurinate the DNA, rinsed with distilled water, denatured, and neutralized (20 min for each treatment). The DNA was then transferred to a nylon membrane with 10x SSC buffer by vacuum filtration. The membrane was exposed to a labeled probe for hybridization, and hybridizing fragments were detected as described above.

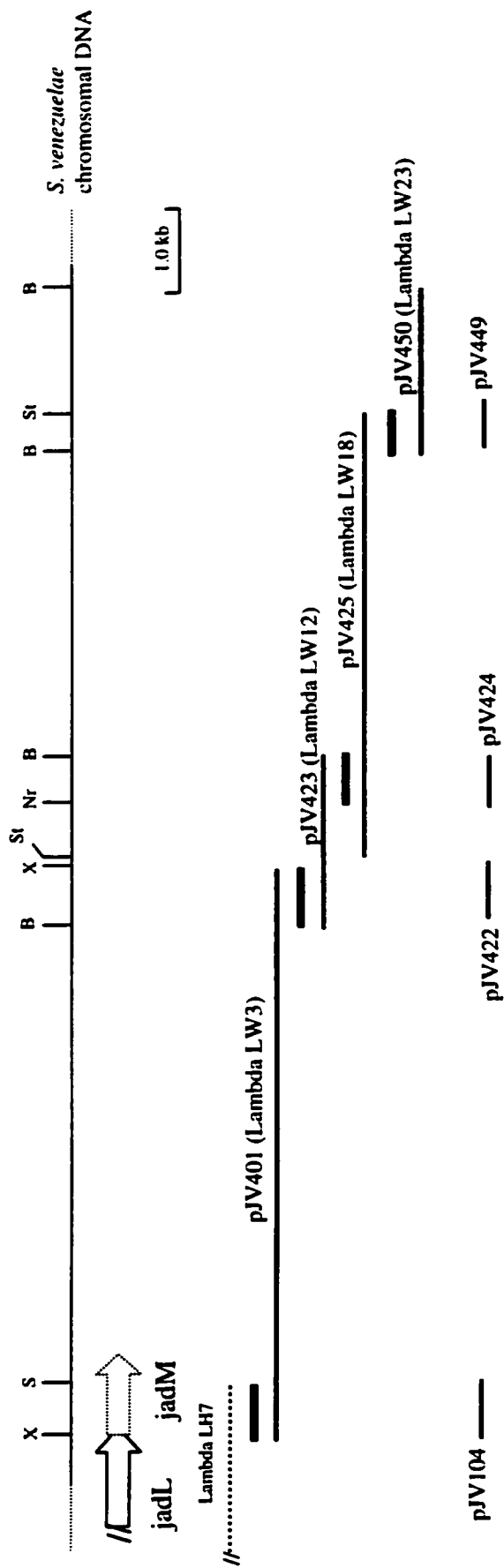
**C. Northern Hybridization:**

For Northern hybridization, RNA was isolated from Gal2I cultures of *S. venezuelae* ISP5230 by using the modified Kirby mix, phenol/chloroform extraction and DNase I treatment (Kieser *et al.*, 2000). It was fractionated under denaturing conditions in an agarose-formaldehyde gel and adsorbed on a nylon membrane (GeneScreen™ & GeneScreen Plus, NEN Life Science) by overnight upward capillary transfer. RNA samples bound to the nylon membrane were immobilized by baking at 80°C for 1-2 h and placed in a hybridization bottle with pre-warmed hybridization solution (0.1% SDS; 50% formamide; 5x SSC; 50 mM NaPO<sub>4</sub>, pH 6.7; 0.1% sodium pyrophosphate; 5x Denhardt's solution; 20 mg/ml salmon testes DNA solution). After pre-hybridization at 42°C for 2-4 h, the membrane was incubated with a <sup>32</sup>P-labelled probe in a fresh pre-warmed hybridization solution at 42°C for 12-18 h. Membranes were washed at room temperature with 2x SSC containing 0.1% SDS, then once with 1x SSC containing 0.1% SDS, and

finally at 55°C for 45 min with 0.2x SSC containing 0.1% SDS. The membrane was exposed to an X-ray film in an intensifying cassette at -70°C for 2-7 days, and the film was developed.

## **X. Chromosome Walking**

A genomic library prepared in the phage vector Lambda GEM-11 (Promega Corp., Wisconsin) from a partial *Sau3AI* digest of *S. venezuelae* ISP5230 DNA (Facey, 1994) was screened for plaques that hybridized with a 0.55-kb *XhoI-SacI* fragment of *S. venezuelae* ISP5230 DNA subcloned in pBluescript II SK+ as pJV104 (McVey, 1998). This probe had been isolated from the 3' end of Lambda LH7 by Han *et al.* (1994) and shown (McVey, 1998) to contain the 3' region of *jadL* and 5' region of *jadM* (Fig.12). Hybridization (Sambrook *et al.*, 1989) with the <sup>32</sup>P[dCTP]-labelled pJV104 insert yielded eight labeled phage clones (Lambda LW1-LW8). When DNA extracted from the phages and digested with *XhoI* was probed with the labeled pJV104 insert, a hybridizing 7.0-kb fragment was detected in six of the eight clones. The fragment from LW3 was inserted in the *XhoI* site of pBluescript II SK(+) to obtain pJV401. A 0.6-kb *BamHI-XhoI* fragment from the 3' *XhoI* end of the pJV401 insert (see Fig. 12) was subcloned as pJV422 and used as the probe in the next round of plaque hybridizations. A library fragment (Lambda LW12) that hybridized with the probe was cloned in pBluescript II SK+ as pJV423. For further chromosome walking a 0.6-kb *NruI-BamHI* fragment of pJV423, subcloned in pJV424, was labeled with <sup>32</sup>P[dCTP] and used to probe the *S. venezuelae* genomic library. A library fragment (LW18) that hybridized with the probe was recloned as a 5.6-



**Fig. 12.** Chromosome walking in the *S. venezuelae* ISP5230 genome. Overlapping recombinant lambda vectors LH7 (3' end only), LW3, LW12, LW18, and LW23 are shown. A restriction map of the 13-kb region of chromosomal DNA is also shown. Thick black lines indicate the inserts in aligned plasmids pJV104, pJV401, pJV422, pJV423, pJV424, pJV425, pJV449, and pJV450 obtained by subcloning the  $\lambda$  DNA fragments in pBluescript II SK(+) and pUC18. Abbreviations: B, *Bam*HI; Nr, *Nru*I; S, *Sac*I; St, *Stu*I; X, *Xho*I.

kb *StuI* fragment in pUC18, giving pJV425. When a 0.6-kb *BamHI-StuI* fragment at the 3' end of pJV425, subcloned as pJV449, was labeled and used to probe the *S. venezuelae* genomic library, it hybridized with library fragment LW23. This was subcloned as a 2.0-kb *BamHI* fragment in pUC18 giving pJV450. Hybridization showed that the inserts in pJV423, pJV425, and pJV450 contained overlapping regions (see Fig. 12). Thus Lambda LW3 overlapped LW12, which overlapped LW18, and LW18 overlapped LW23.

For chromosome walking to obtain genes upstream of the core PKS cluster, lambda 8 (Ramalingam, 1989), which overlapped 5' end of LH7 (Han *et al.*, 1994; see Fig. 2) was digested with *EcoRI/BamHI*. A 4.0-kb *EcoRI/BamHI* fragment overlapping the 5' end of LH7 was subcloned between the *EcoRI* and *BamHI* sites of pBluescript II SK(+) to give pJV429. Restriction mapping and sequencing demonstrated that the pJV429 insert included the 5' end of DNA cloned in LH7.

## **XI. Gene Disruption**

In general, the gene to be disrupted was first cloned in an *E. coli* vector (pUC18/19 or pBluescript II), then inactivated at a restriction site near its center by inserting the  $Am^R$  gene from pJV225 (Chang, 2001). A fragment containing the insertionally inactivated gene was retrieved by digestion with suitable restriction enzymes and recloned in the multicloning site of the conjugal vector pJV326 (He *et al.*, 2001). The recombinant conjugal vector was passaged through the DNA-methylation deficient *E. coli* strain ET12567 (pUZ8002), to avoid streptomycete restriction systems during intergeneric

transfer. Transconjugant *S. venezuelae* strains containing the disrupted gene were selected by plating on MS agar overlaid with apramycin. Colonies that grew were patched on MYM agar containing apramycin for further examination. Strains resistant to apramycin but sensitive to thiostrepton (and therefore containing only DNA integrated by a double crossover) were selected.

#### **A. Disruption of *jadM***

An *NruI* site detected 85-bp upstream of the *jadM* start codon by restriction analysis of the ORF was targeted as the site for insertional inactivation. Because the *NruI* site was only 210 bp from the *XhoI* end of the ORF, the sequence cloned in pJV402 was extended beyond *XhoI* to increase opportunities for double crossovers with chromosomal DNA when the plasmid was introduced into *S. venezuelae*. A 3.4-kb *XhoI-KpnI* fragment retrieved from pJV402 was ligated to a 1.3-kb *XhoI-KpnI* fragment of pJV405 containing the chromosomal region adjoining *jadM*. This region had been initially cloned as a 4.0-kb *SacI* fragment in pJV404A, and subcloned as an *NruI* (blunted)-*XhoI* fragment in the *EcoRV-XhoI* multiple cloning sites of pBluescript II SK(+) to give pJV405. Digestion of pJV405 with *XhoI-KpnI* and ligation with the 3.4-kb *XhoI-KpnI* fragment from pJV402 gave pJV406. Digestion of pJV406 with *NruI*, dephosphorylation with calf intestinal phosphatase, and ligation with the 1.6-kb *EcoRV* cassette containing *apr<sup>r</sup>* (retrieved from pJV225) yielded constructs with *apr<sup>r</sup>* in different transcriptional orientations with respect to *jadM* (opposite in pJV407A; similar in pJV407B). Linearizing pJV407A/B with *EcoRI/EcoRV*, blunting the linear products with SI nuclease, and ligation with the

promiscuous vector pJV326 linearized with *Bam*HI and blunted with SI nuclease, gave pJV408A/B (with the *apr*<sup>r</sup>-fragment in opposite orientations). These constructs were transferred conjugally from *E. coli* to *S. venezuelae* ISP5230 by incubating a mixture of *E. coli* cells harboring pJV407A/B and *S. venezuelae* ISP5230 spores for 4-6 days at 28°C on a selection medium containing 50 ug/ml apramycin. Transconjugant strains VS1075 and VS1076 were obtained.

### **B. Disruption of *jadN***

Restriction enzyme analysis showed an *Nco*I site near the middle of *jadN*. Taking advantage of this, a 1.6-kb *apr*<sup>r</sup>-cassette from pJV225 (Chang, 2001) was inserted into *jadN* in alternative transcriptional directions to give pJV412A/B. The native *jadN* in wild-type *S. venezuelae* was disrupted by the general procedure described for *jadM* (see above). After conjugal transfer of pJV412A/B into *S. venezuelae* ISP5230, the insertionally inactivated transconjugants (VS1077/1078) were selected.

### **C. Disruption of *jadX*, *O*, *P*, *Q*, *S*, *T*, *U*, and *V***

Using the general procedures described above for *jadM* and *jadN*, the cloned genes 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*<sup>r</sup> retrieved from pJV225. In the resulting plasmids



(pJV414A/B) the *apr<sup>r</sup>* disrupting *jadO* was present in opposite transcriptional orientations. Fragments containing the disrupted *jadO* in pJV414A/B were retrieved from agarose gels and ligated with the *oriT*-containing vector pJV326, giving pJV415A/B. The final plasmid constructs were transferred from *E. coli* to *S. venezuelae* ISP5230 by intergeneric conjugation. Using similar methods, *jadX*, *jadP*, *jadQ*, *jadS*, *jadT*, *jadU* and *jadV*, cloned in either pBluescript II SK+ or pUC18, were disrupted by inserting the apramycin resistance cassette in alternative directions at their *EcoRV*, *BstEII*, *MaeII*, *EcoR57I*, *BamHI*, *BstEII* and *EcoR57I* sites, respectively, to give pJV413A/B, pJV416A/B, pJV417A/B, pJV418A/B, pJV419A/B, pJV420A/B, and pJV421A/B, respectively. These constructs were transferred from *E. coli* to *S. venezuelae* ISP5230 by intergeneric conjugation, as in the disruption of *jadO*. Single colonies of the transconjugant strains VS1079/1080, VS1081/1082, VS1083/1084, VS1085/1086, VS1087/1088, VS1089/1090, VS1091/1092, and VS1093/1094, each resistant to apramycin but sensitive to thioestrepton, were selected. In the first member of each transconjugant pair the apramycin resistance 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).

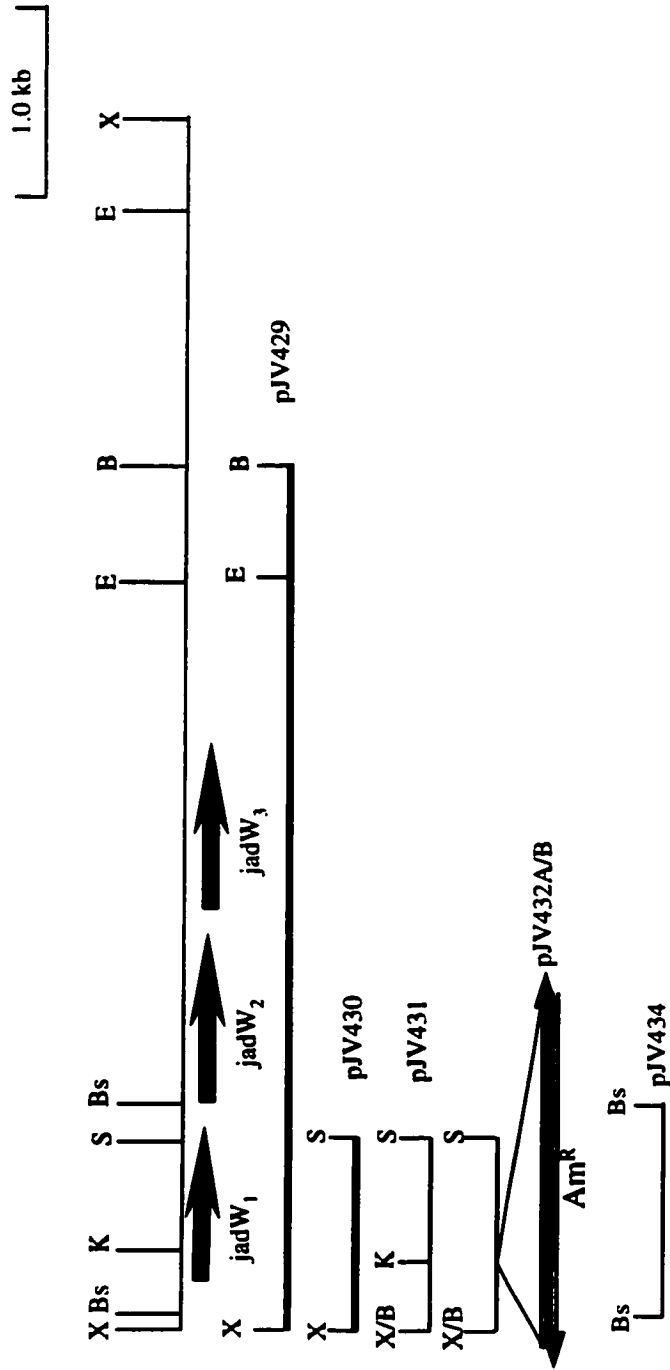
#### **D. Disruption of *jadR*\***

A 3.3-kb *KpnI-StuI* fragment retrieved from pJV425 was recloned in pBluescript SK(+), giving pJV456. pJV456 was digested with *NruI-BamHI* to delete a 0.75-kb fragment containing *jadR*\*, the 1.6-kb *Am<sup>R</sup>* gene retrieved from pJV225 was inserted at the

linearized *NruI*-*Bam*HI (blunted) site, giving pJV457. The 4.1-kb fragment in pJV457 was retrieved by digestion with *KpnI*/*Eco*RI and blunted. The fragment was inserted into the blunted *Eco*RI site of pJV326 giving construct pJV458. Plasmid pJV458 containing the disrupted *jadR\** was used to transform *S. venezuelae* ISP5230 conjugationally. Of the single colonies that grew in the presence of apramycin, one colony (VS1107) sensitive to thiostrepton was chosen.

#### **E. Disruption of *jadW*<sub>1</sub>**

A 1.2-kb *SacI*-*XhoI* fragment containing *jadW*<sub>1</sub> was retrieved from a *SacI*/*XhoI* digest of pJV429 and ligated with *SacI*/*XhoI*-digested pBluecript SK(+) to give pJV430. After pJV430 had been linearized with *XhoI*, it was blunt-ended and then digested with *SacI*. The 1.2-kb *XhoI* (blunted)-*SacI* fragment was retrieved and ligated into the *Bam*HI (blunted)-*SacI* sites of pUC18, giving pJV431. Digesting pJV431 with *KpnI*, blunting-ending the linearized plasmid and ligating it with a 1.6-kb *EcoRV* cassette containing the *Am*<sup>R</sup> gene retrieved from pJV225, gave pJV432A/B, in which the *Am*<sup>R</sup> inserts were oppositely oriented (Fig. 13). From pJV432A/B 2.6-kb fragments containing *jadW*<sub>1</sub> disrupted by the *Am*<sup>R</sup> gene were excised by digestion with *XbaI*/*Eco*RI and ligated into *XbaI*/*Eco*RI-digested promiscuous vector pJV326 to give pJV433A/B. When pJV433A/B were transferred to *S. venezuelae* by conjugation, transconjugants resistant to apramycin but sensitive to thiostrepton were isolated as single colonies with the *Am*<sup>R</sup> gene in alternative orientations (VS1095/1096).



**Figure 13.** Construction of *jadW<sub>1</sub>*-disrupted mutants. Broad filled arrows below the map represent the ORFs (*jadW<sub>1</sub>*, *jadW<sub>2</sub>*, *jadW<sub>3</sub>*) identified in the pJV429 insert, and their orientations. Restriction sites aligned below the map define the inserts in plasmids pJV429, pJV430, pJV431, pJV432A/B, and pJV434. Double line arrows below pJV432A/B represent each orientation of the apramycin resistance gene inserted at the *KpnI* site to disrupt *jadW<sub>1</sub>*. Abbreviations: B, *Bam*HI; Bs, *Bsa*AI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; X, *Xho*I.

### **F. Construction of disruption mutants with *jadW*<sub>2</sub> deleted**

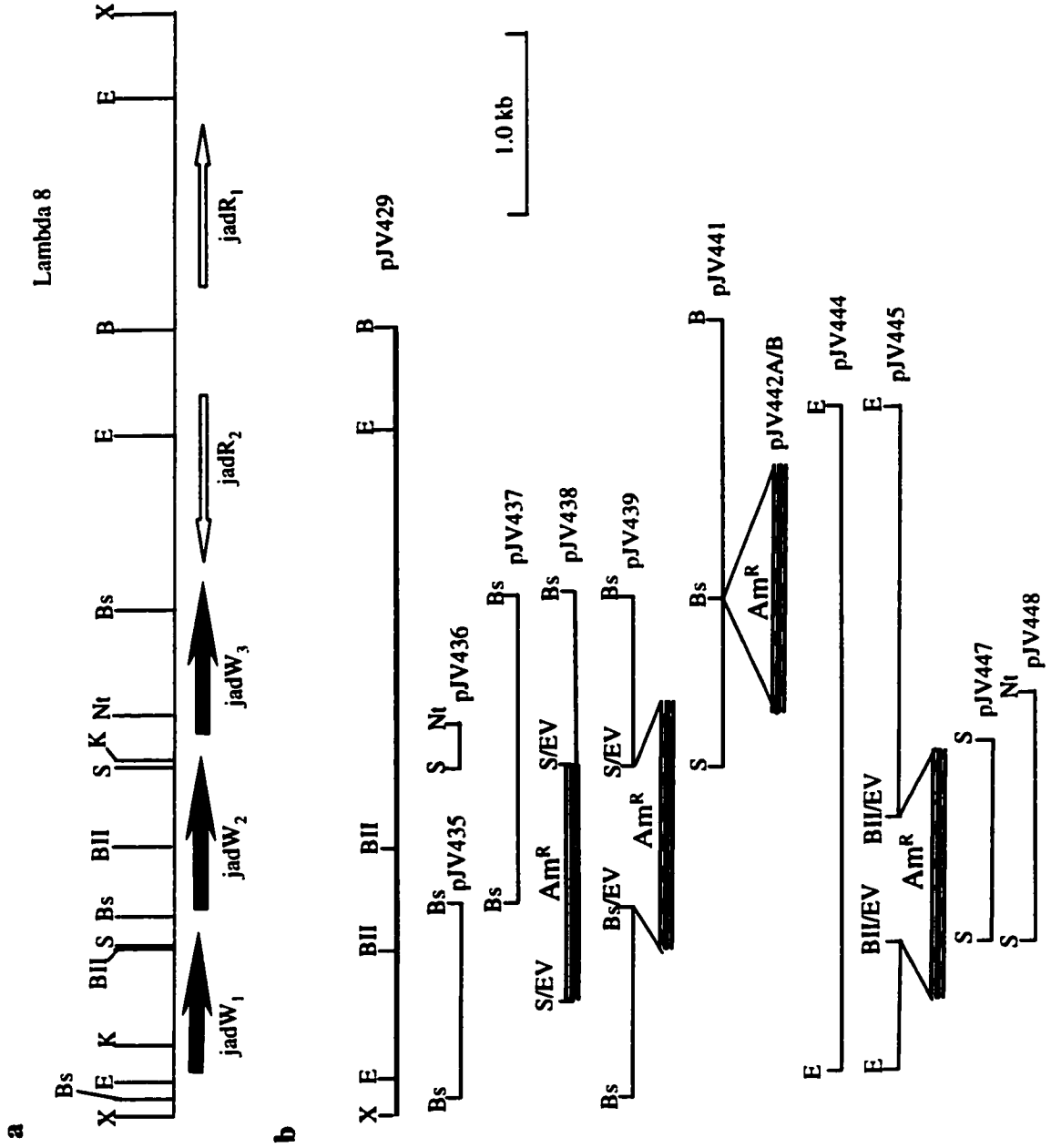
Plasmid pJV429 was digested with *Bsa*AI; a 1.6-kb fragment containing *jadW*<sub>2</sub> and part of *jadW*<sub>3</sub> was retrieved from the digest and ligated with pUC18 linearized with *Sma*I and dephosphorylated. The resulting plasmid (pJV437; Fig. 14) was digested with *Sac*I, excising 0.75 kb of *jadW*<sub>2</sub> DNA from the pJV437 insert. The *Sac*I end was blunted, and ligated with a 1.6-kb *Eco*RV cassette containing the Am<sup>R</sup> gene retrieved from pJV225. The resulting plasmid (pJV438) contained a 2.6-kb insert, which was retrieved by digestion with *Bam*HI/*Eco*RI and ligated with pJV431 linearized with *Bam*HI (see Fig. 13). The two fragments were blunted, and then incubated with T4 DNA ligase to give pJV439. The 3.6-kb fragment of DNA in pJV439 thus contained *jadW*<sub>1</sub> and *jadW*<sub>3</sub> separated by the 1.6 kb Am<sup>R</sup> gene, which had replaced most of the deleted *jadW*<sub>2</sub> (see Fig. 14). pJV439 was recloned in the *Eco*RI site of the promiscuous vector pJV326, giving pJV440. The plasmid was transferred conjugationally into *S. venezuelae* ISP5230, and transconjugants (VS1099/1100) with *jadW*<sub>2</sub> replaced by the Am<sup>R</sup> gene were isolated by selecting single colonies resistant to apramycin but sensitive to thiostrepton.

### **G. Construction of disruption mutants with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>**

A 3.35-kb *Eco*RI fragment from pJV429 (see Fig. 14) was recloned in pBluescript II giving pJV444. Digestion of pJV444 with *Bst*EII deleted a 0.54-kb fragment containing the 109-bp 3'-end of *jadW*<sub>1</sub> and the 420-bp 5'-end of *jadW*<sub>2</sub>; the residual pJV444 insert was blunted and ligated with a 1.6-kb *Eco*RI Am<sup>R</sup> cassette, giving pJV445 (see Fig. 14).

**Figure 14 (a).** Restriction map of *S. venezuelae* ISP5230 chromosomal DNA containing *jadW*<sub>1</sub>, *jadW*<sub>2</sub>, *jadW*<sub>3</sub>, *jadR*<sub>2</sub>, and *jadR*<sub>1</sub>. Arrows below the restriction map show the locations of these genes in the *jad* cluster. Below the arrows, constructs in the inserts of plasmids pJV429, pJV435, pJV436, pJV437, pJV438, pJV439, pJV441, pJV442A/B, pJV444, pJV445, and pJV447 are shown. Sites in the constructs are aligned with sites in the restriction map. locating the genes. Abbreviations: B, *Bam*HI; BII, *Bst*EII; Bs, *Bsa*AI; E, *Eco*RI; Ev, *Eco*RV; K, *Kpn*I; Nt, *Not*I; S, *Sac*I; X, *Xho*I.

**(b).** Inactivation of *jadW*<sub>2</sub>, *jadW*<sub>3</sub>, and both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>. A fragment containing the apramycin-resistance gene (*Am*<sup>R</sup>) was inserted in the region of pJV439 from which most of *jadW*<sub>2</sub> had been excised; *Am*<sup>R</sup> was also inserted at the *Bsa*AI site of pJV441 to disrupt *jadW*<sub>3</sub> or at the *Bst*EII site of pJV444 to construct a mutant with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>. Introducing the plasmids into *S. venezuelae* ISP5230 and isolating double cross-overs conferring an *Am*<sup>R</sup> Thio<sup>S</sup> phenotype gave strains VS1097/1098, VS1099/1100, and VS1101/1102.



The 4.4-kb insert in pJV445 was retrieved by *EcoRI* digestion and ligated into the *EcoRI* site of pJV326 giving pJV446. Plasmid pJV446 was transferred from *E. coli* into *S. venezuelae* ISP5230 by conjugation and single colonies of the transconjugants resistant to apramycin but sensitive to thiostrepton gave strains VS1103/1104, with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>.

#### **H. Disruption of *jadW*<sub>3</sub>**

A 2.3-kb *SacI*-*Bam*HI fragment from pJV429 was recloned in pUC18, giving pJV441. The 1.6-kb Am<sup>R</sup> gene was inserted in the *Bsa*AI site of pJV441 in alternative directions giving constructs pJV442A/B (see Fig. 14). The disrupted insert in pJV442A/B was retrieved and ligated with pJV326, giving pJV443A/B. Mutants VS1101/1102 in which the wild-type *jadW*<sub>3</sub> in *S. venezuelae* ISP5230 has been insertionally inactivated by allele exchange were isolated after conjugational transfer of plasmids pJV443A/B from *E. coli* to the streptomycete.

#### **XII. Construction of a Plasmid Containing *jadW*<sub>1</sub>**

To construct a plasmid containing only *jadW*<sub>1</sub>, pJV429 was partially digested with *Bsa*AI. The 1.2-kb *Bsa*AI fragment containing *jadW*<sub>1</sub> (see Fig. 13) was ligated into the *Sma*I site of pUC18 to give pJV434. The 1.2-kb insert of pJV434 was retrieved by digestion with *EcoRI*/*Bam*HI, and inserted between the *EcoRI* and *Bam*HI sites of pJV326 to give pJV435. Plasmid pJV435, containing *jadW*<sub>1</sub>, was passaged through the DNA-

methylation-deficient *E. coli* ET12567 and transferred conjugally into both *jadW*<sub>1</sub>-disrupted and wild-type *S. venezuelae* host strains. Selection of transconjugants resistant to thiostrepton furnished strains VS1097 from the *jadW*<sub>1</sub>-disrupted mutant, and VS1098 from the wild-type host.

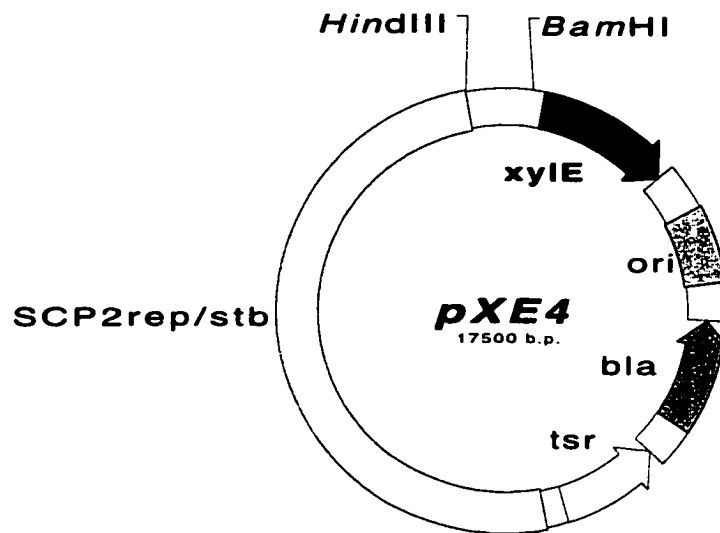
### **XIII. Construction of a plasmid containing *jadW*<sub>2</sub>**

To prepare a donor *E. coli* plasmid for transferring an additional *jadW*<sub>2</sub> gene into *S. venezuelae* ISP5230, pJV436 containing a 0.3-kb 3'-terminal *SacI*-*NotI* segment of *jadW*<sub>2</sub> (see Fig. 14) was digested with *SacI* and dephosphorylated with CIAP. It was then ligated with the 1.0-kb *SacI* fragment from pJV447, giving pJV448. The 1.5-kb insert in pJV448 was retrieved from a *PvuIII*/*EcoRV* digest and ligated into the *Bam*HI (blunted) site in pJV326, giving pJV463. Plasmid pJV463 was transferred conjugationally into *S. venezuelae* ISP5230 and VS1099, and single colonies of transconjugants resistant to thiostrepton were selected. From *S. venezuelae* ISP5230 and VS1099 host strains, transconjugants VS1105 and VS1106, respectively, were isolated.

### **XIV. Construction of pXE4::*apr*<sup>r</sup>**

The Am<sup>R</sup> gene was excised from pJV225 (Chang et al., 2001) as a *HindIII* fragment and ligated into the *HindIII* site of pXE4 (Fig. 15; Ingram et al., 1989) with the same transcriptional orientation as *xylE*. The pXE4::*apr*<sup>r</sup> construct and a pXE4 control plasmid



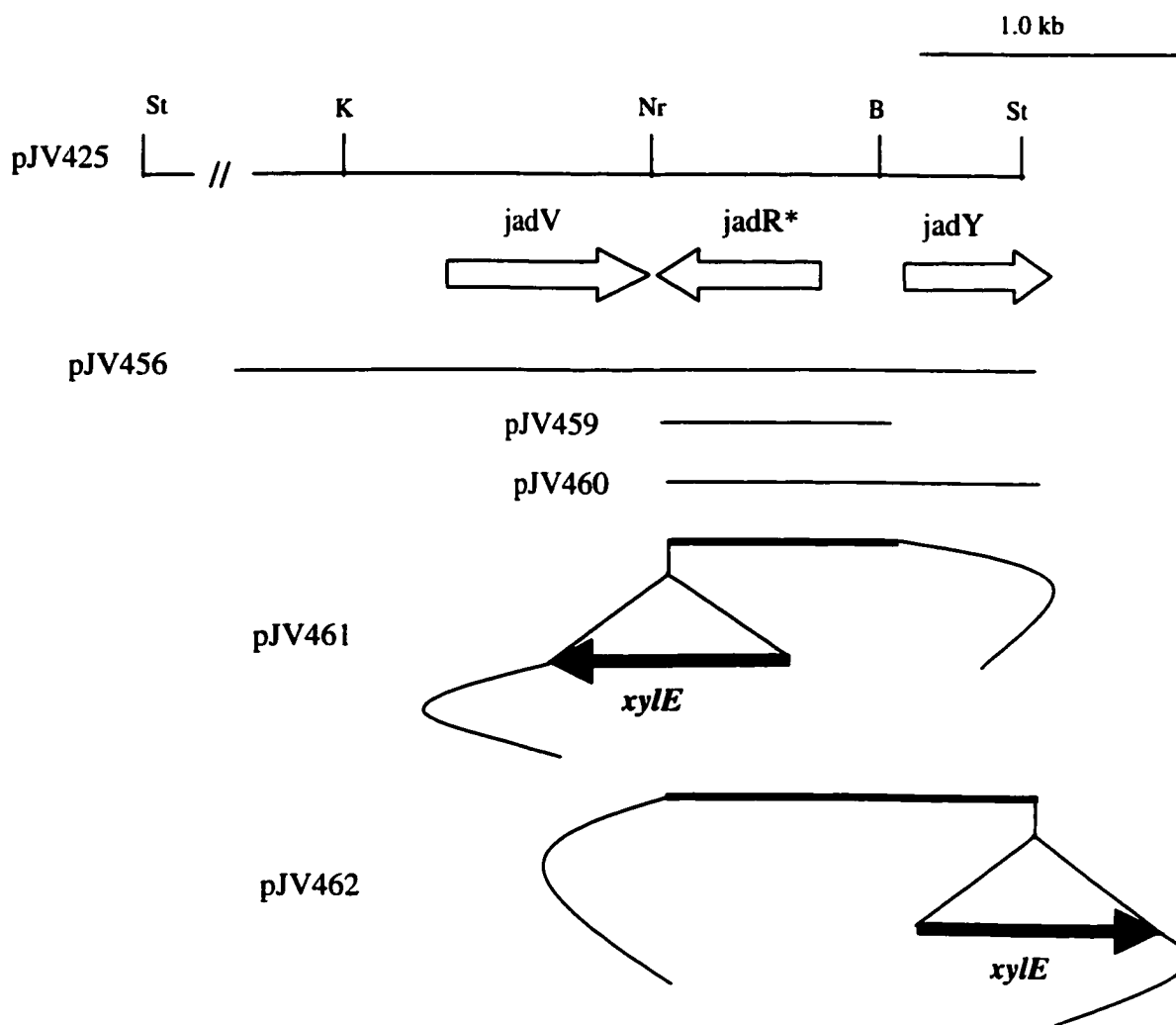


**Figure 15.** Features of the promoter probe plasmid pXE4: *ori*, the ColE1 origin of replication; *tsr*, the thiostrepton resistance gene from *Streptomyces azureus*; *bla*, a  $\beta$ -lactamase gene from *E. coli*; SCP2 *rep/stb*, the replication and stability functions of *Streptomyces* plasmid SCP2\*; *xylE*, a promoterless copy of *xylE* from *Pseudomonas putida* (Ingram *et al.*, 1989).

were passaged through *E. coli* ET12567 before being used to transform protoplasts of *S. venezuelae* ISP5230.

#### **XV. Construction of pXE4::0.75 and pXE4::1.5**

The region of the *S. venezuelae* ISP5230 chromosome containing *jadR\**, *jadY* and the sequence between the two genes consisted of overlapping 0.75-kb *NruI*-*Bam*HI and 1.5-kb *NruI*-*StuI* fragments (Fig. 16). These two fragments were obtained from pJV456, which contained a 3.3-kb *KpnI*-*StuI* fragment from pJV425 (see Fig. 12). Digestion of pJV456 with *NruI*/*Bam*HI and ligation with a *SmaI*/*Bam*HI digest of pUC18 gave pJV459 containing the 0.75 kb *NruI*-*Bam*HI fragment, and digestion of pJV456 with *NruI*/*StuI* followed by ligation with *EcoRV*-digested pBluescript II SK(+) gave pJV460. *EcoRI*-linearized pJV459 was blunted, and the 0.75-kb insert retrieved by further *Hind*III digestion was ligated into the *Hind*III and *Bam*HI (blunted) sites of the promoter probe vector pXE4 to give pJV461. The transcript orientation of *xylE* in pJV461 was the same as that of *jadR\**. The 1.5-kb insert in pJV460 was retrieved with *Hind*III/*Bam*HI and subcloned in pXE4, giving pJV462, in which *xylE* is transcribed in the opposite direction from *jadR\** (see Fig. 16). The resulting plasmids, pXE4::0.75 (pJV461) and pXE4::1.5 (pJV462), as well as pXE4 were passaged through *E. coli* ET12567 and used to transform *S. venezuelae* ISP5230 protoplasts. Transformants resistant to thiostrepton were selected and patched on fresh MYM medium, then incubated at 30°C for 4 days to allow sporulation.



**Figure 16.** Diagram showing construction of pXE4::0.75 and pXE4::1.5. Restriction sites are aligned with those in the map of pJV425, and define the inserts in pJV456, pJV459, pJV460, pJV461, and pJV462. Abbreviations: B, *Bam*HI; K, *Kpn*I; Nr, *Nru*I; St, *Stu*I.

## **XVI. 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 insert in 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 *S. venezuelae* VS1087 (*jadS* disrupted), VS1089 (*jadT* disrupted), VS1091 (*jadU* disrupted) and VS1093 (*jadV* disrupted).

## **XVII. Jadomycin B Production and Analysis.**

Cultures of *S. venezuelae* ISP5230 were grown in 125-ml Erlenmeyer flasks at 30°C on a rotary shaker (220 rpm). A vegetative inoculum was prepared by incubating a spore suspension (20 µl) in 25 ml of MYM medium (Stuttard, 1982) for 24 h. Portions (1 ml) of this culture were used to inoculate 25 ml of Gal2I medium in 125-ml Erlenmeyer flasks. The Gal2I cultures, after incubation on a rotary shaker (220 rpm) at 30°C for 6.5 h, were supplemented with 0.75 ml of absolute ethanol, and incubation was resumed for 48 h. Filtered broths were extracted with ethyl acetate and assayed by high performance liquid chromatography (HPLC) using Beckman System Gold equipment and software. Culture extracts (20 µl) in methanol were injected on a C<sub>18</sub> reverse-phase silica column (50 × 4.6 mm) and eluted at a flow rate of 1 ml/min with a linear gradient from 100% solvent A (acetonitrile:water, 1:1) to 25, 50, 100, and 0% solvent B (100% acetonitrile)

programmed to change after 3, 6.5, 7.5, and 10 min, respectively. Both solvents contained 0.1%(v/v) trifluoroacetic acid; jadomycins in the eluate were monitored at 313 nm; JdB had a retention time of 7.5 min.

The conditions normally used for jadomycin production were used to culture and extract *S. venezuelae* mutants expected to accumulate pathway intermediates. For *jadW<sub>1</sub>*-disrupted mutants (VS1095/1096) and *jadW<sub>2</sub>*-deleted mutants (VS1106), 0.1 cm<sup>2</sup> squares of mycelium from agar cultures were used to inoculate MYM medium. Cultures were incubated for 2-3 days before use as a vegetative inoculum in Gal2I medium. Samples (2.5 ml) were taken aseptically every 12 h from representative flasks of the Gal2I production cultures and centrifuged to remove mycelium fragments. The clear supernants were extracted with equal volumes of ethyl acetate. The extracts were evaporated, and the residues, redissolved in 200 µl of methanol, were analyzed by HPLC under the standard conditions used to measure JdB.

### **XVIII. Bioassay and HPLC Analysis of Chloramphenicol Production.**

The bioassay described by Doull *et al* (1986) was modified; spores of an *S. venezuelae* strain spread evenly on MYM agar in a 9-cm Petri plate were incubated at 30°C for 48-72 h. Plugs were removed aseptically with a cork borer, placed equidistantly on MYM agar in 9-cm Petri Plates, and incubated at 30°C for 12-14 h. They were then overlaid (2.5 ml per plate) with soft GNY agar (Malik and Vining, 1970) seeded (1.0%; v/v) with a *Micrococcus luteus* culture grown overnight in GNY medium. The overlaid plates were

incubated overnight and the diameters of inhibition zones in the agar around the plugs were measured.

To bioassay production of Cm in shaken cultures, ethyl acetate extracts (100  $\mu$ l) from measured volumes of culture filtrate were absorbed on sterile paper disks, dried at room temperature in a sterile air flow, and placed on MYM agar. The disks and agar were overlaid with GNY agar seeded (1.0 % v/v) with a culture of *M. luteus*. After incubation at 30°C overnight, zones of inhibition in the agar around the paper disks were measured. Production of Cm in shaken cultures was also measured by HPLC analysis of samples of the culture filtrate extracted with ethyl acetate and chromatographed as described by Brown et al. (1996).

#### **XIX. Expression of *jadM* in *E. coli*.**

A 1.0-kb *XhoI-PvuII* fragment containing *jadM* was retrieved from pJV406, blunt-ended with SI nuclease, and ligated (T4 DNA ligase) to pET-21(+), also linearized with *BamHI* and blunt-ended with SI nuclease. Transformation of *E. coli* BL21 (DE3) with the ligation mixture yielded a colony from which the construct pJV409 was isolated. Cultures of the transformant and of *E. coli* BL21 (DE3) harboring pET-21(+) were grown to late log phase in LB medium, and the T7 polymerase promoter in pET-21(+) was induced by adding isopropylthio- $\beta$ -galactopyranoside (IPTG) to 1 mM final concentration. Cells harvested by centrifugation were resuspended in gel loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% w/v SDS, 5% v/v 2-mercaptoethanol, 10% v/v glycerol

and 0.025% bromphenol blue; Sambrook, *et al.*, 1989), lysed by heating at 100 °C for 3-5 min, and analyzed by SDS-PAGE (Laemmli, 1970). Total proteins were separated on a 4% (w/v) polyacrylamide stacking gel and a 15% (w/v) separating gel; they were visualized by staining the gel with 0.1% Coomassie brilliant blue R-250.

## **XX. Measurement of Total Protein Concentration**

Test strains of *E. coli* grown overnight in 5 ml of LB broth at 37°C, and also *S. venezuelae* strains grown at 30°C for 24 h in 25 ml of MYM medium or for 24-48 h in Gal2I medium were harvested by centrifugation. They were washed twice in 20 mM phosphate buffer (pH 7.2), resuspended in this buffer and lysed by sonication on ice (4 x 15 s, with 30-s cooling intervals). The sonicate was centrifuged (10,000 rpm, 4°C, 10 min) to remove cell debris. The total concentration of proteins in cell extracts, diluted if necessary, was measured with the bicinchoninic acid reagent as prescribed by the supplier (*Pierce*). Bovine serum albumin (BSA) was used as the standard.

## **XXI. Measurement of Catechol 2,3-Dioxygenase Activity**

Transformants containing *xylE* were selected on MYM agar supplemented with thiostrepton or apramycin, 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). In these procedures, cells were harvested by centrifugation, washed in 20 mM phosphate buffer (pH 7.2), and suspended (final volume 3 ml) in sample buffer. They were lysed by sonication on ice (4 x 15, with 30-s cooling intervals). A 10% solution of Triton X-100 (10 µl/ml) was added, and the extracts were placed on ice for 15 min. After cell debris had been removed by centrifugation, the supernatant solution was mixed with 0.5 ml of assay buffer in a prewarmed (37°C) cuvette, and the change in absorbance at 375 nm was followed. The quantity of cell extract was adjusted to produce a linear change over the measurement times (2, 4, and 6 min). Catechol dioxygenase activity was calculated as the rate of change in optical density at 375 nm per min per mg protein, and converted to mU/mg. Protein in cell extracts was measured with the bicinchoninic acid reagent.

## **XXII. Complementation Tests**

### **A. With an A-factor mutant of *S. griseus***

To test whether streptomycin production in the A-factor-deficient *S. griseus* HHI mutant could be restored by metabolites extracted from *S. venezuelae* ISP5230, the complementation procedure described by Horinouchi *et al.* (1984) was adapted. Mycelium from *S. griseus* HHI was patched on nutrient agar (Difco), and about 1 cm away from each patch the agar surface was inoculated with a drop (1 µl) of *S. venezuelae*



ISP5230 spore suspension. Cultures were incubated at 30°C for 2-3 days, overlaid with soft nutrient agar seeded with *B. subtilis*, and incubated again at 37°C overnight. As an A-factor-positive control, wild-type *S. griseus* 13350 replaced mutant HH1. Inhibition zones around the *S. griseus* patches indicated streptomycin synthesis.

To test whether chloroform extracts of *S. venezuelae* ISP5230 cultures grown in Gal2I medium for 8, 12, 16, 20, 24, 28, 32, 36 and 48 h after ethanol treatment, or without ethanol treatment, restored streptomycin production in *S. griseus* HH1, the complementation procedure was adapted further: the concentrate (10 µl) from chloroform extraction of filtered broth (20 ml) was absorbed on a paper disk, evaporated to dryness and placed 1-cm away from a patch of *S. griseus* HH1 mycelium. The soft nutrient agar overlay seeded with *B. subtilis* was applied and incubated as above.

#### **B. With the *jadW*<sub>1</sub>-disrupted mutant of *S. venezuelae***

To determine whether known butyrolactone effectors or *S. venezuelae* wild-type extracts could restore JdB production in *jadW*<sub>1</sub>-disrupted mutants, 25-ml cultures of the mutant growing in Gal2I medium were supplemented at the time of inoculation with A-factor, IM-2 or nonalactone to give concentrations of 2, 2 and 20 µg ml<sup>-1</sup>, respectively. In addition, parallel tests in which paper disks were impregnated with solutions containing 20 µg of A-factor, 20 µg of IM-2, 40 µg of nonalactone or the chloroform extract from 20 ml of *S. venezuelae* culture filtrate were used to assay for recovery of sporulation by

*jadW<sub>1</sub>*<sup>-</sup> mutants grown on MYM agar. The paper disks were evaporated to dryness and placed 1-cm away from a colony of the *S. venezuelae jadW<sub>1</sub>*<sup>-</sup> mutant.

### **XXIII. Isolation and Assay of Metabolites from *S. venezuelae***

#### **A. Production of a putative $\gamma$ -butyrolactone by *S. venezuelae* ISP5230**

Shaken cultures of *S. venezuelae* ISP5230 were grown under standard conditions (25 ml of Gal2I medium in 125-ml Erlenmeyer flasks with 1.0 ml vegetative inoculum). In one experiment (see Results, page 163) the cultures (total 500 ml) were grown for 17 h without ethanol treatment. In another experiment (Results, page 163), cultures were treated with ethanol (0.75 ml) 6.5 h after inoculation, and incubation was continued for a total of 23.5 h. The cultures were harvested by filtration, and the pooled filtrates were extracted with either an equal volume of chloroform or with three half volumes of ethyl acetate (see Results, page 164). Extracts were back-washed with water and evaporated at 50°C *in vacuo*. The residues, redissolved in 50% aqueous acetonitrile, were each chromatographed on a column (1.6 x 25 cm) of 40-63  $\mu$ m octadecyl-functionalized silica gel (Aldrich). Compounds were eluted with 50 % aqueous acetonitrile and 50 fractions (200 drops each) were collected. The even numbered fractions were dried in an air jet and redissolved in 25 % aqueous acetonitrile. Samples (20  $\mu$ l) were used for HPLC analysis at 210 (Takano *et al.*, 2000), 266, or 273 nm under conditions for Cm or JdB analysis; 100  $\mu$ l samples were bio-assayed using the Cm assay conditions for detecting antibiotic

activity against *M. luteus*, and 20  $\mu$ l samples were used to test for complementation of the *jadW*<sub>1</sub>-disrupted mutant.

### **B. Fractionation of culture extracts**

To purify crude JdB, for <sup>1</sup>H and <sup>13</sup>C NMR analyses and for <sup>1</sup>H-<sup>1</sup>H NOE experiments, the product extracted from production cultures with chloroform was redissolved in chloroform and flash chromatographed on a short (2.3 x 3.5 cm) column of silica gel H eluted initially with chloroform, and then with a gradient of methanol in chloroform. The predominant purple band, which followed a smaller blue-green band containing the aglycone, was eluted with chloroform-methanol (99:1). After evaporation of the solvent, the residue was taken up in acetonitrile. Dilution of the solution with water yielded crystalline JdB. For further purification, a reverse-phase column of silica gel C<sub>18</sub> (1.6 x 22 cm) was used. JdB was eluted with a stepped gradient (50-100 %) of acetonitrile in water. Purple fractions containing JdB (monitored by HPLC analysis) were pooled and dried under a stream of nitrogen for NMR spectroscopy. To determine the structure of JdB from single-crystal X-ray diffraction data, crystals were obtained by slow evaporation of a 50% acetonitrile solution at room temperature.

To detect the presence of a putative  $\gamma$ -butyrolactone (J-factor) and unidentified antibiotics in cultures of *S. venezuelae* ISP5230, 900-1800 ml of pooled filtrates from cultures grown under the standard conditions for JdB production were extracted with an equal volume of chloroform. The extract was evaporated, and the residue was partially

redissolved in acetonitrile (3 ml). The suspension was diluted with water (3 ml) and after 30 min to allow crystallization of JdB, the filtered solution was applied to a reverse phase 40-60  $\mu\text{m}$  silica gel  $\text{C}_{18}$  column (1.6 x 22 mm). Metabolites were eluted with 50 % acetonitrile, and 3-ml fractions were collected. Even-numbered fractions were evaporated to dryness, and the residue was redissolved in 50% aqueous acetonitrile (100  $\mu\text{l}$ ). Samples (10  $\mu\text{l}$ ) were applied to paper disks and bio-assayed against *M. luteus* as described for Cm broth bioassays. Fractions lacking antibiotic activity were pooled, and evaporated to dryness for investigation of J-factor activity.

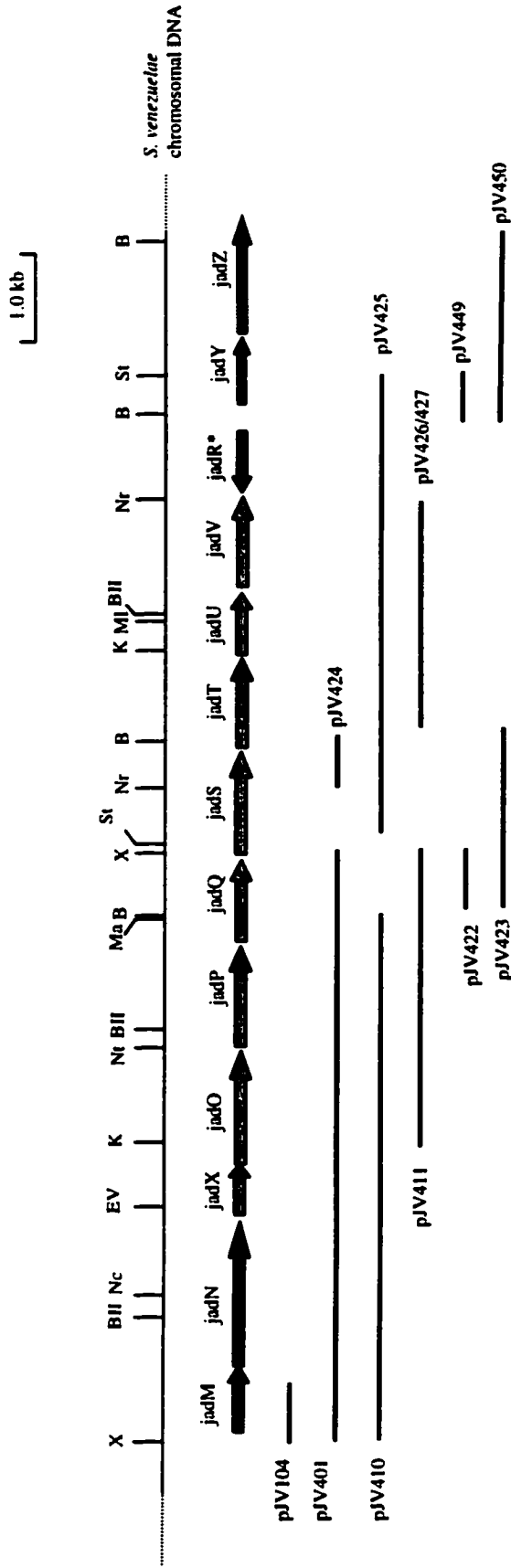
## RESULTS

### I. Cloning Genes Downstream of the Core PKS Cluster

The sequenced region of the *S. venezuelae* chromosome downstream of the core PKS cluster included only the 5'-end of *jadM* (McVey, 1998). Chromosome walking was undertaken to complete the sequence of *jadM*, and investigate the *jad* cluster beyond this gene.

#### A. Sequencing of the *jad* cluster and detection of ORFs

The 0.55-kb *XhoI-SacI* fragment of *S. venezuelae* ISP5230 DNA cloned in pJV104 (McVey, 1998) was used in conjunction with the *S. venezuelae* ISP5230 genomic library prepared in Lambda GEM-11 (Facey, 1994) to initiate chromosome walking (see Fig. 12). Plaque hybridization (Sambrook *et al.*, 1989) with <sup>32</sup>P[dCTP]-labeled pJV104 yielded Lambda phage clone LW3, and further walking gave three additional clones (Lambda LW12, LW18, and LW23). DNA extracted from the phages and digested with various restriction enzymes gave plasmid constructs pJV401, pJV410, pJV411, pJV423, pJV425, pJV426, pJV450 (Fig. 17). Sequencing the inserts in pJV401, pJV423, pJV425, and pJV450 showed that they contained overlapping regions, and detected 13 ORFs (*jadM*, *jadX*, *jadN*, *jadO*, *jadP*, *jadQ*, *jadS*, *jadT*, *jadU*, *jadV*, *jadR\**, *jadY*, and *jadZ*; see Fig. 17) in the region of the *S. venezuelae* chromosome downstream of the PKS core cluster.



**Figure 17.** Restriction map and analysis of *S. venezuelae* ISP5230 genomic DNA downstream of *jadL*. The region was subcloned in the overlapping recombinant lambda vectors LH7, LW3, LW12, LW18, and LW23 (see Fig. 12). Arrows below the restriction map show the locations and orientations of ORFs (*jadM, N, X, O, P, Q, S, T, U, V, R\*, Y, Z*) in the *jad* cluster. The grey arrows represent genes involved in biosynthesis or attachment of the deoxysugar in jadomycin B. Black arrows represent *jad* cluster genes flanking those involved in the deoxysugar biosynthesis pathway. The inserts in plasmids pJV401, pJV410, pJV411, pJV412, pJV422, pJV423, pJV424, pJV425, pJV426/427, pJV449, and pJV450 are defined by restriction sites aligned with 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, *Sau*I; X, *Xho*I.

## **B. Characterization and functional analysis of ORFs**

The features of each ORF were determined and probable functions were deduced from alignments of their protein sequence with functional homologues in other organisms, as well as by determining the phenotypic effects of gene disruptions.

### **a. *jadM***

#### **i. Characterization of *jadM***

*jadM* (GenBank accession No. AF222693), lay immediately downstream of *jadL* and partially overlapped this gene. The putative ATG start codon of the ORF containing *jadM* was located 26 bp inside the 3'-end of the preceding *jadL* ORF (McVey, 1998). A putative RBS (AAGG) for *jadM* could be recognized 10-bp upstream of the start codon, and an in-frame downstream stop codon (TGA) predicted a gene of 786-bp, encoding a protein with 262 amino acids (Fig. 18). The next ORF (containing *jadN*) was oriented for transcription in the same direction as *jadM*, and shared two nucleotides of its ATG start codon with the *jadM* stop codon.

#### **ii. Potential function of *jadM***

Of the deduced amino acids in *jadM*, 36% were similar and 24% identical to those of the HetI sequence in *Anabaena* PCC7120. In this cyanobacterium, *hetI*, *hetM* and *hetN* have

<u>CTCGAGTACCGCGGGACGCGGCCGCCCCGTACGGCCGCGCTCCCGACCC</u>	50
<u>XhoI</u>	
<u>ATCCCCGGGGCCCCGACTCCGACTCCCCAGACCCCGACTCCGACCCCGACT</u>	100
<u>ApaI</u>	
<b>RBS</b>  -----> <i>jadM</i>	
-----> <i>jadL</i>	
<u>CCCCGCA<b>AAGG</b>CTGGTCCGCG<b>ATG</b>CCCGCTCCTGAGGGTCGCGGACGAT<b>GGA</b></u>	150
***	
<u>GCTGCACCTCTGGACCCTCCGCCCGCCCCCGCCGGGGTCCCGCGGCCGCCG</u>	200
<u>NotI</u>	
<u>ACGCCCTCGCGACCGGGGA</u> ACTCGACGGTGCCGAGCGGCGCCGGGCGGAG	250
<u>NruI</u>	
<u>GCGTACGTACGCCCCGGGACCGGGTGCAGTACCTCGCGGCGCACCTCGC</u>	300
<u>GCTGCGCCGGGTCTCGCCCGTACACCGGAGTCCCGGCCGGGCGGCTGC</u>	350
<u>GCTTCGGCCGTGAGGCCGCCGGGCGGCCCGTGCTGCTCGGGGTGGCGGA</u>	400
<u>CCGCCGTCTTCTCGCTGTCGCACAGCCACGGGCTGGTGCTGCTCGGGGT</u>	450
<u>GGCCCGGCGGCCGGTGGGCGTCGACGTCCAGCGGTCCCGCCGTACGCCA</u>	500
<u>CCGCGGAGGTGTGCCGCGGCGCGCTCCACCCGGCCGAGCGGGAGGAGCTC</u>	550
<u>SacI</u>	
<u>GCGGCCCTGCCGAGGACCGTCTCGCGGCGGCCCTTCGCCCGGTGTGGAC</u>	600
<u>CCGGAAGGAGGCTTACCTCAAGGGACTCGGCACGGGACTCGCGCGGGGCC</u>	650
<u>TCGCCACCGACTTCTCGGCACGCCGGGCCACGGCGGGCTCCGGCGGCCC</u>	700
<u>GCCGGTGGACGGTCTCGACCTGCCGGGCGGACCCGACCACGCGGCGGC</u>	750
<u>NotI</u>	
<u>CGCCGCCCTGCACGGCGACACCGCGCCACGGGTACGACGAGGGCCCTGC</u>	800
<u>ApaI</u>	
<u>GCCCCGAGGACCTGACCAGCACCACACGCATCGCACGACACGCAGACA</u>	850
<u>CCGCACGACACGTACGACCCGTACGACACGCAGACCGAAAGGGGAGTGGG</u>	900
----->	
<u>AAGCGC<b>ATGA</b>ACCACGACGTCCACGGCCGGAGGAGCCCTCGCGGACCGTCT</u>	950
***	
-----> <i>jadN</i>	
<u>CACCGAACTGGGCCGGCTCCGGGAGCTCGCCAGGAGGGCCCCGACCCA</u>	1000

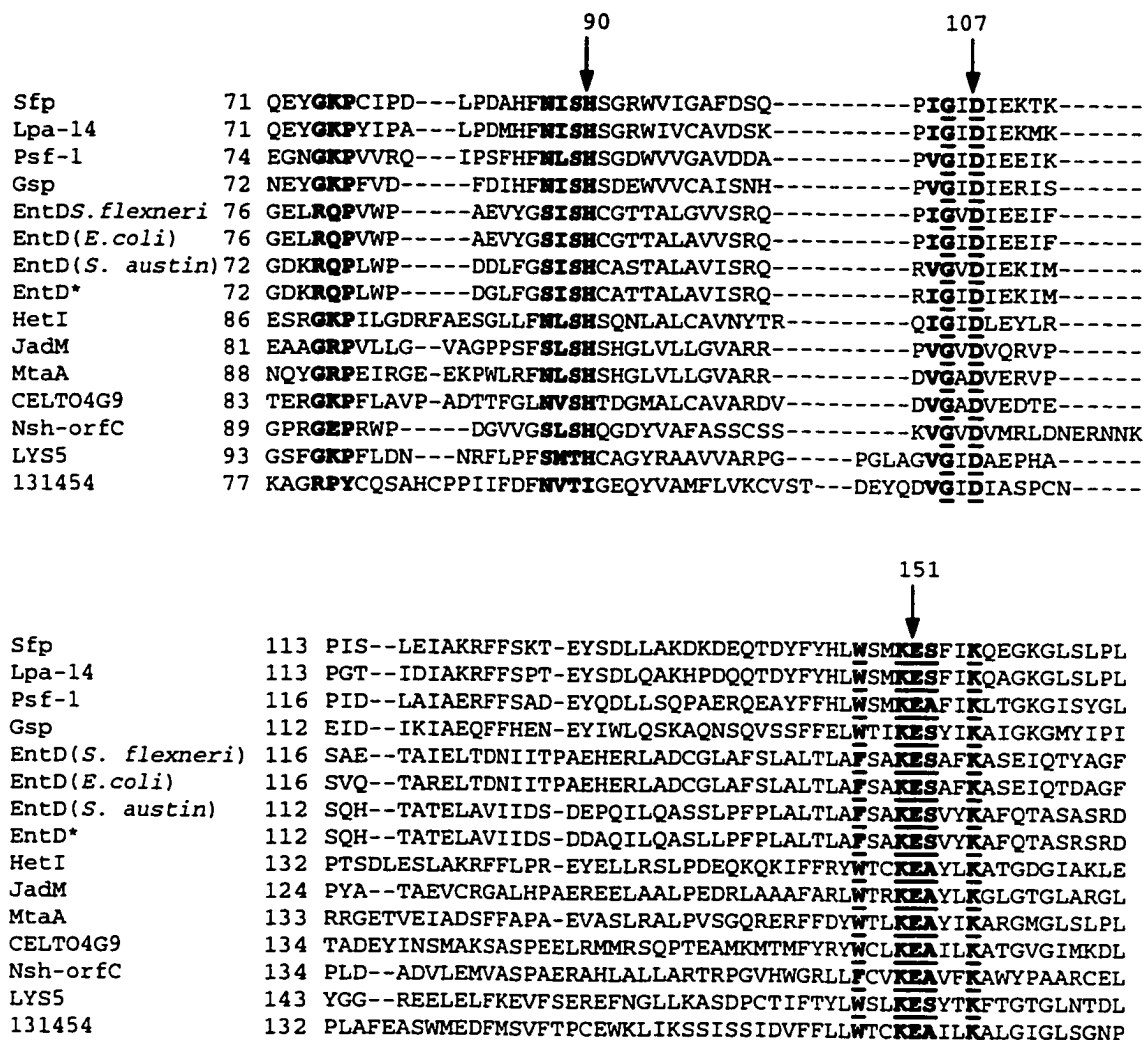
**Figure 18.** The nucleotide and deduced amino acid sequences of *jadM*. The translational start and stop codons for *jadM* and *jadN* are labeled with asterisks; arrows indicate the direction of translation. Significant restriction enzyme recognition sequences are underlined. The GenBank accession No. for *jadM* is AF222693.



been implicated in the production of an unidentified secondary metabolite regulating heterocyst spacing (Black & Wolk, 1994). Sequence analysis indicates that HetM contains an ACP domain, while HetI is similar to several members (Sfp, Gsp and EntD) of the phosphopantetheinyl transferase (PPTase) superfamily (Lambalot *et al.*, 1996; Walsh *et al.*, 1997; Silakowski, *et al.*, 1999). HetI is postulated to be the HetM-specific PPTase involved in synthesizing the secondary metabolite (Black and Wolk, 1994), while HetN is an NAD(P)H-dependent oxidoreductase, similar to some oxidoreductases associated with polyketide and fatty acid biosynthesis. In addition to resembling the HetI sequence in *Anabaena*, the deduced amino acid sequence of *jadM* resembles the sequence of MtaA in *Stigmatella aurantiaca* (29% similar and 22% identical amino acids). This protein is a PPTase activating biosynthesis of the electron transport inhibitor myxothiazol, probably by post-translational modification of MtaB, MtaG and the unique combination of PKSs and nonribosomal peptide synthetases (NRPSs) making up the biosynthetic machinery for myxothiazol (Silakowski *et al.*, 1999). MtaA may also be responsible for transferring phosphopantetheine (Ppan) to proteins involved in the biosynthesis of a variety of secondary metabolites in *S. aurantiaca*.

### **iii. Comparison of JadM with the phosphopantetheinyl transferase superfamily**

To determine whether JadM contained the PPTase consensus, the sequence was aligned with members of the PPTase superfamily (Fig. 19). Amino acids highly conserved in the superfamily were present in JadM, and also some residues considered important in Sfp-type PPTases (Reuter *et al.*, 1999). The crystal structure of Sfp enzymes indicates that

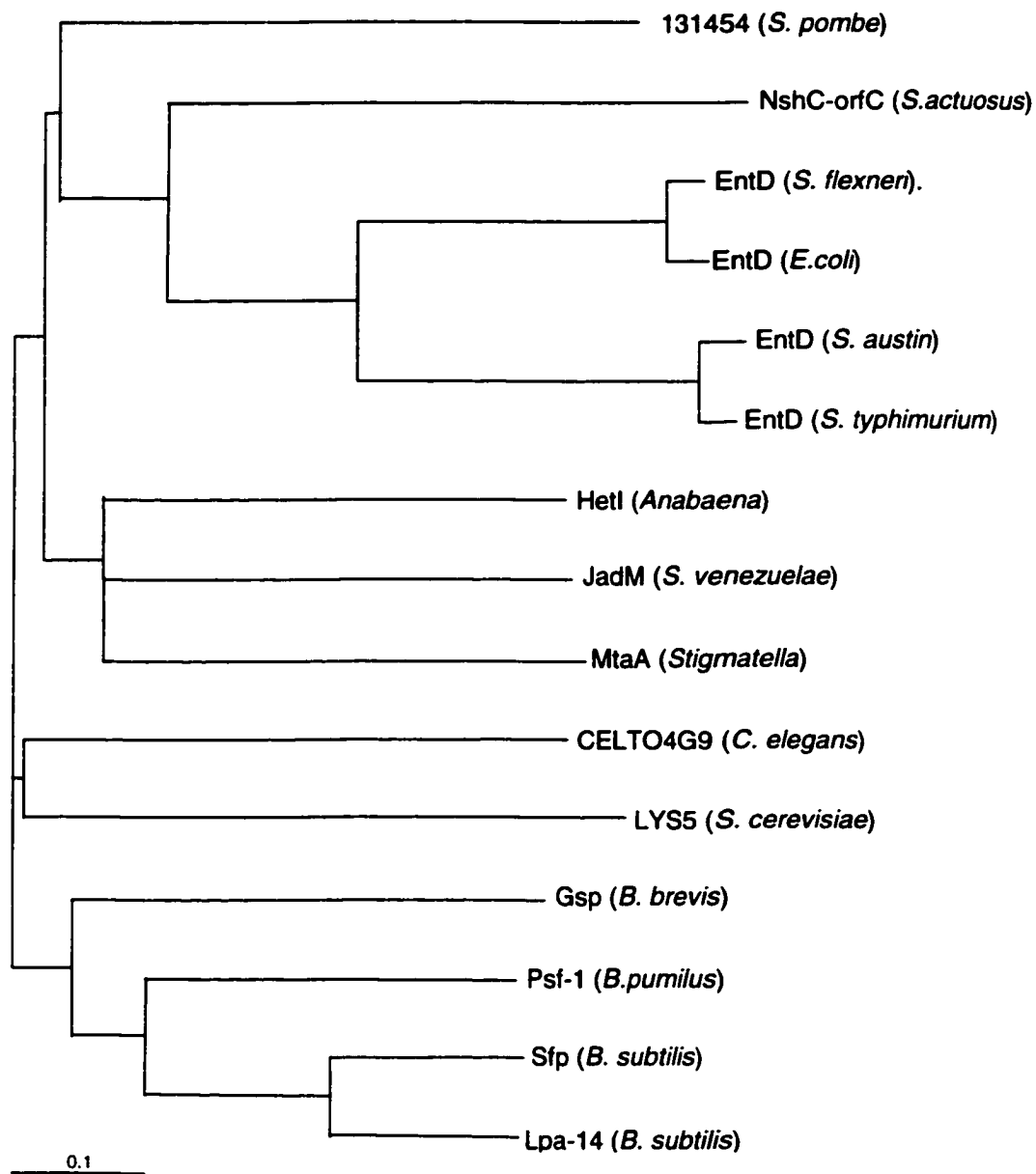


**Figure 19.** Multiple sequence alignment of PPTases with ClustalW. Highly conserved amino acids are in bold letters; conserved sequences in the PPTase superfamily are in bold and underlined. As an aid to locating key regions, the residues at positions 90, 107 and 151 are marked with a vertical arrow. The organisms involved are: Sfp, *B. subtilis*; Lpa-14, *B. subtilis*; Psf-1, *B. pumilus*; Gsp, *B. brevis*; EntD\*, *S. typhimurium*; HetI, *Anabaena* PCC7120; JadM, *S. venezuelae*; MtaA, *S. aurantiaca*; CELTO4G9, *C. elegans*; Nsh-orfC, *S. actuosus*; LYS5, *S. cerevisiae*; 131454, *S. pombe*.

the active site accommodates a magnesium ion complexing with the pyrophosphate group in coenzyme A, the side chains of three acidic amino acids and one water molecule (Reuter *et al.*, 1999). These highly conserved regions may interact with PCP substrates. The Mg<sup>2+</sup>-liganding residues Asp107 and Glu151 in Sfp are highly conserved in every enzyme listed in Fig. 19, including JadM. The  $\alpha$ -phosphate of coenzyme A binds to Lys155 and His90. In JadM and all PPTases investigated except 131454, Lys150 and His90 were highly conserved, while position 105 was invariably glycine for spatial reasons. The sequence motif Gly74-Lys75-Pro76 involved in binding the adenine base of coenzymeA was preserved in JadM, but with the Lys75 replaced by arginine. This is an acceptable substitution because Lys75 forms only hydrogen bonds, and not a salt bridge with the main-chain carbonyls of Ile104, Lys155 and Gln156 (Reuter *et al.*, 1999). A ClustalW alignment of the deduced amino acid sequence of *jadM* with the sequences of PPTases from other organisms supported assignment of the gene to the PPTase superfamily. The phylogenetic tree in Fig. 20 indicates that JadM, HetI and MtaA have a common ancestor, and that these three genes are more closely related to one another than to other members of the family. Comparisons of the deduced amino acid sequence of *jadM* with database sequences are consistent with such a conclusion.

#### **iv. Temporal expression of *jadM***

Cultures of *S. venezuelae* ISP5230 grown in Gal2I medium for 24, 48, 72 or 96 h, either with or without ethanol supplementation at 6 h, were assayed for JdB, and total RNA extracted from the mycelium was used for Northern hybridizations (data not shown).



**Figure 20.** Phylogenetic tree showing the relationship of JadM to members of the PPTase superfamily.

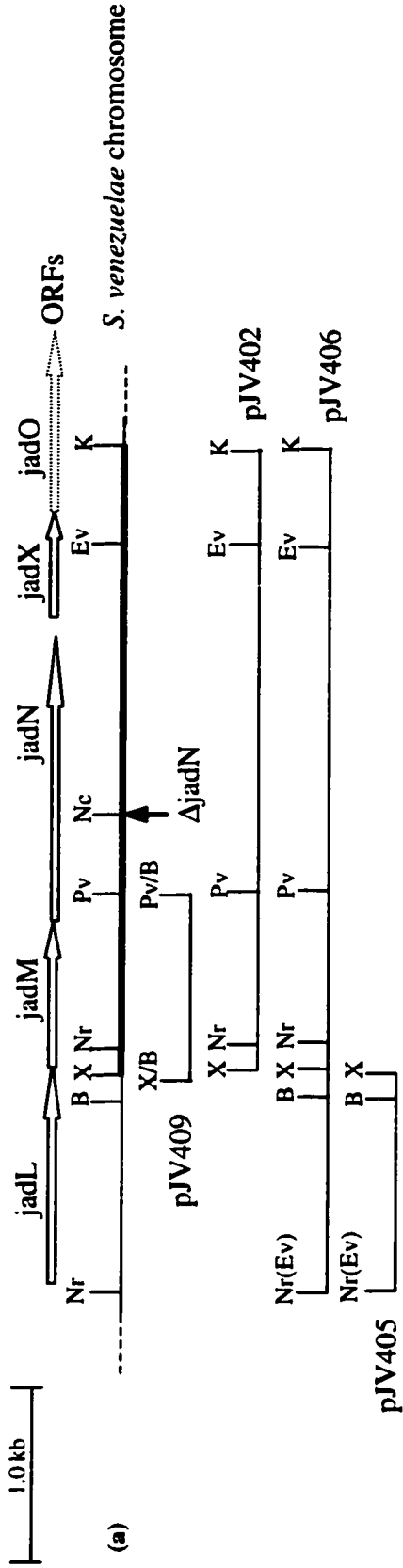
Gels from electrophoresis of 40- $\mu$ g RNA samples were probed with the [ $^{32}$ P]dCTP-labeled 3.4-kb *XhoI-KpnI* insert from pJV402 containing *jadM*. Expression of *jadM* mRNA was strongest 24 h after ethanol supplementation, and was still detectable at 48 h, but could not be detected at 96 h. The JdB titre was highest 48 h after ethanol treatment, as reported by Doull *et al.* (1994), and had fallen to near zero in 72-h cultures. Neither JdB nor *jadM* mRNA was detected at any stage in the growth of cultures not treated with ethanol.

#### **v. Analysis of *jadM*-disrupted mutants**

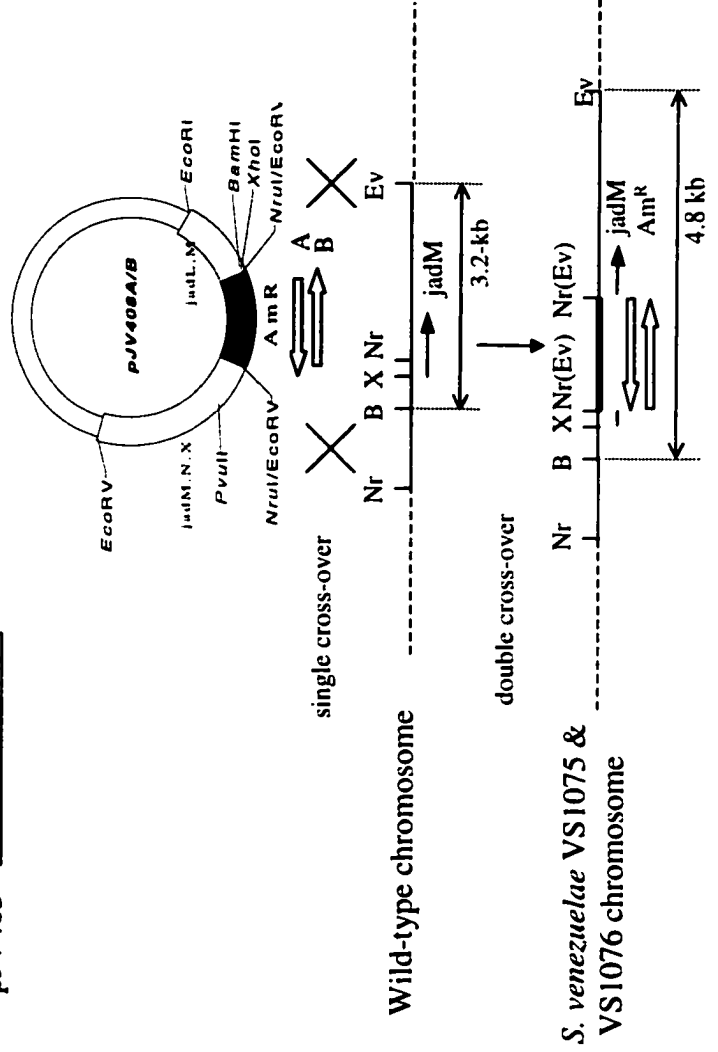
*jadM* was disrupted by inserting the AmR gene in alternative orientations at its *NruI* site (Fig. 21), giving pJV408A/B. Conjugal transfer of pJV408A/B into ISP5230 and selection for allele exchange yielded two independent colonies (VS1075/1076) with the AmR gene in opposite orientations. Genomic DNA for *S. venezuelae* VS1075/1076, and ISP5230 digested with *EcoRV/BamHI* and probed by Southern hybridization with the  $^{32}$ P labeled 3.2 kb *EcoRV-BamHI* fragment of *S. venezuelae* DNA from pJV406 (see Fig. 21) gave a single strong signal at 4.8 kb from VS1075/VS1076, and one at 3.2 kb from *S. venezuelae* ISP5230 (Fig. 22). When the 1.6-kb *apr*-fragment was used to probe the *EcoRV/BamHI* digests, a single strong signal at 4.8 kb was obtained only from the disrupted strains VS1075 and VS1076. These were the results expected for double-crossover mutants. Cultures of VS1075 and VS1076 grew normally on minimal agar. In Gal2I medium optimized for JdB production, the JdB titre was severely reduced (2-5% of the wild-type amount), irrespective of the direction in which the *apr* gene cassette was

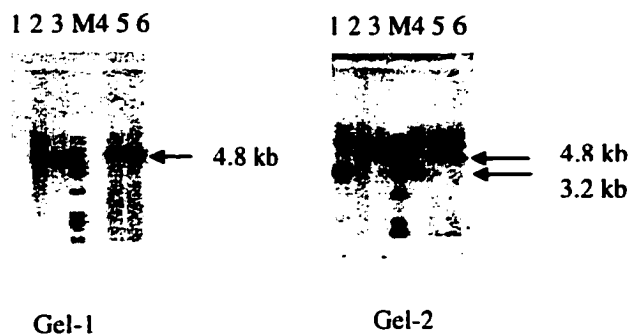
**Figure 21 (a).** Restriction map of the 4.6-kb fragment of *S. venezuelae* ISP5230 genomic DNA subcloned from Lambda LW3. The 3.4-kb *XhoI-KpnI* fragment retrieved from Lambda LW3 and subcloned in pBluescript II SK(+) is shown as a thickened line. Arrows above the restriction map show the locations and orientations of ORFs (*jadM*, *N*, *X*, *O*) in the *jad* cluster. Restriction sites in the pJV402, pJV405, pJV406 and pJV409 inserts are aligned with the chromosomal map. Abbreviations: B, *Bam*HI; Ev, *Eco*Rv; K, *Kpn*I; Nc, *Nco*I; Nr, *Nru*I; Pv, *Pvu*II; X, *Xho*I. Am<sup>R</sup>, apramycin resistance.

**(b).** Disruption of *jadM*. A cassette containing the apramycin-resistance gene in alternative orientations (A or B) was inserted at the *Nru*I site of *jadM* cloned in pJV326. Introduction of the plasmids into *S. venezuelae* gave strains (VS1075 & VS1076) with double crossovers selected for their Am<sup>R</sup> Thio<sup>S</sup> phenotype.



(b)





**Figure 22.** Southern hybridization of genomic DNA digested with *Bam*HI/*Eco*RV. Lane 1 and 4, *S. venezuelae* ISP5230; lane 2, VS1077; lane 3, VS1078; lane M, DNA size marker; Lane 5, VS1075; Lane 6, VS1076. Gel-1 probed with labeled 1.6-kb *Am*<sup>R</sup> gene; Gel-2 probed with labeled 3.2-kb *Bam*HI-*Eco*RV fragment.



inserted. From these results it was concluded that the major role for JadM is in JdB biosynthesis. The small amount produced by *jadM*-disrupted mutants could be due to the activity of a primary metabolic phosphopantetheinyl transferase.

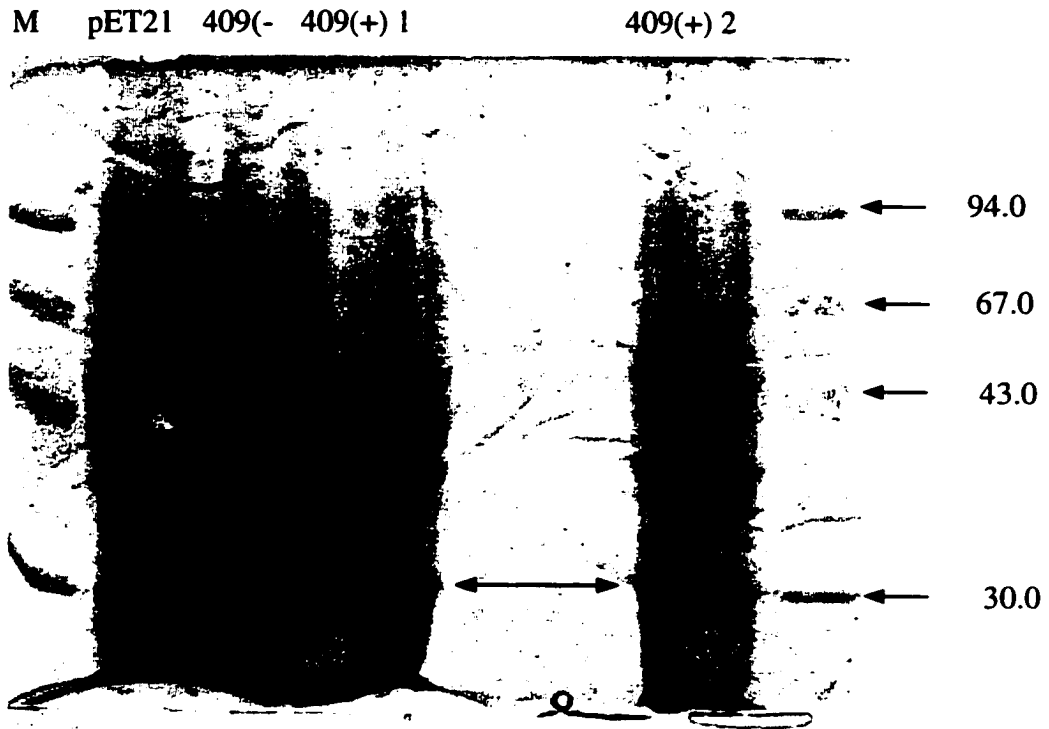
#### **vi. Expression of *jadM* in *E. coli***

Examination of the *jadM* ORF identified possible RBS (AAGG), start codon (ATG) and stop codon (TGA) sequences (see Fig. 18). To determine whether these correctly defined *jadM*, a 1.17-kb *XhoI-PvuII* fragment was cloned in pET 21+. The recombinant plasmid, pJV409, was used to transform *E. coli* BL21 (DE3). The transformant was grown in LB medium, and proteins extracted from the cells were analyzed by SDS-PAGE. The amount of a 29.1 kDa protein increased substantially in extracts of *E. coli* BL21 (DE3) harboring pJV409 (see Fig. 21 for the construction, Fig. 23 for the results). This protein was not detectable in extracts of *E. coli* BL21 (DE3) harboring only the pET 21+ vector, either before or after induction. The results suggested that synthesis of the 29.1-kDa protein depended on the RBS and start codon of *jadM*, expected to be present in transformants carrying pJV409, but absent from transformants carrying only the pET21+ vector.

#### **b. *jadN***

##### **i. Characterization of *jadN***

Analysis of the sequence immediately downstream of *jadM* located a large potential



**Figure 23.** Analysis of *jadM* expression in *E. coli* by SDS-PAGE. Lanes: M, molecular markers (kDa); pET-21(+), *E. coli* BL21 (DE3) transformed with pE-21(+) and induced with IPTG; 409(-), *E. coli* BL21 (DE3) transformed with pJV409 and not induced; 409(+), 1 & 2, two samples of *E. coli* BL21 (DE3) transformed with pJV409 and induced with IPTG. The 29.1-kDa protein is indicated by the double-headed arrow.

ORF, designated *jadN*, with a high third-base G+C content. It was oriented for transcription in the same direction as *jadM*, and two nucleotides of its ATG start codon overlapped the *jadM* stop codon. The ORF was predicted to terminate 1794 bp downstream of its starting ATG. In a BlastP search, the deduced amino acid sequence of *jadN* showed extremely high similarity to many decarboxylases, including malonyl-CoA decarboxylases. The product of *jadN* showed strongest resemblance (81% identical, 88% similar amino acids) to a decarboxylase (*lanP*) of *S. cyanogenus* (AF080235) and to methylmalonyl-CoA decarboxylase (EC 4.1.1.41) of *S. coelicolor* A3(2) (81% identical, 87% similar amino acids; T29065). Other closely related gene products were the putative decarboxylase SimA12 (80% identity; AF324838) from *Streptomyces antibioticus*, the putative decarboxylase PgaI (80% identity; AY034378) in *Streptomyces* sp. PGA64, and the  $\beta$ -subunit of propionyl-CoA carboxylase, (55% identity; NC\_000853) from *Thermotoga maritima*. An NCBI Blast P CDD search located a carboxyl transferase domain in JadN (Fig. 24). This places JadN in a family of biotin-dependent carboxylases with a carboxyl transferase domain that carries out transcarboxylation from biotin to an acceptor molecule, which for all members of the carboxytransferase family is an acyl-CoA.

Malonyl-CoA is one of the most common chain-extender units for the biosynthesis of polyketide antibiotics in streptomycetes (Hopwood & Sherman, 1990), and its use in the synthesis of JdB would be expected. Therefore *jadN* may function as a malonyl-CoA decarboxylase in polyketide condensation reactions leading to JdB. The supply of malonyl-CoA needed for these reactions is potentially available as the product of another

```

      10      20      30      40      50      60
consensus  1 LNKIGMGGGEKAVKPEHPRGKLTARERIDLLDPGSFGELEDLPHRATEFGR---K--R 55
JadN      23 elaqegpdpsgppnastAKGKLTARERIELLLDKGSFTEIEQLRRHRAQGFG---EakK 79
PH1287    14 KKKILQMGGEAIAKKQHEKGLTARERLELLDPGSFVEIGMFVKHRAFGMD--K--R 69
SC1C2.16  25 RAAALAGPSEKATAAQHAKGKLTARERIELLLDPGSFVEVEQLRRHRATGFGLeakK--P 82
PCCASE    32 NHEAVHAGSERAVAKQHAKGKRTARERIDMLLEDEGSFVELDEHARHRSTNFGMDadr--P 89

      70      80      90      100     110     120
consensus  56-LPGDGVVTGSGAIIGRAVEVVAQDFTVFGGSLGPEHGFKITRAMETAIKEGEPLIGIND 114
JadN      80-PYTDGVITGNGTVEGRTVFVYAHDFRIFGGALGEAHATKIHKIMDMAIAAGAPLVSLND 138
PH1287    70eLPADGVITGYGTINGRLVFVYAQDFTVMGSSLGENHANKIKRIMELALEAGAPVIGLND 129
SC1C2.16  83--YTDGVITGNGTVEGRTVFVYAHDFRIFGGALGEAHATKIHKIMDMAIAAGAPLVSLND 140
PCCASE    90--YDGVVTGNGTVDGRRVCFVSQDFTVFGGSLGEVFGKIVKVMDLAMKTGCPLVGIND 147

      130     140     150     160     170     180
consensus 115SGGARIgQEGVENLRGYGLIFGANSRASGVIPQISLVTGPCAGGGAYSPALTDFFIIMVKG 174
JadN     139GAGARI-QEGVSALAGYGGIFQRNTRASGVIPQISVNLGPCAGGAAYSPALTDFFVMVRE 197
PH1287   130SGGARI-qEGVDSLKGYGEIFKNTILSGVVPQITAIMGPCAGGAVYSPAIGDFILMVDN 188
SC1C2.16 141GAGARI-qEGVSALAGYGGIFQRNTRKASGVIPQISVNLGPCAGGAAYSPALTDFFVMVRD 199
PCCASE   148SGGARI-qEGVAALGLYAEIFKRNTHASGVIPQISLINGPCAGGAVYSPAITDFTVMVDQ 206

      190     200     210     220     230     240
consensus 175T-SFMFLTGPDVVIKVTGEEVVTSEELGGADQEMTKSGVAHLTADDDLDALALIRRLLSYL 233
JadN     198T-SQMFITGPDVVKAVTGEETIQNGLGGADVHAGVSGVSHFVYDDEETCLAEVRYLLSML 256
PH1287   189psTFMFITGPDVVKAVTGEVTFVQLGGAMVHAQRSQGAHLIGKSDDEVIMLIKLLSYL 248
SC1C2.16 200t-SQMFITGPDVVKAVTGEETIQNGLGGADVHAETSGVCHFAYDDEETCLAEVRYLLSL 258
PCCASE   207t-SHMFITGPDVIKVTGEDVSPEDLGGARTHNERSGNAHYLATDEDDAISYVKELLSFL 265

      250     260     270     280     290     300
consensus 234PpaSNMEPPPIVPTGDFPDRP-----SIVPDDPNRFPYDVREVIAGIVD--- 277
JadN     257P--QNNRDLPFVMPSSDPADRPGdr-----l1DLVPADGNRSYDVREVIIEELVD--- 303
PH1287   249P--SNnmeKPPRVKPKDEPFRtrp-----elyDIVPDdpnKGYDVRQVIYTIIVDRde 298
SC1C2.16 259P--QNNrenPPRAESSDFVDRrsd-----t11DLVPA dgnRFPYDMTKVIEELVD--- 305
PCCASE   266P--SNnlsSSPVFPGAEEVEEGsvadgvgdadleldALVPDspnQPYDMREVITRLVDE-- 321

      310     320     330     340     350     360
consensus 278--EGSFLETKAGWAKNVVTFARLGGIPVGVVAGQPRVLAGVLDPDSADKAARFIRDCDA 335
JadN     304--DSDYMEVHAAWAPNLVCAFPARLDGHVVGIVANQPAAAGVLDIKASEKGARFVQFCDA 361
PH1287   299ngNPDFLELQPYFAPNAVIGFGRINGQPVGIVANNPIHLAGVLDIDSSDKIARFVRFCD 358
SC1C2.16 306--EGEYLEVHERWARNIICALARLDGRVVGIVANQPQALAGVLDIEASEKAARFVQMCDA 363
PCCASE   322---gEFLEVSALFAPNMLCGFGRIEGASVGVVAGVANNQPMQLAGTLDIDASEKAARFVRFCD 378

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          370      380      390      400      410      420
      .....|.....|.....|.....|.....|.....|
consensus 336FNIPLVILVDVPGFLPGDQQYGGIIRHGAKLLYALAEATVPKITVIPPKLYGGAYVMD 395
JadN      362FNIPIITLVDVPGFLPGVDQEHDGIIRHGAKLLYAYCMATVPRISVVLRKAYGGAYVMD 421
PH1287    359FNIPIVTFVDVPGYLPGVDQESRGIIRHGAKVLYAYAEATVPMVTIILRKAYGGAYLAMG 418
SC1C2.16 364FNIPIITLLDVPGFLPGVDQEHHGGIIRHGAKLLYAYCMATVPRISLILRKAYGGAYVMD 423
PCCASE    379FNIPVLTLLDVPGFLPGTQDWNAIIRHGAKLLYAYAEATVPLVTVITRKAYGGAYDVMG 438

          430      440      450      460      470      480
      .....|.....|.....|.....|.....|.....|
consensus 396SKILGADFMAAWPTARIAVNGPEGAVGIKFRKELAAAAMRRKDLEALRKQKIAEYEEELS 455
JadN      422SRSIGADISLAWPTHEIAVNGAEGAANVFRREIAAAD----DPEAMRAQKIQEYREELV 477
PH1287    419SKHLGAdFVFAWpTAEIAVNGPEGAANIIFRKEIAKAE----NPEEFRRQKIQEYREKFPA 474
SC1C2.16 424SQSIGAdLTYAWpTHEIAVNGAEGAANVIFRRQIADAE----DPEAMRARMVKEYKSELM 479
PCCASE    439SKHLGAdINLAWpTAQIAVNGAQGAANILYRRQLAEAAERGEDVEALRARLQEYEDTLC 498

          490      500      510      520
      .....|.....|.....|.....|.....|
consensus 456 PPYVAAARGFADAVIDPGRTRAKLVIALALLWTKPRFFPWRKHGNI 501
JadN      478 HPYVAAERGLVDDVIDPRETRQVLCRSVAMLAAKHADLPRRKHGNp 523
PH1287    475 NPYVAAARGYIDDVIDPAETRAKIVMALEALENKRVKLPPKHGNI 520
SC1C2.16 480 HPYVAAERGLVDDVIDPAETREVLITSLAMLHTKHADLPSRKHGNP 525
PCCASE    499 NPYVAAERGYVDSVIPPSHTRGHVARALRMLADKREALPAKKHGNI 544

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**Figure 24.** Blast P CDD search of JadN and carboxyl transferases. Bold letters indicate consensus amino acids in the carboxyl transferase domain. PH1287, methylmalonyl-CoA decarboxylase alpha chain in *Pyrococcus horikoshii*; SC1C2.16, putative carboxyl transferase in *S. coelicolor* A3(2); PCCASE, propionyl-CoA carboxylase beta chain in *Sac. erythraea*.

*jad* cluster gene, *jadJ*, that encodes a biotin carboxylase-biotin carboxyl carrier protein able to convert acetyl-coenzyme A to malonyl-CoA (Han *et al.*, 2000).

## ii. Analysis of *jadN*-disrupted mutants

Genomic DNA from *jadN*-disrupted mutants (VS1077/1078), with the *apr*-cassette inserted into *jadN* in opposite orientations, was analyzed by Southern hybridization (see Fig 22). When Southern blots of *EcoRV/BamHI*-digested genomic DNA from *S. venezuelae* ISP5230, VS1077 and VS1078 were probed with the <sup>32</sup>P labeled 3.2 kb *EcoRV-BamHI* fragment of *S. venezuelae* DNA from pJV406, VS1077 and VS1078 gave signals at 4.8 kb, whereas the signal given by *S. venezuelae* ISP5230 was at 3.2 kb. When the 1.6-kb *apr*-fragment was used to probe the *EcoRV/BamHI*-digested genomic DNA samples, the signal at 4.8 kb was obtained only from the disrupted strains. These were the expected hybridization patterns for double-crossover mutants. Since cultures of VS1077/1078 grew normally on minimal agar, *jadN* is not essential for fatty acid biosynthesis. In the optimized fermentation medium for producing JdB, each mutant culture gave only 10-25% of the wild-type JdB titre. Based on this result, *jadN* is presumed to be used in JdB biosynthesis; the residual 10-25% present in *jadN*-disrupted mutants may represent the activity of a primary metabolic enzyme.

## c. *jadX, O, P, Q, S, T, U, V, R\*, Y, Z*

Sequencing and analysis of the inserts in pJV411, JV423, pJV425, and pJV449 detected eleven ORFs (*jadX, O, P, Q, S, T, U, V, R\*, Y, and Z*: see Fig. 17). Their properties are

summarized in Table 2. Ten of the ORFs (*jadX, O, P, Q, S, T, U, V, Y* and *Z*) were transcribed in the same direction; *jadR\** was transcribed in the opposite direction.

#### **i. Characterization of *jadX***

A Blast P search showed only that the sequence of the *jadX* product was 26% identical to that of MmyY (GenBank AJ276673), a protein of undetermined function encoded by a gene in the methylenomycin biosynthesis cluster, and located in the large linear plasmid SCP1 of *S. coelicolor* A3(2) (Kinashi *et al.*, 1987). The *jadX* product also showed similarity to an unknown protein of *Polyangium cellulosum* (GenBank U24241), to a hypothetical protein of *Mycobacterium tuberculosis* (GenBank A70928), and to the product of ORF5 (GenBank AF127374) from *Streptomyces lavendulae*, but as the probability of chance identity in all of these matches was high, the function of the *jadX* product is uncertain.

#### **ii. Characterization of *jadO***

In BlastX searches, the deduced amino acid sequence of *jadO* showed similarity to genes for many 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. However, *JadO* also resembled dTDP-4-keto-6-deoxy-L-hexose 2,3-dehydratase encoded by *aveBVI* of *S. avermitilis* (59% identical aa). The D-olivose, D-oliose and D-mycarose 2,3- dehydratases (*MtmV*) of *S. argillaceus* (51% identical aa), and the products of *lanS*

**Table 2. Features of ORFs downstream of *jadL* in the *jad* cluster of *S. venezuelae***

ORF*	Size of product (aa) #	Distance (bp) from preceding ORF	%G+C	Putative function
<i>jadM</i>	262	26 <sup>#</sup>		PPTase
<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 <sup>†</sup>	73.8	<i>tet-R</i> homologue
<i>jadY</i>	238	15	69.5	dehydrogenase (quinone)
<i>jadZ</i>	exceeds 430		71.4	oxidoreductase

\* Genes are listed in the order their ORFs occur in the *jad* cluster. # The putative start codon for *jadM* was located 26 bp inside *jadL*. The putative start codons for *jadP* and *jadV* are GTG; other ORFs start with ATG. † *jadR\** is transcribed towards *jadV*; The distance between the *jadR\** and *jadV* stop codons is 16 bp.



in *S. cyanogenus* (68% identity), ORF10 in *S. violaceoruber* (47% identity), TylCVI in *S. fradiae* (46% identity) and *eryBVI* in *S. erythraea* (44% identity) were also closely related. Alignment of 2,3-dehydratase amino acid sequences omitting JadO showed four highly conserved areas (regions 1-4 in bold letters, Fig. 25). 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. 25 can participate in the synthesis of two or three different deoxysugars, or of a trideoxyhexose and an aminosugar (Hoffmeister *et al.*, 2000).

### iii. Characterization of *jadP*

The deduced amino 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 *S. 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 *S. galilaeus*, and dTDP-3,4-diketo-2,6-dideoxyglucose 3-ketoreductase (38% identity) used by *S. antibioticus* for C-2 deoxygenation during dTDP-L-oleandrose biosynthesis.

	region 1	region 2
LanS	81 <b>HRSGRFFSVEGLHV</b>	146 <b>QLSPTVQATRSN</b>
UrdS	77 <b>HRSGRFFTVEGLHV</b>	147 <b>QLSPTVQATRSN</b>
AveBVI	80 <b>HRSGRFFSVEGLHV</b>	145 <b>QLSPTVQATRSN</b>
EryBVI	94 <b>HESGRFFSIEGLHV</b>	159 <b>QLSPTLQATRSN</b>
TylCVI	43 <b>HESGRFFSIEGLRV</b>	108 <b>QISPTVQATRSN</b>
Orf10	63 <b>HTSGRFFSIEGLRV</b>	128 <b>QLSPTVQATRSN</b>
MtmV	82 <b>HDSGRFFTVEGLRA</b>	147 <b>QLSPTVQATRSN</b>
JadO	75 <b>PPQRPLLHRRGPAR</b>	141 <b>EPAPAVADRPGR</b>
	. : . *	: : * : .
	region 3	region 4
LanS	408 <b>YEAVHSEEGGR-FLNAESRYLLIE</b>	454 <b>LVRHGHYVNVQARTLLACLNA</b>
UrdS	407 <b>YEAVHSEEGGR-FLNAESRYLIVD</b>	453 <b>LVRHGHYVNVQGR TLLACLNA</b>
AveBVI	403 <b>YAAVHSEEGGR-FLHAQARYLFVE</b>	449 <b>LLRYGRYVNVQARTLLSLLTT</b>
EryBVI	443 <b>YDAWHSEEGGR-FYRNENRYMLIE</b>	488 <b>LLGHSHYVNIQLRSIIACASA</b>
TylCVI	389 <b>YDTVQSEEGGR-FYHARNRYAIVE</b>	440 <b>LLAHGNYVNVVELRTLIIACAH</b>
Orf10	409 <b>YDTLQSEEGGR-FHHAENRYVVVE</b>	455 <b>LLHHSNYVNVVEARSLVACTQA</b>
MtmV	430 <b>YDAVQSEEGGR-FYRALSRYLVE</b>	474 <b>LLQHSNYCNIQARSLIACLQS</b>
JadO	455 <b>YEAVHSEEGGRGFLQTPRAATFLG</b>	511 <b>TTRHGHYLVNVQARTLLACVHA</b>
	* : : * * * * * * . . :	: . . * * : : * : : : :

**Figure 25.** Alignment of JadO and NDP-hexose 2,3-dehydratase sequences by Clustal W. Highly conserved amino acids are in bold letters, 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 *S. cyanogenus*; UrdS: *urdS* in *S. fradiae*; AveBVI: *aveBVI* in *S. avermitilis*; EryBVI: *eryBVI* in *Sac. erythraea*; TylCVI: *tyl-orf6* in *S. fradiae*; MtmV: *mtmV* in *S. argillaceus*; Orf10: *orf10* in *S. violaceoruber* Tü22; JadO: *jadO* in *S. venezuelae*.

#### iv. Characterization of *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 *S. griseus* (75% identical aa). Other relevant gene products were glucose-1-phosphate thymidyltransferase of *S. avermitilis* (71% identical aa), dTDP-1-glucose synthase of *S. galilaeus* (70% identical aa),  $\alpha$ -D-glucose-1-phosphate thymidyltransferase of *S. venezuelae* ATCC15439 (67% identical aa) and glucose-1-phosphate thymidyltransferase of *S. 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 and Thorson, 1994; Kirschning *et al.*, 1997). In an alignment of *JadQ* with NDP-hexose phosphate transferases (Fig. 26), these regions (marked with bold letters) can be recognized at two locations. The region near the N-terminus is present in all sequences, and is associated with activator binding. The 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 (bold letters in Fig. 26; Katsube *et al.*, 1991; Thorson *et al.*, 1994). The difference in location is consistent with the role of *AscA* in catalyzing the first step in biosynthesis of a 3,6-dideoxysugar instead of a 2,6-analogue. That pathways to deoxysugars may be initiated with specific NDP-sugars is indicated by the presence in *S. 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).

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UrdG      MKALVLAGGSGTRLRPFYSMPKQLIPIANTFVLVHVLNRNLRELGVTEAGVIVG-NRGPE 59
LanG      MKALVLSGGSGTRLRPFSSMPKQLIPIANKPVLEHVLNRNVRDLGVTEIGVIVG-DRAEQ 59
LanZ2     MKALVLSGGTGTLLRPLTHSLPKQLIPLAGRPFVVAHVLDSDVRDLGVRETGIVVT-DGGEQ 59
Orf8      MKALVLAGGSGTRLRPIHTSAKQLVAVANKPVLFYGLEAIAAAGITDVGLIVG-DTAGE 59
StrD      MKALVLAGGTGTLLRPIHTSAKQLVAVANKPVLFYGLEAIRAAGIIDVGIVVG-DTAGE 59
AveBIII   MKGIVLAGGTGSRLYPLTRALSKQLLPVYDKPMIYYPLSVLMLGGIKDILVISSPDHLEQ 60
JadQ      VKGIFLAGGHGTLLRHPITLGTSKQMLFPVYDKPMIYYPLSVLMLAGIRDIQIISSPDDIEN 60
AscA      VKAVILAGGLGTLLSEETVVKPKPMVEIGGPKILWHIMKLYSSYGINDFVICCG-YKGYV 59
          :*:.*:** **:* : . * :: : . *:: : : *:: : : *:: : :

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UrdG      ISAVLGDGAIEFGMRVYIIPQDAPRGLAHTVAITRDFLCDDDFVMYLGDNMLPDGVVDMRG 119
LanG      IQAVIGDGSALGVEITYIPQEAPLGLAHCVELARPFLGDDDFVMYLGDNMLPEGVERIAD 119
LanZ2     IEQALGDGSRGSLSTYFRQDTPRGFGHALSLARDFLGDDDFVYRGDTLVTESIADRAA 119
Orf8      VRAAVGDGAKFGLDITYIEQSRPLGLAHAVLIAHTYLGDDDFVMYLGDNFIVGGIDDLVR 119
StrD      IVAAVGDGSRFGLKVSYPQSKPLGLAHCVLISRDFLGEDDFIMYLGDNFVVGVEDSVR 119
AveBIII   FRRLLDGDSRLGLNIDYAAQQRPGGIAEAFBIGADFIGQDQVSLVLGDNIFHGMGFSHLL 120
JadQ      FRRLLDGDSPLGISLSYAVQEQPRGLAEAFILISADHIGDSDVALVLGDNIFHGPFGAGIL 120
AscA      IKEYFANYFMHMSDITFCMRDN-----EMVVHQKRVPEWNVTLVDTGEDSMTGGRLRRVK 114
          . . : : : : . . : : * : .

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UrdG      GIHRPS-AGGQVVVYKVPDRSFGVAELGPGQEVLRVLEKPRDPRSDMALVGVYFFTAAI 178
LanG      EFTAGR-PAAQVVVHKVPDPRAFGVAELAADGRVERLVLEKPEPKSDLALIGVYFFTPAI 178
LanZ2     AFAAER-PAAGVVVP-----EAAPDVELV--HFFTPAV 149
Orf8      TFRDGRPPAARILLTHVSDPSAFGVAELDDDDGRVVGLEEKPRHPKSDLALVGVYFFTPAI 179
StrD      EFRAAR-PDAHMLTRVPEPRSFGVAELSDSGQVLGLEEKPAHPKSDLALVGVYLFSPA 178
AveBIII   RSHTRDVGCVLFGYAVTDPERYGVGEVDASGKLLSVEEKPTAPRSNLAITGLYLYDNDV 180
JadQ      QDKAVDVGCVLFGYPVRDPERYGVGEVDADGRLVLSLEEKPEHPRSDLAITGLYFYDNDV 180
AscA      DYVKDDEAFCTYGDGVSD-----VNIAELIAFHKSHGK 148
          : : .

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UrdG      HDAAVAIEPSARG-ELEITD-AIQWLVSAGDVRASQYDGYWKDTGNVEDVLECNRHLLD 236
LanG      HKAAVAIEPSARG-ELEITD-AIQWLVANGDRFKANEYKGYWKDTGRIEDVLECNRKLLD 236
LanZ2     HEAAVAALADRPGDAVTPAG-AAQWLRAGGGDVRTGTYTGYWRRIASAEDVLAHRTLLD 208
Orf8      HEAVRAIEPSWRG-ELEITH-AIQHLIDNGADIQSMVIEGYWKDTGNVADMLEVNRTVLE 237
StrD      HEAVAAITPSWRG-ELEITD-AVQWLIDAGRDRVSTVISGYWKDTGNVTDMLEVNRLVLE 236
AveBIII   IEVARGIRSSARG-ELEITD-VNRAYLAEGRARLVDLGRGFTWLDAGTHDSLHAGQYVQ 238
JadQ      IDIAKNLTPSARG-ELEITD-VNRIYLERGKAEVLSLGRGFVWLDAGTHDALTEAGQYVQ 238
AscA      QATLTATYPPGRFGALDIKDKQVRSFPEKPKGDGALINGGYFVLSPKVIDLIDGDKSTWE 208
          . : : * : * :

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**Figure 26.** Alignment of the sequences of JadQ and NDP- hexose phosphate nucleotidyl transferases. UrdG: *urdG* in *S. fradiae* Tü2717 ; LanG and LanZ2: *lanG* and *lanZ2* in *S. cyanogenus* S136; Orf8: *orf8* in *S. violaceoruber* Tü22; StrD: *strD* in *S. griseus*; AveBIII: *aveBIII* in *S. avermitilis*; AscA: *ascA* in *Yersinia*. Bold letters mark the highly conserved activator-binding and substrate-binding regions. Highly conserved amino acids are identified below the alignment as in Fig. 25.

#### **v. Characterization of *jadS***

The deduced amino acid sequence of *jadS* matched the sequences 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 antitumor agent elloramycin. The *JadS* sequence also resembled (35-42 % identical aa) those of glycosyltransferases used in the biosynthesis of nogalamycin by *Streptomyces nogalater*, of the angucycline antibiotics landomycin and urdamycin by *Streptomyces fradiae*, of daunorubicin by *Streptomyces peucetius*, and for transfer of forosamine to the aglycone by *Saccharopolyspora spinosa*. Genes for glycosyltransferases have been identified in a number of clusters for polyketide antibiotic biosynthesis. Where examined, the gene products catalyzed 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 (Westrich *et al.*, 1999; Kunzel *et al.*, 1999). Since *JadS* closely resembles *UrdGT2*, *DnrS* and *OleGI*, it is probably a family-1 glycosyltransferase acting by the characteristic mechanism that inverts configuration at the reaction site (Campbell *et al.*, 1997).

#### **vi. Characterization of *jadT***

The *jadT* product showed 68% sequence identity to the TDP-glucose-4,6-dehydratase used in streptomycin biosynthesis by *S. 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 *S. cyanogenus*, *S. fradiae*, *S. galilaeus*, *S. violaceoruber*, *Sac. erythraea*, and *S. avermitilis*. In actinomycetes, genes for 4,6-dehydratases are more highly conserved than those for glycosyltransferases (Liu and Thorson, 1994), and fragments of *strD*, *E*, *L* and *M* from *S. 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).

#### **vii. Characterization of *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 *S. galilaeus*. It also resembled the putative epimerase (58% identical aa) used by *S. peucetius* to form the daunorubicin precursor thymidine diphospho-L-daunosamine, the NDP-4-keto-6-deoxy-epimerase (58% identical aa) used in by *S. avermitilis* for avermectin biosynthesis, and the TDP-4-keto-6-deoxyhexose 3,5-epimerase (56%

identical aa) used by *M. megalomicea subsp. nigra* for biosynthesis of the anti-parasitic agent megalomicin.

#### **viii. Characterization of *jadV***

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

#### **ix. Characterization of *jadR\****

The deduced amino acid sequence of the 612-bp ORF (*jadR\**) downstream of *jadV*, but transcribed in the opposite direction, showed similarity in a BlastP search of GenBank to TetR-family transcriptional regulators. These included TetR of *S. coelicolor* A3(2) (27% identical aa; AL355832), TetR/AcrR-like proteins encoded by *yixD* and *yhgD* of *B. subtilis* (24% identity; NC-000964), PA1226 of *Pseudomonas aeruginosa*, and putative TetR regulatory proteins in *Prauserella rugosa* and *Mycobacterium leprae*. A domain present in these bacterial regulatory proteins can be recognized in the N-terminal region of *JadR\**, together with a helix-turn-helix motif similar to that in DNA-binding regions of bacteria (Fig. 27).

		10	20	30	40	
		.....*	.....*	.....*	.....*	.....*
consensus	1	<b>ILDAALELFAER</b> GYDATT <b>VREI</b> AKEAGV <b>SKGALYR</b> HFPSKEELL <b>LAL</b>				47
jadR*	22	<b>ILDALYEQLRSAPSEAVGLDRIARTAGVSRSTVY</b> TFGSRSG <b>LDAL</b>				68
1BJY_A	8	<b>VIDAALELLNETGIDGLTTRKLAQKLGIEQPTLY</b> WHVKNK <b>RALLDAL</b>				54
SC7H1.26	76	<b>ILATARRELG-RNPD-TTLEELARAAGVVRRTLFG</b> HFP <b>GRAALLEAL</b>				120
RV2250C	45	<b>ILRAAASCVV</b> DYGVDR <b>VTLAEIARRAGVSRPTVYR</b>				91
SC7H1.21	23	<b>VLLAAGDALAADGFDALDLGEIARRAGVGKTTVYR</b>				69
Rv0067c	19	<b>VLEVAYQTF</b> AA <b>DGLS-VFVDEIARRAGVGAGTVYR</b>				64
BETI	14	<b>LVDAALRVIGDQGT</b> LAV <b>TMSEIARTAGVSPALAH</b> YFGSKEQL <b>LIAT</b>				60
Rv1219c	12	<b>IREAAIEQFGRHGFGVG-LRAIAEAAGVSAALVIH</b> FGSKEGL <b>RKAC</b>				57

**Figure 27.** A Blast P CDD search of JadR\* and TetR proteins. Bold letters indicate the TetR family domain; underlined bold letters represent helix-turn-helix region. The proteins are: 1BJY\_A, *Escherichia coli* chain A; SC7H1.26, an unknown protein of *Streptomyces coelicolor* A3(2) containing a helix-turn-helix motif at aa 91-112; RV2250C, hypothetical transcriptional regulator of *Mycobacterium tuberculosis* containing a DNA-binding region at aa 62-81 (probable helix-turn-helix motif); SC7H1.21, a putative transcriptional regulator of *S. coelicolor* A3(2); Rv0067c, a possible transcriptional regulator of *M. tuberculosis* H37Rv with a probable helix-turn-helix motif at aa 35 to 56; BETI, a regulatory protein of *Sinorhizobium meliloti*, with a DNA-binding region (helix-turn-helix motif) at aa 31..50; Rv1219c, a hypothetical protein of *M. tuberculosis* H37Rv, with a helix-turn-helix motif at aa 28-49.



Tetracycline repressors participate in the most widespread resistance mechanism against this antibiotic in Gram-negative bacteria. They are homodimeric proteins regulating resistance at the transcriptional level (Orth *et al.*, 1999). The helix-turn-helix region is important for inducibility by tetracycline (Berens *et al.*, 1997).

#### **x. Characterization of *jadY* and *jadZ***

In the sequenced region beyond *jadR\**, a complete ORF designated *jadY*, and a partial ORF (*jadZ*) were detected. They were transcribed in the opposite direction from *jadR\**, and were thus oriented in the same direction as most of the ORFs in the *jad* cluster. The deduced amino acid sequence of *jadY* showed strongest similarity (53% identity) to a putative reductase of *S. coelicolor* A3(2) (AL132824), but close resemblance also to a hypothetical protein of *Brevibacterium linens* (46% identity, AF030288), and to a probable reductase of *Mesorhizobium loti* (36% identity, NC\_002678). A BlastP CDD search showed an NAD(P)H dehydrogenase (quinone) domain close to the N-terminal region of JadY (Fig. 28). Proteins with this feature are primarily bacterial and eukaryotic enzymes (EC:1.6.99.2) catalyzing NAD(P)H-dependent two-electron reductions of quinone redox cofactors, and protect cells against damage by free radicals and reactive oxygen species. The enzymes also use FAD as a co-factor.

The deduced amino acid sequence of part of a large ORF (*jadZ*) detected downstream of *jadY* was notably similar to an oxidoreductase-like protein of *Streptomyces aureofaciens* (49% identity, AY033994), to a polyphenol oxidase of *Acremonium murorum* (35%

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          10      20      30      40      50      60
    .....*.....|.....*.....|.....*.....|.....*.....|.....*.....|.....*.....|
consensus  1  LLLAGAIHPRSSSESKINKTLLDLAKENFPVLTHELYKDYPDPSIDVAE-QEKLLLEADLI 59
JadY      59  veeaaagrHEAIKAGGATVELVDIAEFGLPLLDESVPAAFGQYANEHTKRwSEKIASFDAP 118
IQBG_A   39  DLYAMNFNPIISRKDITGKLKDPANFQYPAESVLAYKEGH-LSPDIVAE-QKKLEAADLV 96
IQR2_A   40  DLYAMNFEPRATDKDITGTLSNPEVFNYGVTHEAYKQRS-LASDITDE-QKKVREADLV 97
mda66    5  LIINGAKAFGSSGGKLNETLTDHAKRTLESLGLEVDTTIVdKGYEHAQE-VEKVFSADAT 63

          70      80      90      100     110     120
    .....*.....|.....*.....|.....*.....|.....*.....|.....*.....|.....*.....|
consensus  60  VFQFPLNWYSVPALLKGWIDRVLTHG----FAYG-----PGGNYLKGGGLM-GKKLMLSI 108
JadY     119  VFVTPEYNRSVPAALKNAIDYlyaewqnksagfvsygvqggtraidhlrvtlaevkvadv 178
IQBG_A   97  IFQFPLQWFGVPAILKGWFERVFIGE----FAYT-----YAAMYDKGPFR-SKKAVLSI 145
IQR2_A   98  IFQFPLYWFSVPAILKGWMDRVLCQG----FAFD-----IPGFYDSGLLQ-GKLLALLSV 146
mda66    64  INQMPGWNMGEPWIVKKYIDEVFSVGhgklyASDgrssqnPTKNYKGGGLMqGKKYMLSL 123

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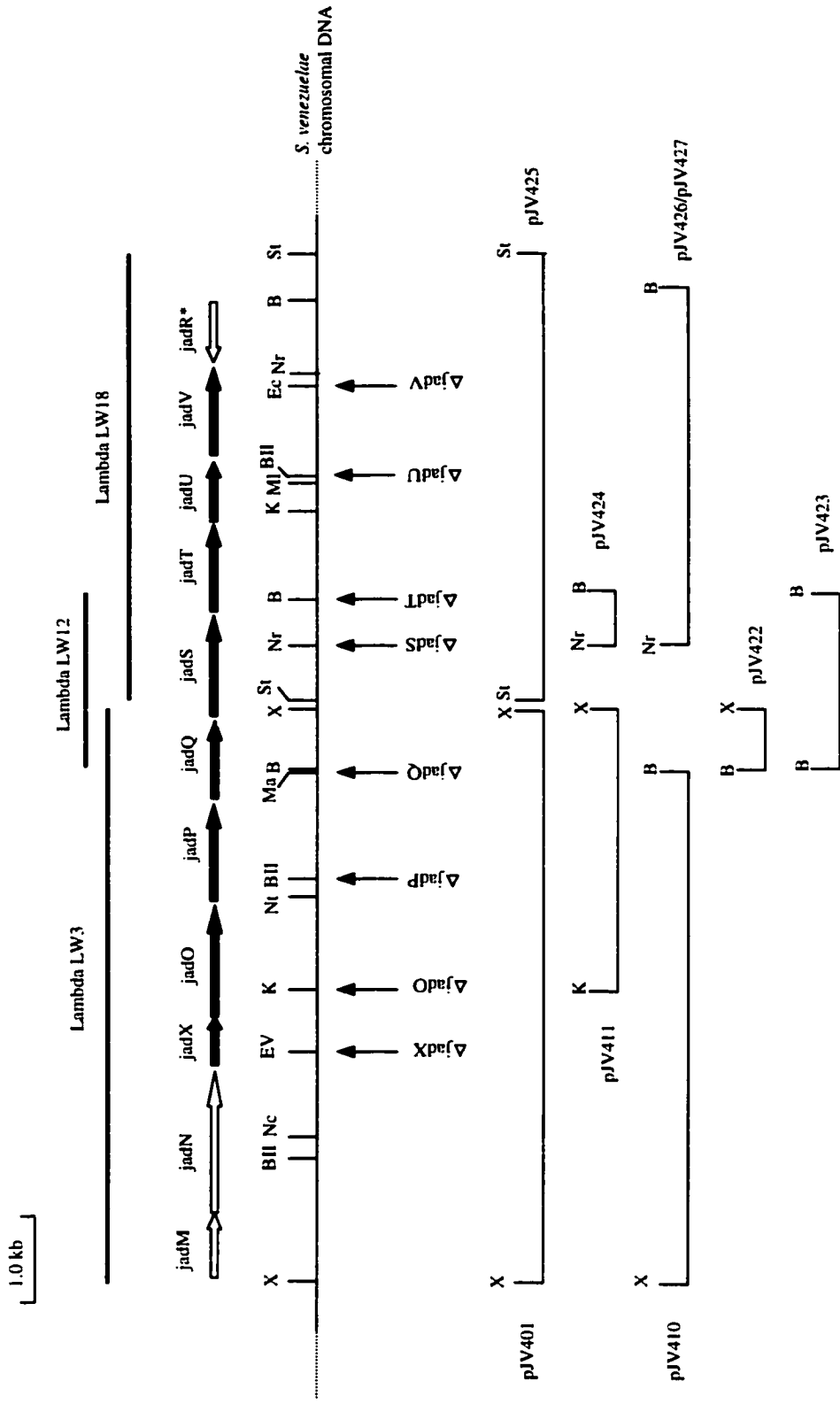
**Figure 28.** Results of a Blast P CDD search of JadY and proteins containing an NAD(P)H dehydrogenase (quinone) domain. IQBG\_A, chain A of human dt-diaphorase NAD(P)H oxidoreductase of *homo sapiens*; IQR2\_A, chain A, human quinone reductase type 2; mda66, a drug activity modulator of *Helicobacter pylori* 26695.

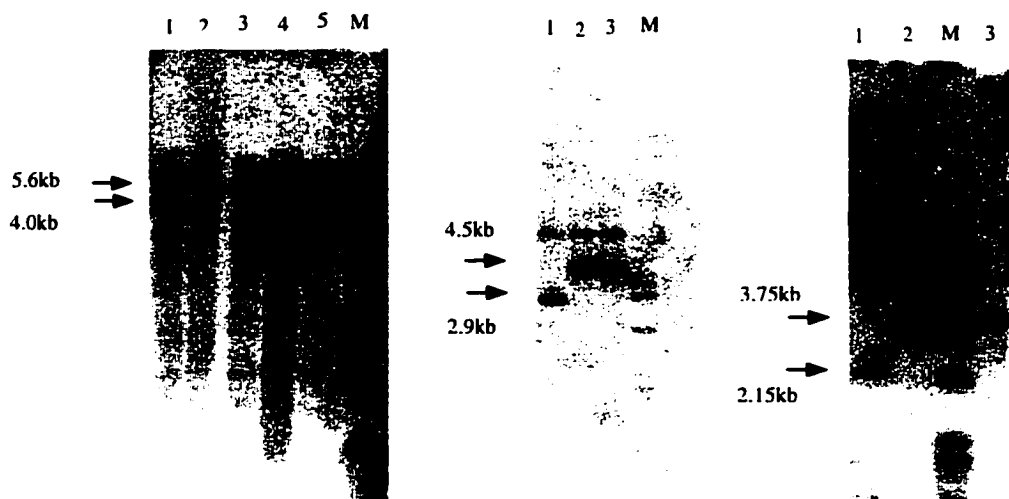
identity, AJ271104), to a spore coat outer protein of *B. subtilis* (33% identity, NC\_000964), and to a CotA protein involved in brown pigmentation during sporogenesis of *B. subtilis* (33% identity, U51115). These results suggested genes *jadY* and *jadZ* are involved in electron transport pathways of primary metabolism, and thus that *jadR\** is the last gene at the right-hand end of the *jad* cluster.

#### **xi. Analysis of mutants disrupted in *jadX*, *O*, *P*, *Q*, *S*, *T*, *U* or *V***

The *jadX*, *O*, *P*, *Q*, *S*, *T*, *U* and *V* ORFs were each disrupted by inserting the Am<sup>R</sup> gene in its two possible orientations in their *EcoRI*, *KpnI*, *BstEII*, *MaeII*, *NruI*, *BamHI*, *BstEII* and *EcoR57I* sites, respectively (Fig. 29). Genomic DNA from *S. venezuelae* ISP5230 and from the *jadX*, *O*, *P*, *Q*, *S*, *T*, *U* and *V*-disrupted mutants (VS1079, VS1081, VS1083, VS1085, VS1087, VS1089, VS1091, and VS1093, respectively) was analyzed by Southern hybridization. Probing genomic DNA digests from VS1079, VS1081, VS1083 and VS1085 with the 4.0-kb *NcoI*-*BamHI* fragment from Lambda LW3 (see Fig. 29) gave a hybridizing band at 4.0 kb from *S. venezuelae* ISP5230, and at 5.6 kb from VS1079, 1081, 1083, and 1085, in which *jadX*, *O*, *P*, and *Q*, respectively, had been disrupted (Fig. 30a). When genomic DNA from *S. venezuelae* ISP5230, VS1087 and VS1089 was probed with the 2.9-kb *NruI* fragment from Lambda LW18 (see Fig. 29), a hybridizing band was located at 2.9 kb from *S. venezuelae* ISP5230, and at 4.5 kb from the strains disrupted in *jadS* or *jadT* (Fig. 30b). Genomic DNA from *S. venezuelae* ISP5230, VS1091 and VS1093 probed with the 2.15-kb *MluI*/*BamHI* fragment from

**Figure 29.** Disruption of sugar genes in the *jad* cluster. The restriction map shown the *S. venezuelae* ISP5230 genomic DNA subcloned in recombinant lambda vectors LW3, LW12, and LW18. Arrows above the restriction map locate and orient ORFs (*jadM,N,X,O,P,Q,S,T,U,V,R\**) in the *jad* cluster. Filled arrows represent genes involved in biosynthesis or attachment of the deoxysugar in JdB. Unfilled arrows represent *jad* cluster genes flanking the deoxysugar pathway genes. Vertical arrows below the restriction map point to sites where an apramycin resistance 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, pJV426 are aligned below the map. Abbreviations: B, *Bam*HI; BII, *Bst*EII; Ec, *Eco*R57I; Ev, *Eco*RV; K, *Kpn*I; Ma, *Mae*II; Ml, *Mlu*I; Nc, *Nco*I; Nr, *Nru*I; Nt, *Not*I; St, *Stu*I; X, *Xho*I.





**Figure 30.** Southern hybridization of *S. venezuelae* genomic DNA

(a) digested with *NcoI/BamHI* and probed with the 4.0-kb *NcoI/BamHI* fragment from Lambda LW3 (see Fig. 29). Lane 1, *S. venezuelae* ISP5230; lane 2, VS1079; lane 3, VS1081; lane 4, VS1083; lane 5, VS1085; lane M, DNA size markers.

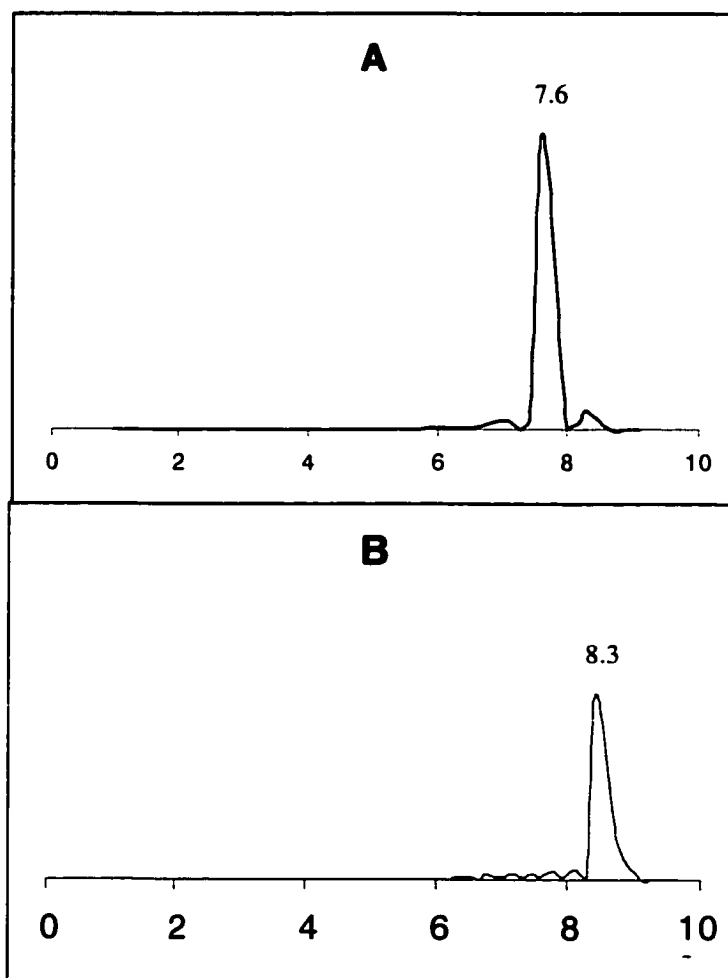
(b) digested with *NruI* and probed with the 2.9-kb *NruI* fragment from Lambda LW18 (see Fig. 29). Lane 1, *S. venezuelae* ISP5230; Lane 2, VS1087; Lane 3, VS1089.

(c) digested with *MluI/BamHI* and probed with the 2.15-kb *MluI/BamHI* fragment from Lambda LW18. Lane 1, *S. venezuelae* ISP5230; Lane 2, VS1091; lane 3, VS1093. The fragment at 5.6 kb in lane 1 is ascribed to incomplete digestion of the 5.6-kb *BamHI* fragment by *MluI* (see Fig. 29).

Lambda LW18 (see Fig. 29) gave a hybridizing band at 2.15 kb with DNA from *S. venezuelae* ISP5230, but at 3.75 kb with DNA from the strains carrying disrupted *jadU* or *jadV* (Fig. 30c). These are the results expected from double cross-overs disrupting *jadX*, *O*, *P*, *Q*, *S*, *T*, *U* and *V*. Cultures of each *jad*-disrupted transconjugant strain grew normally on MYM agar and in jadomycin-production medium. Analysis of culture extracts by HPLC, and comparison with JdB 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 (Fig. 31). With the conditions used for HPLC, the retention time for JdB was 7.6 min, whereas for the aglycone it was 8.4 min. The pooled extracts (omitting those from *jadX*) were purified by flash chromatography on silica gel H and examined by  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectroscopy (Table 3). The major signals corresponded in chemical shift values to the resonances in  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectra published for jadomycin aglycone (Ayer *et al.*, 1991). Cultures of *jadX*-disrupted mutants with the apramycin-resistance gene in either orientation accumulated both JdB and the aglycone; the amounts of each product were similar, but each represented only 10-20% of the JdB 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.

## **xii. Investigation of a potential polar effect**

To determine whether failure to make the dideoxysugar or to transfer it to jadomycin aglycone after an insertional inactivation event was due to a polar effect preventing expr-



**Figure 31.** HPLC analysis of culture extracts from *S. venezuelae* strains. (A), ISP5230; (B) VS1082 (disrupted in *jadO*). Strains VS1083, VS1085, VS1087, VS1089, VS1091 and VS1093 gave results similar to VS1082. The X axis shows retention time (min).



**Table 3. Jadomycin signals detected by  $^{13}\text{C}$  and  $^1\text{H}$  NMR spectroscopy\***

Position	$^{13}\text{C}$ NMR <sup>a</sup> $\delta$ (ppm)	$^1\text{H}$ NMR <sup>b</sup> $\delta$ (ppm)
1	62.7	5.29 (d, J = 2.8 Hz)
2	170.0	
3a	87.2	6.14 (s, 1H)
3b	129.9 <sup>c</sup>	
4	114.5	6.80 (bs, 1H)
5	143.4	
6	120.9	6.89 (bs, 1H)
7	154.1	
7a	111.1	
7b	122.1 <sup>c</sup>	
8	183.5	
8a	133.1	
9	120.9	7.79 (apparent d, splitting of 7 Hz, 1H)
10	137.7	7.65 (apparent t, splittings of 8.3 & 7.7 Hz, 1H)
11	124.4	7.23 (apparent d signal, obscured by $\text{CHCl}_3$ )
12	162.0	
12a	113.4	
13	185.8	
13a	142.2	
1'	40.0	1.81 (m, 2H) (H1' & H2')
2'	25.3	1.55 (m, 1H)
3'	11.9	1.02 (t, J = 7.2 Hz, 3H)
4'	13.8	0.43 (d, J = 6.7 Hz, 3H)
5'	21.3	2.36 (s, 3H)
7-OH		10.16 (s, 1H)
12-OH		11.70 (s, 1H)

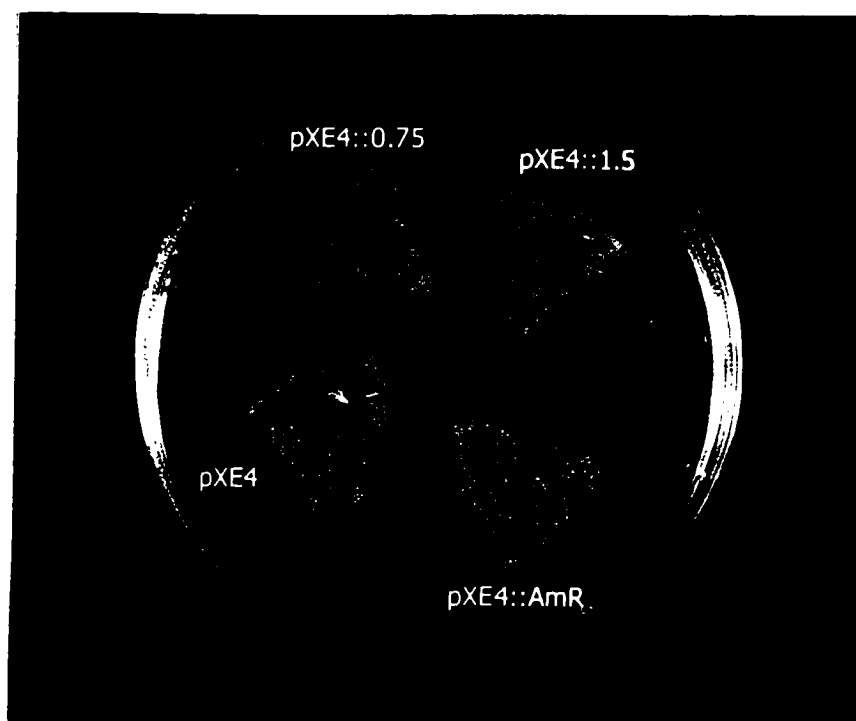
\* see footnotes on following page.

-expression of downstream genes, the promoter probe vector pXE4 containing *xylE* was used. The *xylE* gene product is a catechol dioxygenase, which converts its colorless substrate to the intensely yellow hydroxymuconic semialdehyde. The promoterless copy of *xylE* present in the bifunctional vector pXE4 (Ingram *et al.*, 1989; see Fig. 15) can be expressed only if DNA containing a promoter is inserted between the *Hind*III and *Bam*HI sites in front of *xylE*. The level of *xylE* expression driven by the inserted promoter reflects intracellular RNA levels, and can be assessed by the intensity of the yellow color generated when catechol is provided as a substrate. It was used here to determine whether disruption of *jadS* (encoding glycosyl transferase) in the *jad* cluster of *S. venezuelae* by inserting an Am<sup>R</sup> gene prevented expression of downstream genes by a polar effect. Since construction of the Am<sup>R</sup> cassette in pJV225 (Chang *et al.*, 2001) from the *E. coli* plasmid pKC203 (Rao *et al.*, 1983; Kaster *et al.*, 1983) retained the Am<sup>R</sup> 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. On LB agar, colonies of *E. coli* transformed with pXE4::*apr*<sup>r</sup> (but not controls transformed with pXE4) became bright yellow when sprayed with 0.5 M catechol (Fig. 32). Similar results were obtained on MYM agar with colonies of *S. venezuelae* ISP5230 (those transformed with pXE4::*apr*<sup>r</sup>, but not controls transformed with pXE4, became bright yellow when sprayed with

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Footnotes for Table 3.

<sup>a</sup> Referenced to CDCl<sub>3</sub> at  $\delta$  77.0 ppm; <sup>b</sup> referenced to CHCl<sub>3</sub> at  $\delta$  7.24 ppm; <sup>c</sup> tentative assignments of Ayer *et al.* interchanged. The jadomycin sample (approx. 1.1 mg) was dissolved in CDCl<sub>3</sub>; <sup>1</sup>H (400.1 MHz) and <sup>13</sup>C (100.6 MHz) NMR spectra were acquired on a Bruker AMX 400 spectrometer.



**Figure. 32.** Patch cultures of *E. coli* transformed with pXE4, pXE4::*apr'*, pXE4::0.75, and pXE4::1.5. The inserts in the latter two plasmids were 0.75 and 1.5-kb DNA fragments from the region between *jadR*\* and *jadY* cloned in pJV456; they are oppositely oriented in the pXE4 vector (see Fig. 16)

0.5 M catechol). Mycelial extracts from shaken cultures of the pXE4 transformants grown in MYM medium averaged 0.7 ( $\pm 0.6$ ) mU mg protein<sup>-1</sup> of catechol 2,3-dioxygenase activity, comparable to the 1-6 mU mg protein<sup>-1</sup> reported in *S. lividans* and *S. coelicolor* A3(2) mycelium carrying pXE4 (Ingram *et al.*, 1989), and contrasting with the 19.0 ( $\pm 1.2$ ) mU mg protein<sup>-1</sup> in extracts from cultures of pXE4::*apr*<sup>r</sup> transformants. Extracts from cultures of the pXE4::*apr*<sup>r</sup> transformants in Gal2I medium gave 28.6 mU mg protein<sup>-1</sup>. This level of catechol dioxygenase activity implied expression of *xylE* from the Am<sup>R</sup> gene promoter.

### **xiii. Complementation of dysfunctional genes by merodiploid construction.**

To verify that failure to produce JdB after insertional inactivation of *jadS* was due to the mutation in *jadS* rather than 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 *S. venezuelae* strain VS1087, in which *jadS* had been inactivated by insertion of the oppositely oriented Am<sup>R</sup> gene. A transconjugant selected for thiostrepton resistance (indicating the presence of free or integrated pJV427) was grown in Gal2I medium under conditions favourable for JdB production, but produced only jadomycin aglycone. The results implied that introducing intact copies of *jad* genes downstream of a disrupted *jadS* behind the

functional promoter in pJV326 did not restore JdB biosynthesis. They were consistent with the results obtained from disruption of *S. venezuelae* with the Am<sup>R</sup> 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 were 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<sup>R</sup> gene. Selection of thiostrepton-resistant transconjugants and HPLC analysis of extracts from cultures grown in Gal2I medium showed that all three transconjugants produced JdB exclusively, implying complementation of the disrupted genes by expression of the intact copies in pJV427.

#### **xiv. Disruption of *jadR*\***

A 0.75-kb fragment of DNA was excised from *jadR*\* cloned in pJV456 by digesting the plasmid with *NruI/BamHI*, and the 1.5-kb Am<sup>R</sup> gene from pJV225 was inserted in its place to form pJV457. Conjugal transfer of the construct into *S. venezuelae* ISP5230 gave the *jadR*\*-disrupted mutant VS1107. Southern hybridization of *KpnI/StuI* digested-genomic DNA from *S. venezuelae* VS1107 and ISP5230 with a <sup>32</sup>P labeled 3.3-kb *KpnI-StuI* fragment from pJV425 detected hybridizing bands at 3.3 and 4.1-kb in the ISP5230 and VS1107 digests, respectively. This was the result expected for a double cross-over replacing wild-type *jadR*\* with the disrupted allele constructed as described. Cultures of VS1107 grew normally on MYM agar and sporulated well. When they were grown in

Gal2I medium, the cultures produced 150% as much JdB as did ISP5230, indicating that an intact *jadR\** product represses JdB biosynthesis.

#### xv. Expression of *xylE* from the region upstream of *jadR\**

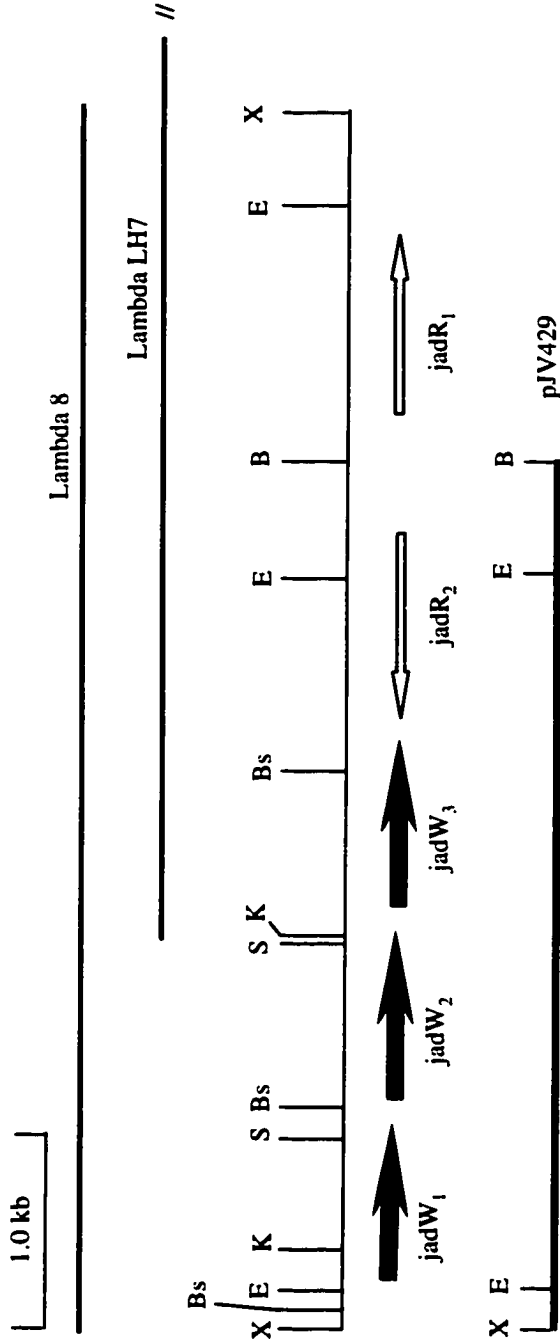
To determine whether the chromosome upstream of *jadR\** contained sequences acting as promoters of *jadR\** transcription, the region was cloned as 0.75-kb and 1.5-kb fragments in the promoter probe vector pXE4, giving pXE4::0.75 (pJV461) and pXE4::1.5 (pJV462), respectively (see Fig. 16). Colonies of *E. coli* transformed with pJV461 or pJV462 (but not the control transformed with pXE4) became bright yellow when sprayed with 0.5 M catechol (see Fig. 32). A similar response was obtained with *S. venezuelae* ISP5230 transformed with pJV461 or pJV462. Mycelial extracts from shaken cultures of the pXE4 transformants grown in Gal2I medium averaged 0.7 ( $\pm 0.6$ ) mU mg protein<sup>-1</sup> of catechol 2,3-dioxygenase activity, whereas extracts from cultures of pJV461 and pJV462 transformants gave 14.7 ( $\pm 1.2$ ) and 13.1 ( $\pm 1.0$ ) mU mg protein<sup>-1</sup> respectively. The high catechol dioxygenase activity implied that two promoters, transcribed in opposite directions, were present between *jadR\** and *jadY*. Extracts from cultures of the pJV461 and pJV462 transformants in *E. coli* DH5 $\alpha$  grown in LB broth gave 3.9 ( $\pm 0.4$ ) and 4.4 ( $\pm 0.4$ ) mU mg protein<sup>-1</sup>. The results indicated that the promoters upstream of *jadR\** and *jadY* showed higher activity in a *Streptomyces* host system than in *E. coli*, implying that the inserted *S. venezuelae* sequences were not adventitious promoters.

## II. Cloning Genes Upstream of the PKS Core Cluster

Sequencing and subcloning *S. venezuelae* chromosomal DNA upstream of the core PKS cluster identified, in addition to genes (*jadI* and *jadJ*) involved in PKS chain extension and cyclization (Han *et al.*, 2000), a pair of divergently transcribed regulatory genes, *jadR*<sub>1</sub> and *jadR*<sub>2</sub>, implicated in the stress requirement for JdB biosynthesis (Yang *et al.*, 1995; 2001). Further upstream of the core PKS sub-cluster, Yang *et al.* (1995) detected an ORF with sequence similarity to sugar dehydratases, but did not investigate its function. The possibility that this ORF might encode a dehydratase involved in sugar biosynthesis acquired relevance when a complete sub-cluster of genes for biosynthesis of the dideoxyhexose component of JdB was identified elsewhere in the *jad* cluster. To obtain this and other genes upstream of the core PKS cluster by chromosome walking, a 4.0-kb *XhoI*-*Bam*HI fragment of the DNA cloned in Lambda 8 (Ramalingam, 1989) was subcloned between the *XhoI* and *Bam*HI sites of pBluescript II SK(+) to give pJV429 (see Fig. 13). Sequencing demonstrated that pJV429 overlapped the left-hand end of the DNA insert in Lambda LH7 (Fig. 33), and corrected errors in the sequence previously reported (Yang *et al.*, 1995).

### A. The *jadW* genes

Analysis of the nucleotide sequence of the pJV429 insert detected three complete ORFs (*jadW*<sub>1</sub>, *jadW*<sub>2</sub>, and *jadW*<sub>3</sub>; Fig. 33). Each ORF was oriented for transcription in the same



**Figure 33.** Restriction map of the 4.0-kb fragment of *S. venezuelae* ISP5230 genomic DNA cloned in pJV429. Broad filled arrows below the map represent ORFs (*jadW*<sub>1</sub>, *jadW*<sub>2</sub>, and *jadW*<sub>3</sub>) identified in the pJV429 insert and their orientations. pJV429 and the *EcoRI* restriction sites used to excise a hybridization probe from this plasmid are aligned below the map. Abbreviations: B, *Bam*HI; Bs, *Bsa*AI; E, *Eco*RI; K, *Kpn*I; S, *Sac*I; X, *Xho*I.



direction as the PKS gene cluster and the *jadR*<sub>1</sub> response regulator gene, but in the opposite direction from the neighbouring *jadR*<sub>2</sub> repressor gene. The gene immediately downstream of *jadR*<sub>2</sub> was designated *jadW*<sub>3</sub>; its stop codon was separated by 80 bp of non-coding DNA from the stop codon of *jadR*<sub>2</sub>. The average G+C content of *jadW*<sub>3</sub> was 72.2 mol%, and the sequence encoded 254 amino acids. Upstream of *jadW*<sub>3</sub> was *jadW*<sub>2</sub> with an average G+C content of 77.7%, and a third codon G+C content of 92.7%. It was separated from *jadW*<sub>3</sub> by 13 bp and encoded 315 amino acids. The third ORF (*jadW*<sub>1</sub>), with an average G+C content of 71.8 mol%, encoded a protein of 309 amino acids; its stop codon was separated from the start codon of *jadW*<sub>2</sub> by 15 bp.

## **B. Features of the *jadW* genes**

### **a. *jadW*<sub>1</sub>**

Aligning the deduced amino acid sequence of *jadW*<sub>1</sub> (Fig. 34) with matching proteins from a BLASTP search of GenBank showed strong similarity to the products of *barX* (42% identity) from *S. virginiae*, *scbA* and *mmfL* (42 and 30% identity, respectively) from *S. coelicolor* A3(2), *afsA* (41% identity) from *S. griseus*, and *farX* (40% identity) from *Streptomyces* sp. FRI-5. Each of the matching gene products is associated with regulation by  $\gamma$ -butyrolactones of morphological or physiological differentiation in streptomycetes.

```

BarX. -----MTSTVPRELVHRAAVAEVFLTGWSRTAENRFALTAQWPRAH 41
FarX. -----MVHRTSTAQVLLTDWQRLDDARFSVTARWPLSH 33
JadW1. -----VHTTSRGEFVHRADPADIIPTDWTQLRQNRFSVSARVACLR 41
ScbA. MPEAVVLINSASDANSIEQTALPVPMALVHRTRVQDAFPVSWIPKGGDRFSVTAVLPHDH 60
AfsA. -----MDAAEVVHPVGIEMVHRTRPEDAFPRNWVRLGRDRFAVEAVLPHDH 47
          :***:  : : . *      **:: * . :

BarX. SYFTPVN-GCYDPLLASETIRQVGTLLSHAEFGVSFGDQFLMWDLHS-VRPEQAGVGAA 99
FarX. AFFTPVGDGYDPLMCAETIRQIAYLLGHAEFAVPFGHQFVLWDLSVSVVRPELLRVGLV 93
JadW1. SLLLSRAGARHDPLVAETIPETSMLVAHAELGVPLDEQFVWDLSYS-ADSEALTVDGL 100
ScbA. PFFAPVHGDRHDPLLIAETLRQAAMLVFHAGYGVPVGYHFLMATLDYT-CHLDHLGVSGE 119
AfsA. PFFAPVGDDLHDPLLVAEAMRQAAMLAFHAGYGIPLGYHFLLTELDYV-CHPEHLGVGE 106
          . : .      :***: :*: : .      ***      ..... :*: *      :      * .

BarX. PADLELDVICSDIRRRGRLAGMRYEVTLYCGGQVIATGGAAFDCTSPAVYQRLRGDRVG 159
FarX. PATVDLAITCVEIKRRAGRLSGLGYEAVVRRDGQVVATGRASVTCTSPAVYQRIRPEHVL 153
JadW1. SSDVTVDVVCSDITRRGSRLRNLRTTVVLTRDDRLLATGSGTARCTSALAYRRMRGERME 160
ScbA. VAELEVEVACSQLKFRGGQPVQQVDWAVRRAGRLATGTATTRFTSPQVYRRMRGD--F 177
AfsA. PTEIGLEVFCSDLKWRAGLPAQGRVGWAVHRGDRLAATGVAATRFSTPKAYRRMRGD--V 164
          : : : : * : : * .      . :      . : : *** : :      . : . : * : : :

BarX. ATGVRPLP--QPLAPASVGRFLTTDVVLSATERPLEWQLRVDEQHPVLFDHPVDHVPGMV 217
FarX. TPEHRPLPLTAPAAPQSVARLSPTDVVLSPLDRENRWQLRVDTNHPVLFDHWVDHVPGMV 213
JadW1. ALG--RVPLIPGVHPRLVGRARTEDDVVLAPGNRPDQWQLRVNTAHTLFRRPNDHVPGMV 219
ScbA. ATPTASVPGTAPVPAARAGRTRDEDVVLSASSQQDTWRLRVDTSHPTLFQRPNDHVPGML 237
AfsA. PVEGISLPETAPVPASPAGRARVEDVVLSGTGREGVWELRVDTRHPTLFQRPNDHVPGML 224
          .      . : *      .      . : *      ****: :      * . ***: * . ** :      *****:

BarX. LMESARQAAQAIDPSRPLFTTMRSEFSRYAELDRPCWIQAEPLPADNG-DRQVRVTGH 276
FarX. LMEAARQAAASALGRPSFMLGVAGEFKRYVELDAPCVIESERLFQDVPGAEVVRVTGH 273
JadW1. LLEAARQAATATTGSAAYLPTDLSVSPLRYVELDSPCWIEAESVPTPDPS-TTTIRVTGH 278
ScbA. LLEAARQAACLVTGPAPFVPSIGGTRFVRYAEFDSPCWIQATVRPGPAAG-LTTVRVTGH 296
AfsA. LLEAARQAACLVAGPAGIVPVEARTRFHRYSEFGSPCWIGAVVQPGADED-TVTVRVTGH 283
          **: *: *****      : *      * * * : .      *** :      .      : *****

BarX. QDDTVFSCLIGTRGAE----- 294
FarX. QNGELTFVGTVTASSYGY----- 291
JadW1. QDGSPVFRCTLTSPSRELSVATAGLDTRLAG 309
ScbA. QDGSLVFLTTLSGPAFSG----- 314
AfsA. QDGETVFSTVLSGPRAHG----- 301
          * : .      . *      :

```

**Figure 34.** Clustal W alignment of the deduced amino acid sequence of JadW<sub>1</sub> with related proteins in GenBank. 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). Sources of the sequences: BarX: *barX* in *S. virginiae*; FarX: *farX* in *Streptomyces*. sp FRI-5; ScbA: *scbA* in *S. coelicolor* A3(2); AfsA: *afsA* in *S. griseus*.

**b. *jadW*<sub>2</sub>**

Database searches with BlastX showed that the deduced amino acid sequence of *jadW*<sub>2</sub> is most similar to the product of ORF5, an dNDP-glucose dehydratase (with 42% identical aa.) of *S. virginiae*. It also resembles (30% identity) an oxidoreductase of *S. coelicolor* A3(2), the probable oxidoreductase PA4361 of *P. aeruginosa*, and many eukaryotic steroid dehydrogenases. Further database searching with BlastP produced significant alignments with the 3- $\beta$ -hydroxysteroid dehydrogenase/isomerase (3- $\beta$ -HSD) family and the NAD-dependent epimerase/dehydratase family. *JadW*<sub>2</sub> contained conserved domains of a 3- $\beta$ -HSD or NAD-dependent epimerase in its N-terminal region (Fig. 35).

**c. *jadW*<sub>3</sub>**

A BLASTP search of GenBank with a refined sequence for *jadW*<sub>3</sub> showed that the deduced protein less closely resembled a sugar dehydratase, as reported by Yang *et al.* (1995), than it resembled (67% identity) an *S. coelicolor* A3(2) reductase containing the family signature (PS00061) for short-chain dehydrogenase/reductases. This reductase is identical to the product of ORFX (GenBank AJ007731) previously reported from *S. coelicolor* A3(2), and postulated to be a 3-ketoacyl-ACP/CoA reductase potentially associated with a  $\gamma$ -butyrolactone autoregulator. It was also similar to PgaM (AY034378), the oxygenase-reductase in *Streptomyces* sp. PGA64 that brings about migration of the double bond in reactions catalyzed by the 3-ketosteroid isomerase/reductase (AF164960) of *S. fradiae*, and the oxygenase-like protein (AY033654) of *S. aureofaciens*.

```

consensus      1 DMKGYSCLVTGGGGFLGRHIVRLLLEGE-LREVRVFDLRHSPELDE-DSSKVQVIT-IK 57
JadW2          1 --mgpriLITGATGFIGGRVAAAAATARE-PAHVRLLARGPLTGPAA-AAGPTETA-V- 54
M. auratus    1 -mPAWSCLVTGAGGFLGQRIIRMLAQEKe-LQEVRTLFRSFTPKHRE-ELSKLQTKkVT 57
musculus      1 -mTGWSCLVTGAGGFLGQRIIRLLVKEKe-LKEIRVLDKAFGPELRE-EFSKLQNKTkLT 57
H. sapiens    2 SLAGVSCLVTGAGGFLGQRIVRLLLEEdeALAEIRLLDKAFSREALW-SFGKFQGKTeVK 60
G. gallus     6 QVPALVYLVTGGCGFLGEHIVRMLLEWeprLRELRVFDLHLSWLEELKTGPVQVT---- 61
Vaccinia      1 -MKVY--AVTGGGGFIGSYIVRALLQCErtLIELRVIDVRNGTKV---SSRNVVV---- 50

consensus     58 YIEGDVRDKDDLDAALQGVDVVIHTACIIDVFG-----KATRDRIMKVNVKGTQNVLDAC 112
JadW2         55 ---GDLRDPASLRRACEGVDVLIHCASAIGDA-----ELAR----AVNDEGTRNLVDAA 102
M. auratus    58 VLEGDILDAQCLRRACQGISVVIHTAAIIDVFG-----AIPRQTVIDINLKGTQHLLDAC 112
Musculus      58 VLEGDILDEPFLKRACQDVSVIHTACIIDVFG-----VTRESIMNVNVKGTQLLLEAC 112
H. sapiens    61 ILEGDIRDVEFLHRACQGVSLVIHTASIIDTLG-----LVEKQLLWEVNVTGTQMLLEAC 115
G. gallus     62 AIQGDVTQAHEVAAAMAGSHVVIHTAGLVDVFG-----KASPETIHKVNVQGTQNVLDAC 116
Vaccinia      51 YIYCDVCDTARLCAALEGVDVLIHTAGLVDVMG-----EYSEDEIYRANVHGTHSALSAC 105

consensus     113 VEAGVRVLVYTSSMEVVGPNSrGQPIVNGDETPYEST--HQDAYPESKALAEKLVLKAN 170
JadW2         103 VRAGATRIVALSTASVhgaapsappppapcrspparpaapgrpasgtswtpagpycdrtw 162
M. auratus    113 IGARVPVFIYSSVAVAGPNSyKVIIQNGSEENHEST--WSDPYAYSSKMAEKAVLAAN 170
Musculus      113 VQASVPVFIYTSSIEVAGPNSyKEIIQNGHEEEPLENT--WPAPYPHSKLAEKAVLAAN 170
H. sapiens    116 AHCNVQHFIYTSSTIEVAGPNcrGDPIFNGDETPYEST--SKFPYAQSKRLAEECVLKAD 173
G. gallus     117 VQTGTRLLVYTSSMEVVGPNVkGHPFYRGNEDTPYEAI--HRHPYPCSKALAEQLVLEAN 174
Vaccinia      106 VCAGVRFVYTSSMEVVGPNmrAEPFBV-GDEKTEYESC--HQHCYPRSKAEELVLSSN 162

```

**Figure 35.** Amino acid alignments from a Blast P search for similarity between the *jadW2* product and 3- $\beta$ -hydroxysteroid dehydrogenase/isomerase family domains. *M. auratus*, 3- $\beta$ -hydroxysteroid dehydrogenase type III (EC number:1.1.1.145) in *Mesocricetus auratus*; *Musculus*, 3- $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5\rightarrow4}$ -isomerase type I in *Mus musculus*; *H. sapiens*, 3- $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5\rightarrow4}$ -isomerase type I in *Homo sapiens*; *G. gallus*, 3- $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5\rightarrow4}$ -isomerase in *Gallus gallus*; *Vaccinia*, 3- $\beta$ -hydroxysteroid dehydrogenase/ $\Delta^{5\rightarrow4}$ -isomerase in *Vaccinia virus*. Bold letters indicate the consensus sequence of the 3- $\beta$ -HSD domain.

## C. Characterization of *jadW* mutants

### a. *jadW*<sub>1</sub>

#### i. Analysis of *jadW*<sub>1</sub>-disrupted mutants

*jadW*<sub>1</sub> was disrupted by inserting the Am<sup>R</sup> gene with each orientation into its *KpnI* site, giving pJV432A/B (see Fig. 14). After conjugal transfer of pJV432A/B into ISP5230, transconjugant colonies (VS1095/1096) were selected. Genomic DNA of the *jadW*<sub>1</sub>-disrupted mutants (VS1095/1096) digested with *EcoRI* and probed for hybridization with a <sup>32</sup>P-labeled 3.35-kb *EcoRI* fragment of pJV429 containing *jadW*<sub>1</sub>, *jadW*<sub>2</sub> and *jadW*<sub>3</sub> (see Fig. 33) gave a single strong signal at 4.9 kb, whereas probing the comparable digest of *S. venezuelae* ISP5230 gave a signal at 3.35 kb (Fig. 36). The hybridization results implied that a double crossover had replaced the native *jadW*<sub>1</sub> with an insertionally inactivated allele containing the 1.5-kb apramycin resistance gene.

#### ii. Phenotype of the *jadW*<sub>1</sub> mutants

Strains VS1095 and VS1096, containing disrupted *jadW*<sub>1</sub> with the apramycin resistance gene in alternative orientations, both grew much more slowly than the wild type, and gave bald colonies on MYM agar. Cultures grown in MYM medium as a vegetative inoculum for JdB production were incubated for 3-5 days to reach cell densities obtained in the wild type after 24 h (Wang *et al.*, 2001). Analyses by HPLC of cultures grown in



**Figure 36.** Southern hybridization of *S. venezuelae* genomic DNA digests probed with the 3.35-kb *Eco*RI fragment from pJV429. Lane 1, VS1095; lane 2, VS1096; lane 3, *S. venezuelae* ISP5230; lane M, DNA size markers.

Gal2I medium from this inoculum, and incubated for 48 h after the standard ethanol treatment at 6.5 h (Wang *et al.*, 2001) showed no JdB production in either strain VS1095 or strain VS1096. Bioassays of the disrupted and wild-type strains grown on MYM agar under conditions supporting Cm production by the wild type (Wang *et al.*, 2001) indicated that disruption of *jadW*<sub>1</sub> markedly decreased overall antibiotic activity in both disrupted strains. Analysis by HPLC of shaken cultures grown for 5 and 7 days in GI medium (Doull *et al.*, 1985) did not detect Cm in VS1095 or VS1096, whereas the wild type cultures contained 18-24 µg ml<sup>-1</sup>.

The *jadW*<sub>1</sub><sup>-</sup> mutant gave bald colonies on MYM agar, but sporulated profusely on TO agar. Moreover, spores generated on TO agar gave sporulating colonies even when serially patched on MYM agar for ten generations. If spores of the *jadW*<sub>1</sub><sup>-</sup> mutant from TO agar were washed extensively with water before they were incubated on MYM agar, colonies appeared 2-5 days later than on controls inoculated with unwashed spores, and were bald. Serial patching on MYM agar of the washed spores also gave bald colonies which, when used to inoculate Gal2I broth cultures, failed to produce either JdB or Cm. In contrast to the absence of JdB production when cultures grown in Gal2I broth were inoculated with washed spores or bald mycelium, cultures inoculated with spores from TO agar produced JdB in 40-70% of the wild-type yield.

### **iii. Complementing *jadW*<sub>1</sub>-disrupted mutants with pJV435**

The 1.2-kb *Bsa*AI fragment containing *jadW*<sub>1</sub> (see Fig. 14) was recloned in the promiscuous vector pJV326, giving pJV435. After conjugal transfer of pJV435 into *S. venezuelae* ISP5230 and its *jadW*<sub>1</sub>-disrupted mutant VS1095, Ts<sup>R</sup> transconjugants VS1098 and VS1097, respectively, were selected. When these were used to inoculate Gal2I broth, all of the cultures, whether derived from the mutant or the wild-type host, gave yields of JdB 180-250% of those in normal wild-type cultures. Addition of thiostrepton to the Gal2I culture medium at final concentrations of 10, 25, or 50 µg ml<sup>-1</sup> did not affect JdB titres, discounting selection of Ts<sup>R</sup> strains as a factor in the increased titre. This implied that in the absence of selection, pJV435 was not rapidly lost from the culture, and therefore may have integrated into the chromosome. When transconjugant strains VS1097 and VS1098 grown on MYM agar were bioassayed for Cm production, the inhibition zones were smaller ( $0.9 \pm 0.1$  versus  $1.2 \pm 0.1$  cm diameter) than those given by the wild type. Consistent with this, HPLC measurements of Cm in shaken cultures after 5-days incubation in GI medium averaged 65% of wild type titres. Accompanying Cm at 7 days was 3-O-acetyl-Cm (20-74% of the amount of Cm, estimated from peak areas). The acetyl derivative does not have antibiotic activity against *M. luteus*, and was identified by co-injection of an authentic sample during HPLC analysis.

## **b. *jadW*<sub>2</sub>**

### **i. Phenotype of *jadW*<sub>2</sub> mutants**



A mutant in which most of *jadW*<sub>2</sub> had been deleted from the wild-type chromosome was constructed by selecting for a transconjugant (VS1099) in which a double crossover had replaced DNA containing *jadW*<sub>2</sub> with the insert from pJV442 (see Fig. 14). Cultures of VS1099 grew well in MYM and jadomycin-production media, but on MYM agar gave white spores instead of the grey-green spores given by the wild type. Whereas the *jadW*<sub>1</sub>-disrupted mutants (VS1095/1096) did not produce JdB, cultures of VS1099 in Gal2I medium produced JdB without ethanol treatment. Cultures subjected to ethanol shock by the standard procedure produced JdB earlier than wild-type cultures and after 48-h incubation contained 5-10 times more than the wild-type. However, cultures in Cm production (GI) medium produced only about one third as much of this antibiotic as the wild-type strain.

#### **ii. Phenotype of mutants with deletions in *jadW*<sub>1</sub> and *jadW*<sub>2</sub>**

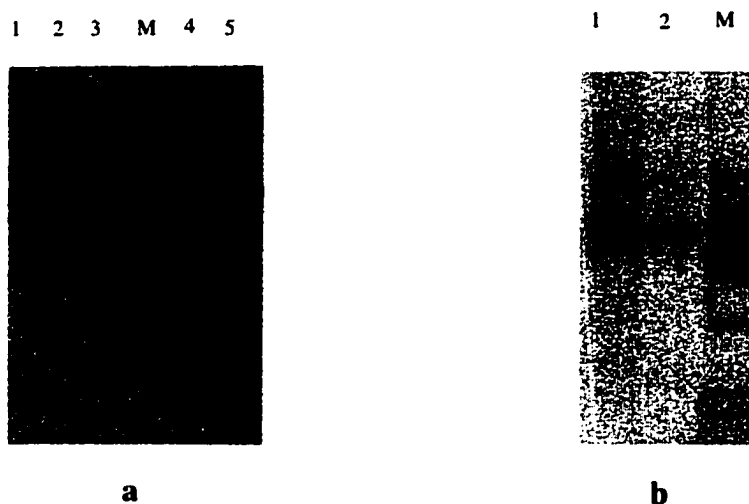
Plasmid pJV446, which contained a fragment with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>, was transferred conjugally into ISP5230 and mutants VS1103/1104 were separately isolated from transconjugant colonies. Cultures of VS1103 and VS1104 grew normally in Gal2I and GI production media as well as in MYM medium. On MYM agar, the strains sporulated as abundantly as the wild type, but gave white spores, lacking the grey-green pigmentation of wild type spores.

#### **iii. Southern hybridization of strains VS1099/1100, 1101/1102 and 1103/1104**

When genomic DNA was extracted from *S. venezuelae* ISP5230 (wild type strain), VS1099/1100 (*jadW*<sub>1</sub>-disrupted mutants), VS1101/VS1102 (*jadW*<sub>3</sub>-disrupted mutants, see later), and VS1103/1104 (mutants with deletions in *jadW*<sub>1</sub> and *jadW*<sub>2</sub>) digestion of the extracts with *Eco*RI and probing with a <sup>32</sup>P-labeled 3.35-kb *Eco*RI fragment of pJV429 containing wild-type *jadW*<sub>1</sub>, *jadW*<sub>2</sub>, and *jadW*<sub>3</sub> (see Fig. 32), gave single strong signals at 3.35, 4.2, and 4.9 kb, respectively (Fig. 37). These are the results expected from double cross-overs replacing native with disrupted alleles containing *jadW*<sub>2</sub>, *jadW*<sub>3</sub>, and *jadW*<sub>1</sub>/*jadW*<sub>2</sub>, respectively.

#### **iv. Comparison of JdB production by the wild-type and by mutants with deletions in *jadW*<sub>2</sub> or in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>**

Spore suspensions of *S. venezuelae* ISP5230 (wild type), VS1099/1100 (*jadW*<sub>2</sub>-disrupted mutants), and VS1103/1104 (mutants with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>) were used at equal concentrations and volumes to inoculate Gal2I medium. From visual observation, the strains initially grew at similar rates; cultures supplemented with ethanol (3% v/v at 6.5 h after inoculation) grew more slowly after supplementation. All cultures were analyzed for JdB production 18.5, 30.5, 42.5 and 54.5 h after inoculation. At the last sampling (54.5 h), wild-type cultures not treated with ethanol were unpigmented, whereas cultures of VS1103/1104 (mutations in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>) were pink and VS1099/1100 (mutation only in *jadW*<sub>2</sub>) were red. Because members of the VS1099/VS1100 and VS1103/VS1104 pairs were independently isolated transconjugants with the same genotypes except for Am<sup>R</sup> gene orientation, and showed similar pheno-



**Figure 37.** Southern hybridization of *S. venezuelae* genomic DNA. The DNA samples were digested with *EcoRI* and probed with the 3.35-kb *EcoRI* fragment from pJV429. Lane M contains size markers.

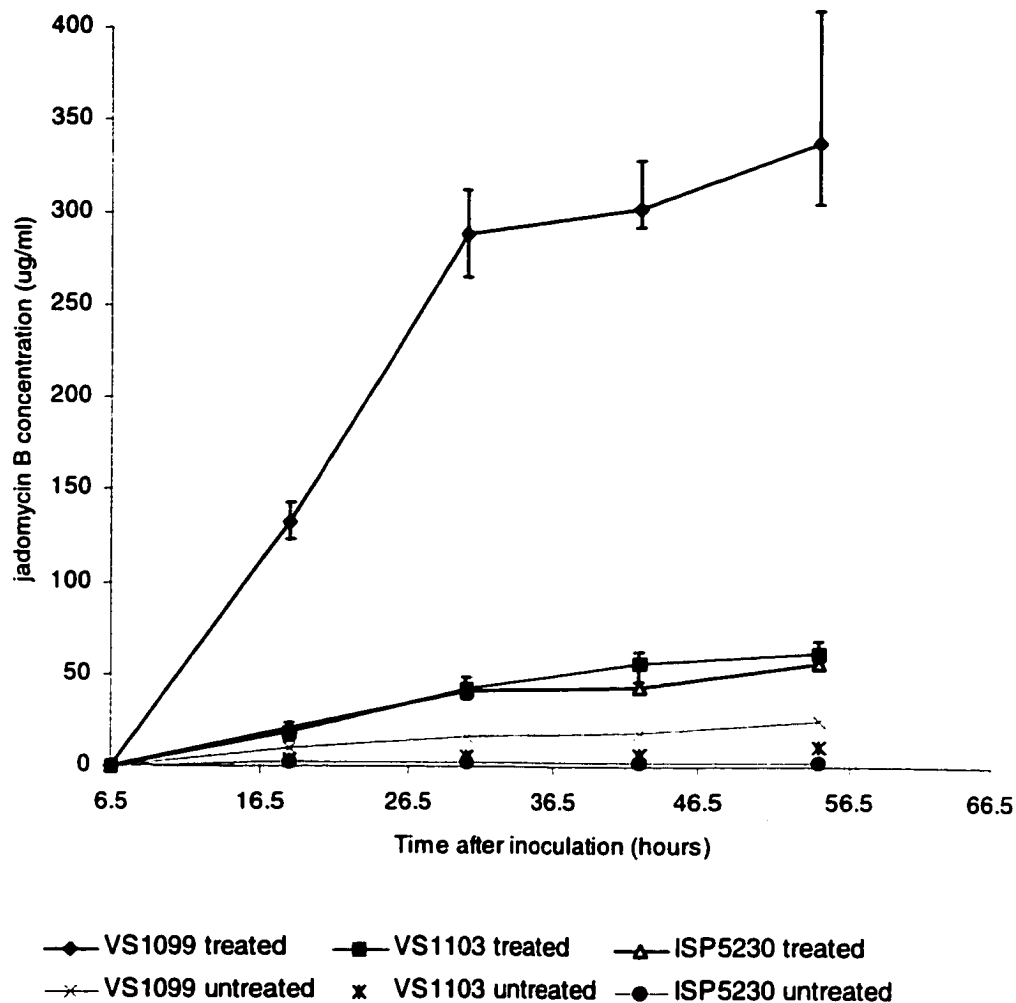
**(a)** Lane 1, 2, 3, 4, 5 contain DNA from VS1099, VS1100, ISP5230, VS1101, and VS1102, respectively. The increased size of VS1099 and VS1100 (4.2 kb) compared with ISP5230 (3.35 kb) is accounted for by integration of the 1.6-kb  $Am^R$  gene into sites from which a 0.75-kb fragment of *jadW*<sub>2</sub> had been deleted. The increased size (4.9 kb) in lanes 4 and 5 is due to insertion of the 1.6-kb  $Am^R$  gene into the *BsaAI* site of *jadW*<sub>3</sub>.

**(b)** Lane 1 and Lane 2 contain DNA from VS1103 and VS1104, respectively. The increased size (4.4 kb) compared to the 3.35 kb wild-type fragment (a, lane 3) is due to insertion of the 1.6-kb  $Am^R$  gene at the *jadW*<sub>1</sub>-*W*<sub>2</sub> site from which a 0.54-kb *BstEII* fragment containing 105-bp of DNA from *jadW*<sub>1</sub>, and 420-bp from *jadW*<sub>2</sub> had been deleted.

types, only one strain (VS1099 and VS1103) of each pair was used for JdB analyses (Fig. 38). Analysis of the cultures by HPLC confirmed that without ethanol treatment the wild-type culture did not produce JdB whereas untreated VS1099 produced 24.5 µg/ml JdB (see Fig. 38). The response of wild type *S. venezuelae* ISP5230 cultures to ethanol closely resembled that reported by Doull *et al.* (1994), but ethanol treatment of the *jadW*<sub>2</sub> mutant (VS1099) increased the JdB titre 5-10 fold. In the mutant cultures, JdB was produced rapidly after ethanol stress; 85-95% of the final titre had accumulated by the 30.5-h sampling whereas in wild type cultures only 67-75% of the final JdB titre had been produced at this time (see Fig. 38). Mutants VS1103 and VS1104 (with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub>) grew like the wild type in Gal2I medium, and when treated with ethanol produced 100–150% of the wild type JdB titre. They resembled the *jadW*<sub>2</sub>-disrupted mutant only in producing a small amount of JdB without ethanol treatment (see Fig. 38). The mutants with deletions in both *jadW*<sub>1</sub> and *jadW*<sub>2</sub> (VS1103/1104) grew normally on GI (Cm production) medium, and by HPLC analysis contained 45-50% of the wild type Cm titre; cultures of the *jadW*<sub>2</sub>-disrupted mutants VS1099 and VS1100 produced 30-47% of the wild type Cm titre.

#### **v. Transformation of VS1099 and ISP5230 with pJV448**

The influence of *jadW*<sub>2</sub> on the phenotype of *S. venezuelae* was assessed in part by introducing additional copies of the gene cloned in pJV448 into the wild-type, and into a mutant (VS1099) from which *jadW*<sub>2</sub> had been deleted. Transformation of VS1099 with pJV448 yielded a strain (VS1105) that grew normally in MYM, Gal2I and GI media, and

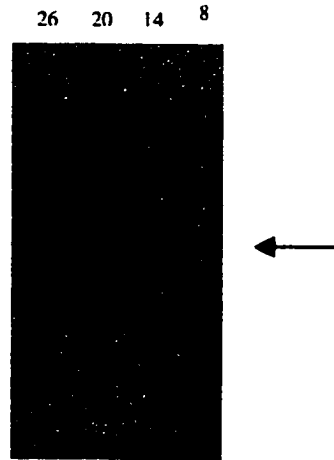


**Figure 38.** Production of jadomycin B by various *S. venezuelae* strains. ISP5230 is the wild type; VS1099 is a *jadW*<sub>2</sub>-disrupted mutant; VS1103 is a mutant with deletions in *jadW*<sub>1</sub> and *jadW*<sub>2</sub>. Cultures were grown in Gal2I medium; treated cultures were supplemented with ethanol (3% v/v) 6.5 h after inoculation. The data are average titres in three replicate cultures (variation is shown only for treated VS1099 and treated VS1103; in treated ISP5230 and all untreated cultures it was less than in treated VS1103).

in Gal2I medium produced 90-120% of the JdB produced by the wild type strain. Including thiostrepton at 10, 30 and 50 µg/ml (final concentration) in the Gal2I medium had little effect on the amount of JdB produced, implying that the transconjugant was thiostrepton resistant, and probably contained an integrated copy of pJV448. In contrast, transformation of *S. venezuelae* ISP5230 with pJV448 gave a strain (VS1106) that grew very slowly on MYM medium. Colonies patched on MYM agar and incubated at 30°C for one month did not sporulate, whereas the wild type gave abundant spores in four days. HPLC analysis of VS1106 cultures in Gal2I medium with or without ethanol treatment did not detect JdB production. Inoculating Gal2I medium with 1-cm<sup>2</sup> from agar cultures or growing the vegetative inoculum in MYM medium for up to 6 days did not result in JdB production. To determine if the presence of *jadW*<sub>2</sub> influenced Cm biosynthesis, VS1105 and VS1106 were compared by bio-assay with ISP5230 after growth on MYM agar under conditions suitable for Cm production (Doull *et al.*, 1986). ISP5230 gave the largest inhibition zone (2.1 ± 0.1 cm), VS1105 gave a smaller zone (1.8 ± 0.1 cm) and VS1106 gave no inhibition zone. To assess whether low antibiotic activity was due to poor growth of VS1105 and VS1106 in the vegetative inoculum, the two strains were grown in TSBG medium for up to 2 days to achieve the same mycelium density as the wild type before they were used to inoculate the production medium. HPLC results showed VS1105 produced 30% whereas VS1106 produced only 1.7% of the wild type Cm titre. Thus although inoculum size strongly influenced Cm production, it alone was not responsible for decreased Cm production by VS1105 and VS1106.

#### v. Transcription of *jad* genes in VS1099 and ISP5230

To determine when the JdB biosynthesis genes in wild-type *S. venezuelae* and the *jadW*<sub>2</sub>-deleted mutant are transcribed, cultures of ISP5230 and VS1099 harvested at various times (8 h, 14 h, 20 h, and 26 h) after ethanol treatment, and without ethanol treatment, were analyzed by Northern hybridization. Total RNA (10 µg) extracted from the mycelium was electrophoresed in an agarose gel. When a 0.9-kb *SacI-BsaAI* fragment containing a segment of *jadW*<sub>3</sub>, or the 1.1-kb *BsaAI* fragment of pJV431 containing *jadW*<sub>1</sub>, was used to probe the RNA, no hybridization was detected with any of the cultures. When a 0.9-kb *StuI-NruI* fragment containing part of *jadS* (the dideoxyhexose glycosyltransferase gene) was used to probe the RNA from VS1099, a 1.2-kb band was present in all of the cultures treated with ethanol (Fig. 39). The intensity of the signal at 1.2 kb increased from 8-h to 26-h in both wild type and mutant strains, but in 8-h cultures of ISP5230 it was very faint. As *jadS* encodes 395 amino acids, the 1.2-kb band corresponded to monocistronic transcription of *jadS*. The Northern hybridization results in Fig. 39 show that transcription of a dideoxysugar transferase gene started at least as early as 8 h after ethanol treatment. No signals were detected in culture extracts without ethanol treatment. The results correlate with expression of *jadM* to form a dedicated phosphopantetheinyl transferase at an early stage of JdB biosynthesis. The strongest Northern hybridization signal for *jadM* was found in cultures 24-h after adding ethanol (Wang *et al.*, 2001). The timing indicates the same physiological pattern for transcription of the PKS cluster as for biosynthesis of JdB.



**Figure 39.** Northern hybridization of *jad* mRNA from *S. venezuelae* VS1099. The arrow points to the 1.2-kb hybridizing band present in sampling times (8, 14, 20, 26 h after ethanol supplementation).



### **c. *jadW*<sub>3</sub>**

#### **i. Phenotype of *jadW*<sub>3</sub>-disrupted mutants**

*jadW*<sub>3</sub>-disrupted mutants (VS1101/1102) grew normally on MYM and Gal2I media and sporulated well, but produced JdB in a smaller amount (50-80%) than the wild type. When GI medium was inoculated with VS1101/1102 the cultures produced a similar amount of Cm to the wild type.

#### **d. Summary of *jadW* mutant phenotypes**

The phenotypes of *jadW* mutants are summarized in Table 4. A photograph of the *jadW*<sub>2</sub>-disrupted mutant and the mutants with deletions in *jadW*<sub>1</sub> and *jadW*<sub>2</sub>, all grown on MYM agar, is shown in Fig. 40.

### **D. Tests for mutant complementation**

#### **a. Bioassay for A-factor production by *S. venezuelae* ISP5230**

Complementation tests using a procedure developed by Horinouchi *et al.* (1984) to detect A-factor production in agar cultures of microorganisms by assaying for diffusible extracellular products restoring sporulation or streptomycin biosynthesis in adjacent colonies of the A-factor-dependent *S. griseus* mutant HHI gave negative results with *S. venezuelae* ISP5230 as the secretor organism. Patch cultures of *S. griseus* HHI assayed

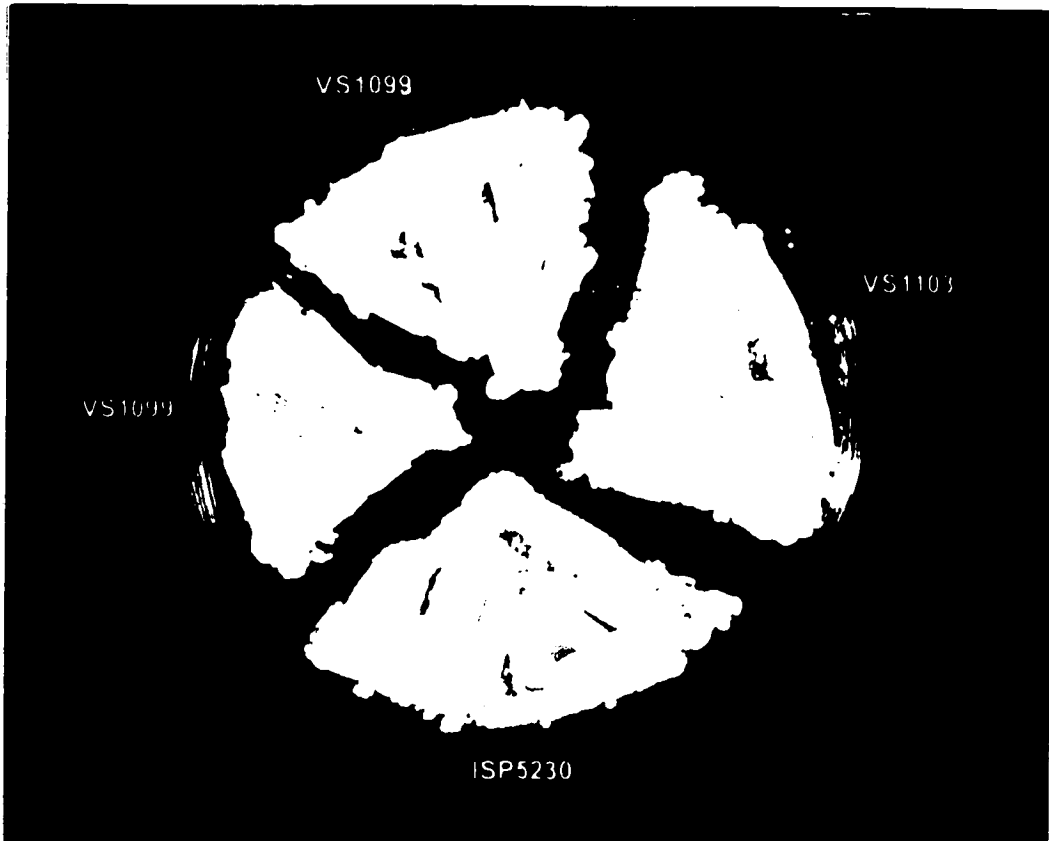
**Table 4. Summary of *S. venezuelae jadW* mutant phenotypes**

	Production of antibiotics*				Sporulation#	Color of spores	Growth of mycelium#
	Cm	JdB					
ISP5230 [wild type strain]	1	1			abundant	Grey/green	good
VS1095/VS1096 [ <i>jadW<sub>1</sub></i> disruptants]	–	–			bald	–	2-5 days slower
VS1097 [VS1095 (pJV435)] <sup>§</sup>	0.3-0.6	1.8-2.5			abundant	Grey/green	good
VS1098 [ISP5230 (pJV435)] <sup>©</sup>	0.3-0.6	1.8-2.5			abundant	Grey/green	good
VS1099/VS1100 [ <i>jadW<sub>2</sub></i> -deletion mutants]	0.3-0.5	5-10			abundant	white	good
VS1101/VS1102 [ <i>jadW<sub>3</sub></i> -disrupted mutants]	~1	0.4-0.8			abundant	Grey/green	good
VS1105 [VS1099 (pJV448)] <sup>&amp;</sup>	~1	1			abundant	Grey/green	good
VS1106 [ISP5230 (pJV448)]	±	–			bald	–	2-5 days slower
VS1103/VS1104 [ISP5230 with deletions in <i>jadW<sub>1</sub></i> and <i>jadW<sub>2</sub></i> ]	0.45-0.5	1.0-1.5			abundant	white	good

\* Normal antibiotic production in the wild type strain is assessed as 1. ±, indicates very low production; –, indicates no production.

# The wild type strain grew in MYM and TSBG media and sporulated on MYM agar. § VS1097 produced acetyl-Cm as well as JdB and Cm.

© pJV435, pJV326 with 1.2-kb *EcoRI-BamHI* fragment containing *jadW<sub>1</sub>*. & pJV448, pJV326 with 1.5-kb *SacI* fragment containing *jadW<sub>2</sub>*.



**Figure 40.** Patch cultures of several *S. venezuelae* strains on MYM agar. ISP5230, wild type; VS1099, mutant with *jadW*<sub>2</sub> disrupted; VS1103, mutant with deletions in *jadW*<sub>1</sub> and *jadW*<sub>2</sub>.

for streptomycin production also failed to respond when exposed to paper disks containing extracts from *S. venezuelae* ISP5230 cultures.

#### **b. Bioassays for complementation of the *jadW*<sub>1</sub>-disrupted mutant**

Patch cultures of the bald *S. venezuelae* VS1095 (*jadW*<sub>1</sub>-disrupted) mutant on MYM agar were tested by the bioassay procedure of Horinouchi *et al.* (1984) for a response to known autoregulators (Yamada and Nihira, 1999) and to potential stimulatory metabolites in wild-type *S. venezuelae* ISP5230 cultures. Paper disks were impregnated with A-factor, IM-2, nonalactone or chloroform extracts from cultures grown on Gal2I medium. Extracts from cultures grown for 8-28 h after ethanol treatment, or for 14-30 h without treatment, stimulated the growth of VS1095 ( $0.6 \pm 0.2$  cm growth zones); cultures extracted later than 28 h after ethanol treatment, or later than 30 h without ethanol treatment gave smaller zones ( $0.1 \pm 0.1$  cm). All of the culture extracts and autoregulators failed to elicit sporulation, but patch cultures of the mutant exposed to A-factor showed zones of growth stimulation similar to those elicited by extracts from wild-type *S. venezuelae*.

#### **c. Effect of A-factor on jadomycin B production in shaken cultures**

A-factor extract (5  $\mu$ l comparable to 50 ml of wild-type *S. griseus* culture; Yamada & Nihira, 1999) was added to 25 ml of Gal2I medium 4 h (Kawachi *et al.*, 2000) after inoculation with *S. venezuelae* ISP5230 or VS1099 (*jadW*<sub>2</sub>-disrupted mutant). HPLC

analysis of JdB production in replica cultures showed a consistent 1.5-fold increase in the A-factor-supplemented cultures at 48 h after ethanol treatment; no effect was detected in cultures not treated with ethanol (data not shown).

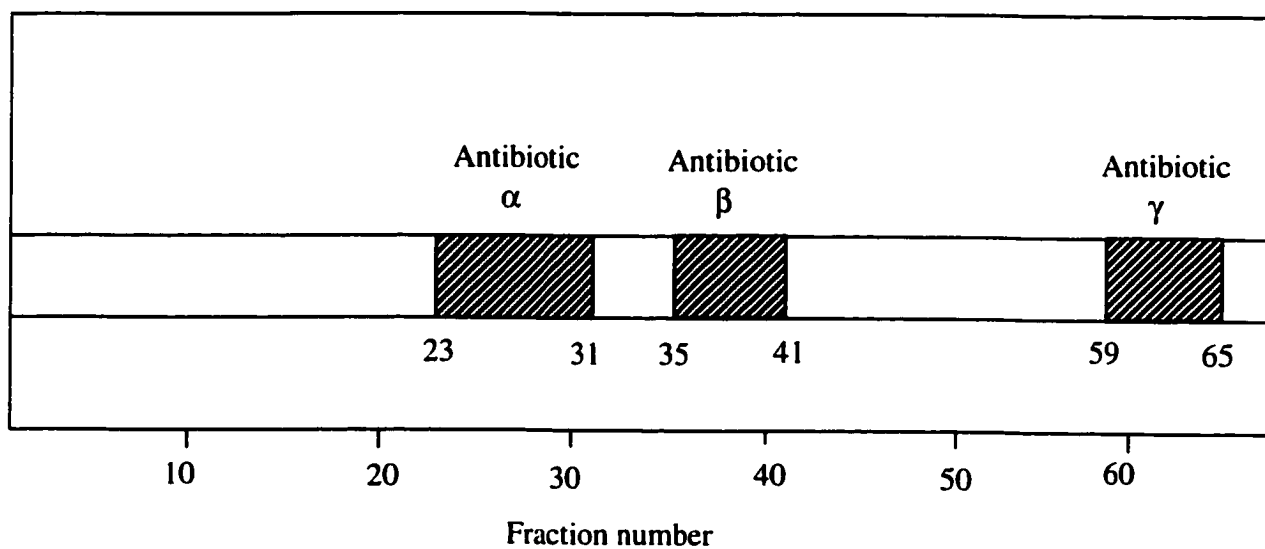
**d. Other experiments to detect a  $\gamma$ -butyrolactone effector and antibiotics in *S. venezuelae* cultures**



In collaboration with Dr. T. Nihira at Osaka University, chloroform extracts from cultures of *S. venezuelae* ISP5230 grown in JdB production (Gal2I) medium were examined for the presence of  $\gamma$ -butyrolactone effectors. In the expectation (Yamada and Nihira, 1998) that biosynthesis of these effectors would precede accumulation of JdB, cultures were extracted 17 h after inoculation (or 23.5 h after inoculation if cultures were treated at 6.5 h with ethanol). The extracts were chromatographed on a column of silica gel C<sub>18</sub> reversed phase absorbent on which  $\gamma$ -butyrolactones are readily eluted with 50% acetonitrile (T. Nihira, personal communication). Consistent with weak retention on silica gel, HPLC analysis on a C<sub>18</sub> column of a chloroform extract from *S. griseus* IFO13350 containing A-factor detected a rapidly eluted peak with UV absorbance at 210 nm, the wavelength at which  $\gamma$ -butyrolactones are detected (Takano *et al.*, 2000). It was anticipated, therefore, that any putative  $\gamma$ -butyrolactone effectors in JdB-producing cultures of *S. venezuelae* ISP5230 would be present in a 50% aqueous acetonitrile eluate from the C<sub>18</sub> silica gel column. However, examination of the eluate by an antibiotic assay procedure detected inhibition of the growth of *M. luteus*. The presence of antibiotic activity in the extract threatened to interfere with the sensitive bioassay procedures

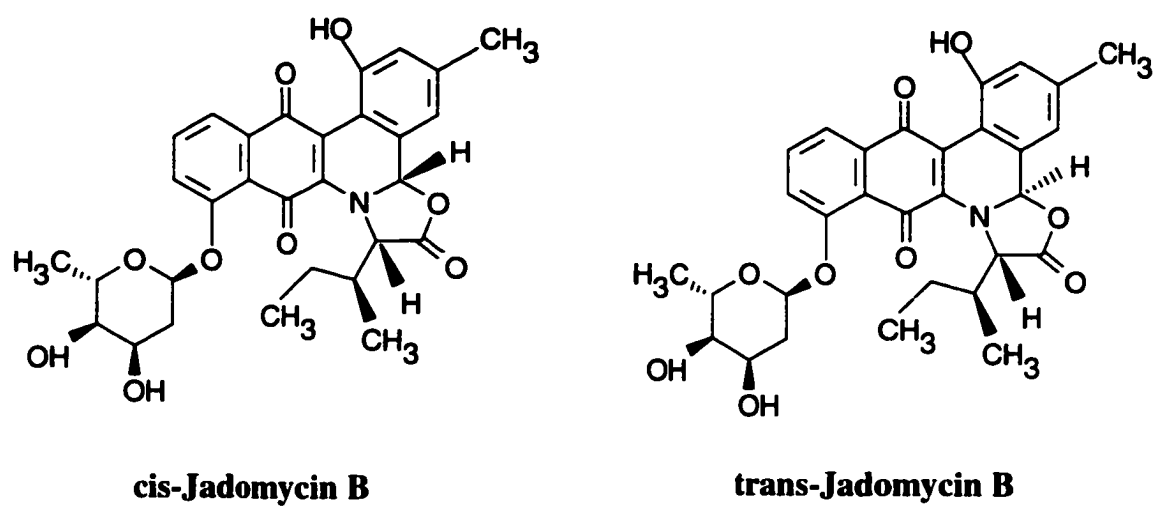
developed in Dr. Nihira's laboratory to detect and identify  $\gamma$ -butyrolactone effectors. Since the latter molecules are relatively stable to heat under acidic conditions, the possibility of selectively inactivating the antibiotic(s) was explored, but autoclaving the acidified (pH 5.0) chloroform extract at 121°C for 20 min failed to completely eliminate the antibiotic activity. As an alternative procedure, the chloroform extract was fractionated by reverse-phase column chromatography. Only fractions devoid of antibiotic activity (Fig. 41) will be bioassayed in Dr. Nihira's laboratory.

### III. Re-examination of the Jadomycin B Structure

Analysis by NMR of metabolites accumulated by mutants disrupted in genes for biosynthesis of the glycosidic moiety of JdB prompted a reexamination of the antibiotic structure. Culture extracts purified by flash chromatograph on silica gel showed paired sets of resonance signals that suggested JdB might be a mixture of isomeric forms. To clarify its structure, the antibiotic was purified further by both normal and reverse-phase column chromatography, and was reexamined by  $^1\text{H}$  and  $^{13}\text{C}$  NMR. It was also crystallized and examined by X-ray crystallography. Spectroscopic and crystallographic analyses carried out in the Chemistry Department at Dalhousie University indicated that the two sets of NMR signals arose from two configurations of JdB; the postulated structures of the major compound designated *cis*-JdB, and the minor component (*trans*-JdB) are shown in Fig. 42. The pH of *S.venezuelae* ISP5230 cultures grown in Gal2I medium and subjected to ethanol treatment was around 6.1-6.4 at 48 h. To investigate whether the presence of two forms of JdB might be due to acid-catalyzed isomerization,



**Figure 41.** Fractionation of *S. venezuelae* ISP5230 extracts. Fractions eluted with 50% aqueous acetonitrile from a  $C_{18}$  silica gel column were bioassayed:  fractions exhibiting antibiotic activity;  fractions devoid of antibiotic activity.



**Figure 42.** The structures of *cis*- and *trans*-jadomycin B in *S. venezuelae* ISP5230.



of an initially unique enzyme product under acidic culture conditions, 0.1 M MOPS buffer was added to a batch of Gal2I medium and the pH adjusted to 7.3. The buffered cultures of *S. venezuelae* maintained a pH of 7.1-7.3 during growth, but produced about 30% less JdB than standard unbuffered cultures. To avoid potential exposure to acid generated in chloroform, the product was extracted from the MOPS-buffered Gal2I medium with ethyl acetate, purified by crystallization from 50% aqueous acetonitrile and dissolved in acetonitrile to obtain its <sup>1</sup>H-NMR spectrum. Despite these precautions, the spectrum showed two isomeric forms of JdB in the same relative amounts as in the product from unbuffered cultures. By slow crystallization, samples of two crystalline forms in the mixture were retrieved, and crystallographic analysis (T. S. Cameron, R. L. White, L. Wang & L. C. Vining, unpublished) clarified the NMR results by showing that the isomers contained *cis*- and *trans* forms of the oxazoline ring in JdB (see Fig. 42). The crystallographic data also revealed that the dideoxysugar attached to the aglycone is L-digitoxose.

## DISCUSSION

*Streptomyces* produce a range of chemically diverse secondary metabolites that not only contribute 70% of the therapeutically useful antibiotics but also play an important role in natural ecological interactions. Clinical and industrial applications of secondary metabolites have generated interest in the molecular genetics of their biosynthesis and fostered numerous investigations of the function, expression, and regulation of antibiotic biosynthesis genes. The results have sustained a persistent curiosity about the relationship between secondary metabolism and cell differentiation. The immediate goal of this study was to understand how and when JdB is synthesized by *Streptomyces venezuelae* ISP5230, but the relationship between cellular differentiation and production of two distinctly different antibiotics, JdB and Cm, was also explored.

### **I. Functional Analysis of Sequenced DNA by Insertional Inactivation of Specific Target Genes**

Gene disruption is now one of the most effective strategies for introducing mutations blocking specific gene functions, and therefore for determining the functions of cloned genes. It is especially useful in identifying genes for antibiotic biosynthesis and regulation. The procedures for insertional inactivation of genes require initial construction of a vector carrying a copy of the target gene into which a marker fragment (such as the Am<sup>R</sup> resistance gene) has been inserted near the middle of its sequence. Introduction of the disrupted gene into a host strain must be followed by two homologous

recombination events. The first crossover generates two alleles of the gene, one of which is the intact native copy, while the other is an inactive allele disrupted by insertion of the marker DNA. The two alleles are separated in the chromosome by vector DNA. The second crossover excises the vector along with either the intact or the disrupted gene, leaving the other copy on the chromosome. When the overall result is allele exchange both parental and mutant strains can be recovered. Intergeneric conjugal transfer of plasmids carrying the inactivated gene from *E. coli* to streptomycetes (Flett *et al.*, 1997) is an efficient technique for introducing constructs containing a disrupted gene into a streptomycete, and was used in this research to disrupt individual genes of the *jad* cluster. Functional changes in phenotype resulting from the specific mutation were then assessed and used to assign the role of the disrupted gene.

One consideration in using the insertional inactivation technique is the potential polar effect, which can give misleading or incorrect results if the disrupted gene is in a biosynthetic pathway having polycistronic gene products. In this case, inactivation of an early gene may prevent expression of downstream genes, confusing assignment of the responsibility for a phenotype change. To take this into consideration, the promoter probe vector pXE4 (Ingram *et al.*, 1989) containing *xylE* was used to detect whether failure to make the glycosidic component of JdB, or to transfer the sugar to the aglycone, was due to insertional inactivation of the gene or to a polar effect preventing expression of downstream genes. The Am<sup>R</sup> cassette from pJV225 (Chang *et al.*, 2001) was normally used to disrupt and tag a target gene. To detect whether the Am<sup>R</sup> gene, originally from the *E. coli* plasmid pKC203 (Rao *et al.*, 1983; Kaster *et al.*, 1983), retained its own promoter

and lacked a following terminator, it was ligated into the promoterless bifunctional vector pXE4 and transferred into *S. venezuelae* ISP5230. The high level of catechol dioxygenase activity in *S. venezuelae* ISP5230 transformed with pXE4::*apr'* (but not in controls transformed with pXE4) implied expression of *xylE* from the Am<sup>R</sup> gene promoter, and indicated that use of the Am<sup>R</sup> cassette for insertional inactivation maintained expression of polycistronically transcribed genes downstream of the insertion site.

## **II. *jadM* Encodes a Jadomycin B-pathway-specific PPTase**

Phosphopantetheinyl transferase activity was first detected in EntD from *E. coli*, and in Sfp from *B. subtilis*. Acyl carrier protein synthetase (ACPS), which catalyzes conversion of the inactive *apo* form of a fatty acid synthase complex to the functional form, was the first PPTase for which a gene was cloned and characterized. Several genes involved in peptide secretion (*entD* in *E. coli*, *sfp* in *B. subtilis* and *gsp* in *B. brevis*) have since been identified, and their products appear to represent a new class of proteins (Borchert *et al.*, 1994). Through refinement of sequence alignments that indicated 12-22% similarity with the ACPS peptide sequence, a PPTase superfamily that included the Sfp/Gsp/EntD group was identified (Lambalot *et al.*, 1996; Gehring *et al.*, 1997). Gsp is present in a locus required for gramicidin biosynthesis; EntD and Sfp were originally reported to activate enterobactin and surfactin synthetase, respectively, and Sfp was recently reported to modify the *apo* form of heterologous recombinant proteins, including the PCP domain of *Saccharomyces cerevisiae* Lys2 (Ehmann *et al.*, 1999), and the *E. coli* ACP domain (Quadri *et al.*, 1998a; Gokhale *et al.*, 1999). Lambalot *et al.* (1996) identified two

consensus motifs shared by PPTase family members; the conserved residues are implicated in enzyme reactions transferring the phosphopantetheinyl moiety of coenzyme A to the hydroxyl of conserved serines in the ACP domain of PKS and the PCP domain of NRPS (Reuter *et al.*, 1999).

Although searching the GenBank database with BlastX failed to identify *E. coli* ACP synthase or the Sfp/Gsp/EntD group of PPTases as homologues of JadM, and showed only 22-24% sequence identity between the deduced amino acid sequences of *jadM* and the two most closely related PPTases (HetI and MtaA), JadM contained residues highly conserved in known PPTase family members, such as the Sfp/Gsp/EntD group. Information now available on the crystal structure of Sfp indicates that these residues most likely interact with the pantetheinylation substrate (Reuter *et al.*, 1999), and suggests a similarity in reaction mechanism. It is reasonable to conclude that JadM contains a Ppan transferase domain and is a member of the PPTase superfamily. It probably converts the inactive *apo*-enzyme form of JadC (the *jad* cluster ACP; Han *et al.*, 1994) to the functional *holo*-enzyme by transferring 4'-phosphopantetheinyl from coenzyme A to the  $\beta$ -hydroxy group of the conserved serine in JadC. The newly introduced -SH group of the Ppan prosthetic group would act as a nucleophile in acylations by coenzyme A esters catalyzed by a PKS (Lambalot *et al.*, 1996). The severely decreased JdB titre after insertional inactivation of *jadM* with an *apr'* cassette indicates that the *jadM* product has a major role in JdB biosynthesis. The residual activity when *jadM* was disrupted might arise from a FAS pantetheinyl transferase. Preliminary evidence from a Northern hybridization indicated that *jadM* is expressed only in cultures

stressed by ethanol treatment, and that expression reaches its maximum in 24 h, then decreases rapidly to become undetectable. This would not be expected for a primary metabolite FAS, but is consistent with evidence (Doull *et al.*, 1993; 1994) that JdB is produced by *S. venezuelae* ISP5230 only during a limited period after exposure to stress. Under our experimental conditions, peak expression of *jadM* preceded by 24 h the maximum JdB titre. The difference in timing suggests that stress activation is followed by relatively slow production and excretion of the antibiotic product. Nevertheless, the correlation between *jadM* transcription and JdB production implies that JadM has a specific function in the pathway.

For the biosynthesis of type-II polyketide antibiotics in streptomycetes, each PKS complex must have a dedicated *holo*-ACP, which in turn requires a specific *holo*-ACP synthase (ACPS). The latter enzyme functions as an integral component of the antibiotic biosynthesis pathway, and differs from *holo*-ACPs participating in fatty acid biosynthesis (Hopwood & Sherman, 1990; Hutchinson, 1995). However, both *fren* and *gra apo*-ACPs could be phosphopantetheinylated *in vitro* by purified *E. coli* ACPS. When combined with ACP-deficient *act* ketosynthases  $\alpha$  and  $\beta$  isolated from *S. coelicolor* A3(2), the *holo*-ACPs formed *in vitro* were fully functional in polyketide synthesis (Carreras *et al.*, 1997). Moreover, co-expression of actinorhodin and griseusin ACPs with ACPS in *E. coli* gave high titres of active *holo*-ACPS (Cox *et al.*, 1997), and *E. coli* ACPS efficiently modified post-translationally the *apo*-ACPs involved in biosynthesis of granaticin, frenolicin, oxytetracycline, and tetracenomycin (Gehring *et al.*, 1996). These results imply that *E. coli* ACPS has broad substrate specificity. However, it will not recognize

the *apo*-forms of several PCP and ArCP domains, including the *apo*-PCP domain of *E. coli* EntF and the *apo*-ArCP domain of *E. coli* EntB (Lambalot *et al.*, 1996; Gehring *et al.*, 1996; Quadri *et al.*, 1998a). The evidence that an *S. venezuelae* ISP5230 mutant disrupted in *jadM* is unaffected in the production of Cm, an antibiotic now known to be biosynthesized via a non-ribosomal peptide synthetase (He *et al.*, 2001) indicates that JadM is not required for this process, and could mean that it is not recognized by the PCP domain in the NRPS. In the *cml* cluster there may be a separate PPTase catalyzing the conversion of *apo*-PCP to its *holo* form. Since the *jadM*-disrupted mutants VS1075 and VS1076 grew normally on minimal agar, *jadM* is also not essential for fatty acid biosynthesis, and thus appears to be a JdB-pathway-specific PPTase.

Although Wang *et al.* (2001) were the first to describe the cloning and characterization of a gene encoding a PPTase from a streptomycete, more than 20 examples of PPTases, including ACPS, EntD and O195 of *E. coli*, Sfp of *B. subtilis* and Gsp of *B. brevis*, have been added to the group on the basis of sequence similarity since the superfamily was first recognized (Lambalot *et al.*, 1996; Walsh *et al.* 1997). However, only a few have been extensively characterized. Among these are PPT1 from the type-II fatty acid synthase of *Brevibacterium ammoniagenes* (Stuible *et al.*, 1997), PPT2, which activates mitochondrial ACP in *Saccharomyces cerevisiae* (Stuible *et al.*, 1998), Lys5 from the lysine biosynthesis system in *S. cerevisiae* (Ehmann *et al.*, 1999), MtaA from the polyketide synthase/polypeptide synthetase complex synthesizing mycothiazole in *Stigmatella aurantiaca* (Gaitatzis *et al.*, 2001) and PptT, which is required in the assembly of mycobactin, the peptide-polyketide siderophore of *Mycobacterium*

*tuberculosis* (Gehring *et al.*, 1997; Quadri *et al.*, 1998b). The crystal structure of Sfp has indicated regions likely to be involved in interactions with the substrate of this PCP (Reuter *et al.*, 1999), and since most of these regions are highly conserved among PPTase family members, directed mutations may provide insights into substrate recognition and specificity by PCP, ACP and NRPS enzyme complexes. Already the crystallographic investigations have allowed a catalytic mechanism to be proposed (Parris *et al.*, 2000).

### **III. The Function of *jadN***

The location of *jadN*, partially overlapping *jadM* in the *jad* gene cluster, suggests that it is associated with JdB biosynthesis in *S. venezuelae* ISP5230. The strong similarity of its deduced amino acid sequence to many known carboxylases in *Streptomyces* species, and the evidence from a Blast P CDD search that a carboxyl transferase domain is present in its N-terminal region suggests that the *jadN* product carries out transcarboxylation from biotin to an acceptor molecule. Since acyl-CoA is the acceptor molecule for all members of the carboxyl transferase family, *jadN* may function as a transcarboxylase in jadomycin polyketide condensation reactions. Previous research has shown that JdB is derived from a C<sub>20</sub> polyketide chain presumed to be generated by condensation of an acetyl starter unit with nine C<sub>2</sub> extender units supplied from malonyl-CoA (Meurer *et al.*, 1997; Kulowski *et al.*, 1999). Therefore, JdB biosynthesis would be expected to create a strong demand for malonyl-CoA, which is formed by carboxylation of acetyl-CoA in two distinct reactions catalyzed by a composite enzyme consisting of acetyl-CoA carboxylase and a transcarboxylase. The initial step, catalyzed by the biotin carboxylase (BC) of acetyl-



CoA carboxylase, uses bicarbonate to carboxylate a biotin carboxyl carrier protein (BCCP); a transcarboxylase then catalyzes carboxyl transfer from BCCP to acetyl-CoA, forming malonyl-CoA (Samols *et al.*, 1988). A gene *jadJ*, encoding an acyl-CoA carboxylase, has been identified in the cluster associated with JdB biosynthesis in *S. venezuelae* ISP5230, and specifies the BC and BCCP components in its N- and C-terminal regions, respectively (Han *et al.*, 2000). It is likely, therefore, that *jadN* catalyzes the transcarboxylation reaction supporting chain elongation during JdB biosynthesis, with *jadJ* generating the necessary supply of malonyl-CoA.

The substantial decrease in JdB production when *jadN* was disrupted by inserting the  $Am^R$  gene in either direction indicated that *jadN* has a major role in the biosynthesis of polyketide intermediates. The residual 10-25% of wild-type JdB production in the mutant could be due to complementation by a primary pathway homologue, but the primary *jadN* clearly did not fully complement the defective secondary metabolic gene. As *jadN*-disrupted mutants grew well on MYM medium, and produced Cm normally, the gene is not involved in the fatty acid or Cm biosynthesis pathways, and thus appears to be a JdB pathway-specific carboxylase.

#### **IV. Pathways for Dideoxysugar Biosynthesis**

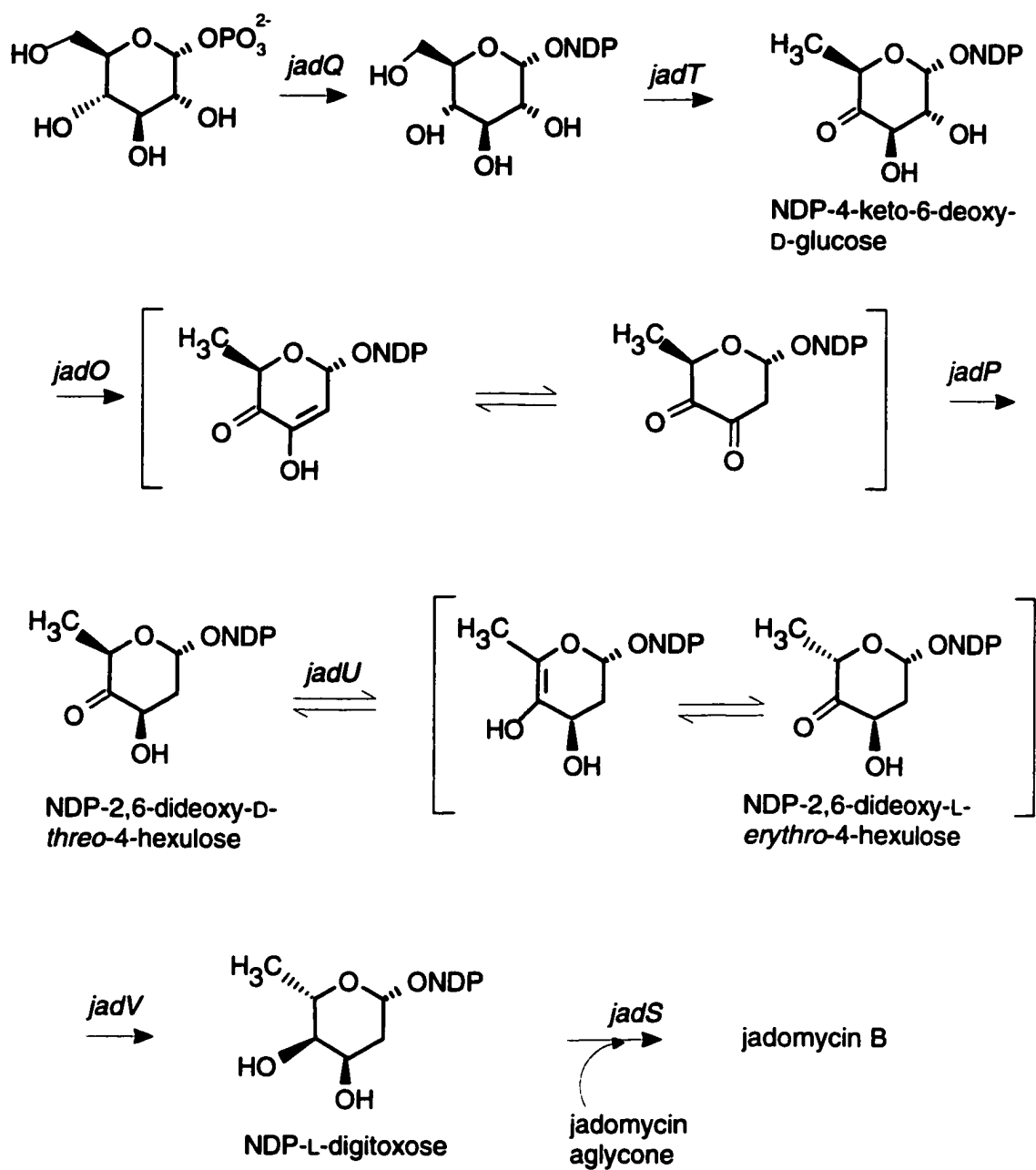
New information on the structure of JdB from the present investigation has identified the 2,6-dideoxysugar moiety as L-digitoxose. The pathway for biosynthesis of 3,6-dideoxysugars has been firmly established (Liu and Thorson, 1994), and the enzymes

involved 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. In the present investigation the sequence of biosynthetic reactions has been predicted from gene sequences that reveal a relationship to enzymes in the existing database. Phenotypic changes accompanying specific gene disruptions can provide further evidence, and is supported by biochemical characterization of intermediates accumulated after gene inactivation. Molecular genetic evidence for enzyme function has cast light on several sugar biosynthetic pathways, and helped in the design of hybrid natural compounds (Zhao *et al.*, 1998; Kunzel *et al.*, 1999; Olano *et al.*, 1999).

The absence of a dideoxysugar component in the substance produced when *jadO,P,Q,S,T,U* and *V* were disrupted implicated the products of these genes in biosynthesis or attachment of the glycosidic moiety. BLAST searches showing marked similarities between the deduced amino acid sequences of the *jad* genes and enzymes for deoxysugar biosynthesis in other species strengthened this conclusion. Based on sequence homologies, *jadO, P, Q, S, T, U* and *V* were assigned to seven biochemical reactions that would be needed to generate the glycosidic moiety of JdB in *S. venezuelae*. The combined evidence from all sources supports the plausible pathway shown in Fig. 43 for biosynthesis of the L-digitoxose component of JdB. First,  $\alpha$ -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 and Thorson, 1994). The subsequent C-2 deoxygenation is catalyzed 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). Participation of such an oxidoreductase in biosynthesis of the 2,6- dideoxyhexose in JdB would be consistent with the biosynthetic pathway for other dideoxysugars 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. The required epimer of the 2,6-dideoxysugar is probably formed by JadU (NDP-4-keto-6-deoxyhexose epimerase) via an enediol intermediate. Presumably JadV (NDP-4-keto-6-deoxyhexose 4-keto-reductase) reduces the NDP-4-keto-6-deoxyhexose 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- bacteria (Liu and Thorson, 1994), 5-epimerization (Eep) and 4-reduction (Ered) constitute the last two steps. The lack of E3 activity (CDP-6-deoxy- $\Delta^{3,4}$ -glucoseen reductases, 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 Gram+ streptomycetes differs from that giving 3,6-dideoxyhexoses in Gram- 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



**Figure 43.** Proposed pathway for biosynthesis of the L-digitoxose moiety.

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 *jadU* gene product. In the absence of this information, the steps showing 5-epimerization preceding 4-ketoreduction in the route to L-digitoxose proposed in Fig. 43 are based only on the similarity of JadU and JadV sequences to those of enzymes in other bacteria, and to the pathways for 2,6-dideoxysugar biosynthesis in which they are believed to participate (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 complete (Katz & Donadio, 1993; Madduri *et al.*, 1998). In doxorubicin biosynthesis, the daunosamine moiety may be transferred either to  $\epsilon$ -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 *S. venezuelae* strains disrupted in individual genes for deoxysugar biosynthesis or in the gene (*jadS*) for glycosyl transfer, (and the evidence that expression of genes downstream of *jadS* was not prevented by a polar effect) strongly suggests that glycosyl transfer by JadS is the final reaction in JdB biosynthesis. Because the enzymes for glycosylation in many post-polyketide reactions

modifying secondary metabolites do not exhibit absolute substrate specificity, a final glycosyltransferase may generate several products (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 been reported for the glycosyltransferases 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 that substrate flexibility for the sugar component can be used to genetically engineer hybrid antibiotics by replacing TDP-desosamine with other substrates to create 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 in the *jad* cluster of *S. venezuelae* ISP5230, the single sugar in the JdB structure, and compact clustering of genes for its biosynthesis, allowed a probable sequence of biosynthetic reactions to be deduced. While 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, 2000b), and is present

in the microbial metabolite ammocidin (Murakami *et al.*, 2001), L-digitoxose has been reported only in microbial products. It occurs in the glycosidic components of the macrocyclic antibiotic kijanimicin from *Actinomadura kijaniata* (Mallams *et al.*, 1981), the tetrocarcin group of antitumor antibiotics from *Micromonospora chalcea* (Tomita *et al.*, 1980), and some antifungal polyene macrolides such as nystatin from streptomycetes (Zielinski *et al.*, 1979).

Molecular genetic analysis of the genes for glycosylation has revealed significant information about the biosynthetic pathways to deoxysugars, and has created a strong interest in their potential for producing new hybrid molecules with valuable pharmaceutical activity. As genes and gene clusters have been cloned, important aspects of deoxysugar biosynthesis, such as deoxygenation and glycosyltransfer have become better understood. Information now available on the programming of glycosylation provides an opportunity for generating novel structures. Genetic alteration of the sugar biosynthesis pathways to allow elimination of unwanted groups, addition of specific moieties, and incorporation of flexible modified substrates could also be an attractive route to novel structures. A better understanding of the specificities of enzymes involved in sugar biosynthesis may allow us, at some time in the future, to select the desired modified aglycone from a gene library and combine it with specific sugar genes to produce new bioactive molecules (Zhao *et al.*, 1999).

## V. Regulatory Genes

Bacterial metabolism is often regulated at the level of gene transcription by factors that determine when particular genes are expressed in relation to the life cycle or to environmental signals (Neidhardt *et al.*, 1987). In wild type *S. venezuelae* ISP5230 expression of the *jad* cluster is influenced by severe environmental stress, as well as physiological stresses imposed by nutritional imbalance. Not surprisingly, therefore, regulation of JdB biosynthesis uses mechanisms different from those encountered in streptomycetes producing antibiotics mainly in response to physiological imbalance (Doull and Vining, 1995). Of the two types of regulatory systems that, on current evidence, have important roles in controlling JdB biosynthesis, one involves genes encoding repressor proteins, and the other is associated with  $\gamma$ -butyrolactone effector molecules.

### A. Repressor gene *jadR\**

The gene *jadR\**, which encodes a repressor and is located upstream of the *jad* PKS cluster, showed high similarity near its N-terminus to TcmR, a repressor controlling tetracenomycin C resistance in the producing organism *S. glaucescens* (Yang *et al.*, 1996). This suggests that the two repressors may have a similar role, and that the tolerance of *S. venezuelae* ISP5230 to JdB may be mediated by an efflux system similar to that postulated for tetracenomycin C (Guilfoile *et al.*, 1992), and involving *jadR<sub>2</sub>/jadR\**. Supporting the function of *jadR\** as a repressor is the result from its



insertional inactivation; the disrupted mutant produced JdB without ethanol supplementation, and with ethanol treatment produced more JdB than did the ethanol-treated wild-type strain. ActII-ORF1 and TcmR repress promoters controlling divergent structural genes (such as *actII-ORF2-3* (Caballero *et al.*, 1991) and *tcmA* (Guilfoile and Hutchinson, 1992)). The structural genes encode proteins for exporting actinorhodin or tetracenomycin C. Similar linkage of antibiotic export genes to genes encoding TetA/TetR-like regulators has been reported in biosynthetic gene clusters for other antibiotics, such as rifamycin (*rifP/rifQ*; August *et al.*, 1998), landomycin (*lanJ/lanK*; Westrich *et al.*, 1999) and pristinamycin (*pip/ptr*; Folcher *et al.*, 2001).

The genetic control mechanisms for tetracycline resistance are well characterized. Expression of *tetA*, which encodes an integral membrane protein of the major facilitator superfamily that exports tetracycline, is controlled by the TetR repressor (Hillen and Berens, 1994). Binding of TetR at a helix-turn-helix motif to two *tet* operators, represses both *tetA* and *tetR*. Only when TetR is released from the *tetR* operator due to association of the operator with tetracycline or its analogues is *tetA* efficiently expressed. Pairs of *tetA* and *tetR* homologues within streptomycete biosynthetic clusters serve to respond to and export the cognate antibiotics.

### **B. Pleiotropic regulatory gene *jadW*<sub>1</sub>**

Pioneering investigations by Khokhlov and co-workers (Zaslavskaya *et al.*, 1979) identified A-factor as a  $\gamma$ -butyrolactone effector pleiotropically regulating streptomycin

production and sporulation in *S. griseus*. Subsequently, Horinouchi *et al.* (1989) discovered *afsA*, encoding a protein responsible for A-factor production. Since a mutation in this gene results in bald strains unable to make streptomycin (Ando *et al.*, 1997), *afsA* is presumed to encode the only pathway-specific enzyme required for biosynthesis of A-factor in *S. griseus*. Homologues of *afsA* are found in *Streptomyces bikiniensis* and some other streptomycetes that produce A-factor-like autoregulators (Hara *et al.*, 1983; Horinouch *et al.*, 1984). Identification of a target gene of the A-factor receptor in *S. griseus* enabled Ohnishi *et al.* (1999) to propose an A-factor regulatory cascade leading to streptomycin biosynthesis.

The close similarity between the deduced amino acid sequence of *jadW*<sub>1</sub> from *S. venezuelae* and the sequence of AfsA from *S. griseus*, revealed by a BlastX search of GenBank, suggests that *jadW*<sub>1</sub> is associated with a  $\gamma$ -butyrolactone regulatory system. This possibility is supported by close resemblance also between *JadW*<sub>1</sub> and products of the *afsA* homologues *barX*, *scbA* and *farX*, which are associated with the biosynthesis and function of  $\gamma$ -butyrolactone autoregulators in other streptomycetes (Table 5; Waki *et al.*, 1997). *BarX* is a pleiotropic regulatory protein controlling the biosynthesis of VB and virginiamycin, as well as resistance to virginiamycin M1 in *S. virginiae* (Kawachi *et al.*, 2000). Even though *afsA* and *barX* exhibit very high sequence similarity, and receptor proteins for A-factor and VB share certain characteristics, there is evidence (Kawachi *et al.*, 2000) that the two genes encode proteins with different functions in their host strains, and that *BarX* does not function as an enzyme for  $\gamma$ -butyrolactone synthesis. In *S.*

**Table 5.** Features of some  $\gamma$ -butyrolactone regulatory genes

Gene	Size aa coded	aa identity to AfsA	Producer	Phenotype of disrupted mutant	
				Sporulation	Antibiotic production
<i>afsA</i>	301		<i>S. griseus</i>	-	-
<i>barX</i>	294	41%	<i>S. virginiae</i>	+	-
<i>scbA</i>	314	63%	<i>S. coelicolor</i>	+	+
<i>farX</i>	291	40%	<i>S. sp.</i> FRI-5	N/R <sup>#</sup>	N/R <sup>#</sup>
<i>jadW1</i>	309	41%	<i>S. venezuelae</i> ISP5230	-	-

<sup>#</sup>N/R, not reported.

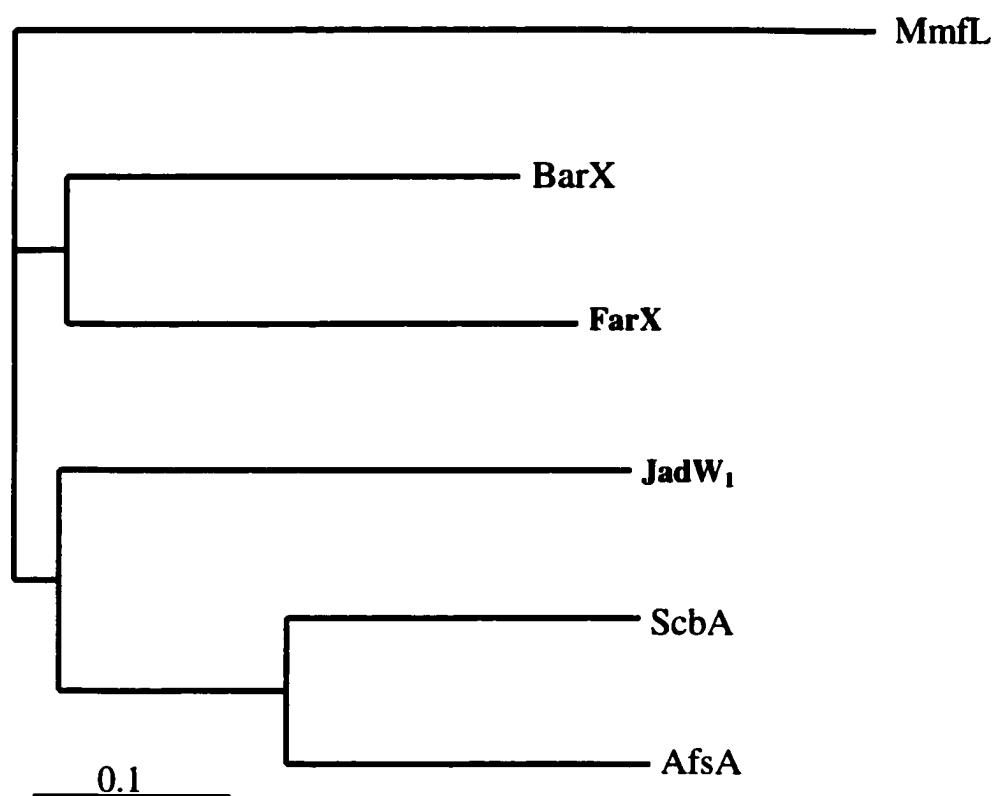
*coelicolor* A3(2) ScbA is a positively acting autoregulator that, like AfsA and BarX, is required for  $\gamma$ -butyrolactone biosynthesis; its gene lies near and is divergently transcribed from *scbR*, encoding a  $\gamma$ -butyrolactone-binding protein (Takano *et al.*, 2001). *Streptomyces* sp. FRI-5 contains *farX*, a homologue of *barX* and *afsA*. The *farX* gene is transcribed in the same direction as *farA*, which encodes a receptor for the autoregulator IM-2 (Waki *et al.*, 1997). A second *afsA* homologue (*mmfL*) in *S. coelicolor* A3(2) shows only 38% sequence identity to *jadW*<sub>1</sub>. It is located at one end of plasmid SCP1, close to two genes encoding proteins that resemble ArpA of *S. griseus*, and encodes a protein involved in biosynthesis of a  $\gamma$ -butyrolactone influencing methylenomycin production (O'Rourke & Chater, 2001).

The absence of either JdB or Cm production by strains disrupted in *jadW*<sub>1</sub>, and restored formation of both products when plasmid-borne copies of *jadW*<sub>1</sub> were introduced, indicated that the gene product activates both antibiotic biosynthesis pathways. The evidence that antibiotic activity measured in bioassays exceeded Cm titres measured by HPLC when plasmid-borne copies of *jadW*<sub>1</sub> were introduced into wild-type *S. venezuelae* implies that the gene product stimulated biosynthesis of at least one additional metabolite with antibiotic activity. That *jadW*<sub>1</sub> regulates biosynthesis of this new antibiotic is implied by the very small inhibition zones detected in bioassays of *jadW*<sub>1</sub>-disrupted mutants. Accumulation of acetyl-Cm when plasmid-borne extra copies of *jadW*<sub>1</sub> were introduced into the wild type could be a response to overstimulation of the Cm biosynthetic pathway; acetyl-Cm is a non-toxic intermediate shielding the producer during Cm biosynthesis (Gross *et al.*, 2002).

Since extracts from wild-type cultures of *S. venezuelae* failed to restore antibiotic biosynthesis and sporulation in *jadW*<sub>1</sub> mutants, the protein encoded by *jadW*<sub>1</sub> is not (like A-factor) the single specific enzyme needed to restore biosynthesis of a  $\gamma$ -butyrolactone effector (here tentatively named J-factor) controlling these activities. It might instead (like BarX) be a positively acting regulatory protein needed for secondary metabolism and differentiation, or alternatively, it might, like ScbA (Takano *et al.*, 2001) have both catalytic and regulatory activities. In either case, failure of extracts from JdB-producing cultures to restore JdB production or sporulation in *jadW*<sub>1</sub>-disrupted strains implies that J-factor, if it was supplied by the wild type extracts in sufficient concentration to be effective, does not compensate for disruption of *jadW*<sub>1</sub>. Reversal of the *bld* phenotype when the mutant was grown on TO agar suggests that TO contains a substance stimulating sporulation. That the substance is sequestered in spores, and is active at a very low concentration was indicated by the densely sporulating lawns obtained on MYM agar with spores from TO agar colonies, even after 10 further serial transfers on MYM agar. Failure of the spores to give sporulating colonies after they were rigorously washed with water implies that the substance, though tightly bound, is indeed a small regulatory molecule. Further evidence is provided by the production of JdB in cultures inoculated with spores of the *jadW*<sub>1</sub> mutant from TO agar, but not when inoculated with bald mycelium collected from MYM agar.

Supplying A-factor at concentrations in the nM range to A-factor<sup>-</sup> mutants of *S. griseus* can restore streptomycin production and sporulation (Miyake *et al.*, 1989). Other  $\gamma$ -

butyrolactones act at similar concentrations in their target *Streptomyces* species; production of VB in *S. virginiae* and IM-2 in *Streptomyces* sp. SRI-5 is estimated to be in the range 0.1 - 1.0 mg per litre of culture (Yamada *et al.*, 1987; Yamada & Nihira, 1999). It is apparent that only small amounts need to be produced *in vivo* to exert regulatory effects. Based on  $\gamma$ -butyrolactone concentrations in other streptomycetes, extracts from the 20 and 50 ml cultures of *S. venezuelae* used here for bioassays and complementation tests would have contained at least 2 and 5  $\mu$ g, respectively, of the putative J-factor. Since bioassays showed that the mutation blocking A-factor biosynthesis in *S. griseus* HH1 was not complemented by these extracts, the putative J-factor is presumed to differ in activity from A-factor. Subjecting the *S. venezuelae* cultures to ethanol stress did not affect the result. Supplementation of *jadW*<sub>1</sub>-disrupted *S. venezuelae* with IM-2 did restore either JdB production or sporulation in the mutant, indicating that J-factor either differs from IM-2 or that intact *jadW*<sub>1</sub> is necessary to express these activities. The possibility that J-factor is a VB-type  $\gamma$ -butyrolactone was not tested. An analysis of relationships between gene products associated with the formation of  $\gamma$ -butyrolactones in streptomycetes (Fig. 44) grouped *JadW*<sub>1</sub> with *AfsA* and *ScbA*, and showed it to be more distantly related to *BarX* and *FarX*. All of these are well separated from *MmfL*. The specificity of receptor proteins for autoregulators (Miyake *et al.*, 1989; Waki *et al.*, 1997; Kinoshita *et al.*, 1997; Kitani *et al.*, 1999) means any variation in lactone ring substituents, hydroxy groups, C-2 alkyl chain length and stereochemistry in the molecules affects their physiological activity (Yamada & Nihira, 1998). This ligand specificity can explain the failure of  $\gamma$ -butyrolactone effectors from other streptomycetes



**Figure 44.** Phylogenetic relationship of JadW<sub>1</sub> to GenBank gene products associated with regulation by  $\gamma$ -butyrolactones.

to complement the *jadW*<sub>1</sub>-disrupted mutant. However, failure to detect complementation with extracts of wild-type cultures grown under various conditions and sampled at various times suggests that the *jadW*<sub>1</sub> mutant phenotype is not merely due to the absence of a small effector molecule. In directly controlling expression of target genes, the *jadW*<sub>1</sub> product could pleiotropically regulate J-factor synthesis as well as morphological differentiation, production of JdB, Cm and unidentified antibiotics in *S. venezuelae* ISP5230.

### **C. Regulatory genes *jadW*<sub>2</sub> and *jadW*<sub>3</sub>**

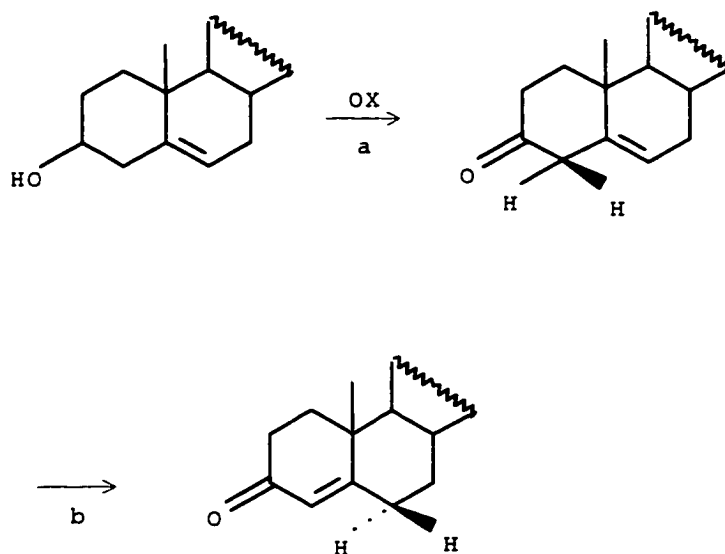
Mutants in which *jadW*<sub>2</sub> was deleted produced titers of JdB 5-10 times higher than the wild type, while introducing an additional copy(s) of *jadW*<sub>2</sub> into the wild type completely abolished JdB production. These results imply that the *jadW*<sub>2</sub> product strongly represses JdB biosynthesis. However, sequence alignments failed to match *JadW*<sub>2</sub> with any known repressor families; instead, the gene product matched in its N-terminal sequence the highly conserved domains of 3- $\beta$ -hydroxysteroid dehydrogenase/isomerases and NAD-dependent epimerase/dehydrases.

In the *jad* cluster *jadW*<sub>2</sub> is located between a pleiotropic positive regulator gene homologous with *afsA*, and a 3-keto ACP/CoA reductase gene. Both of these are implicated in the biosynthesis of  $\gamma$ -butyrolactone regulators of antibiotic biosynthesis and differentiation in *Streptomyces*. *jadW*<sub>2</sub> is transcribed in the same direction as *jadW*<sub>1</sub> and *jadW*<sub>3</sub> so polycistronic expression is possible. However, the *jadW*<sub>2</sub> sequence has no



GenBank homologues associated with regulation by  $\gamma$ -butyrolactones. The 3- $\beta$ -HSD domain in its N-terminal region of *JadW<sub>2</sub>* is a characteristic of dehydrogenase/epimerase pairs involved in the biosynthesis of steroid hormones from cholesterol with NAD(P) as a cofactor. These catalyze the obligatory oxidation and isomerization of 3- $\beta$ -hydroxypregnene and androstene steroid precursors to the 3-ketosteroids needed to form steroid hormones (Fig. 45; Simard, 1991). Known  $\gamma$ -butyrolactones (see Fig. 4; Yamada and Nihira, 1998; Takano *et al.*, 2000) show some structural features of steroids, and might be modified by the products of *jadW<sub>2</sub>* and *jadW<sub>3</sub>*, but further investigation is needed to clarify the role of these genes in *S. venezuelae*.

The complete loss of JdB production in *jadW<sub>1</sub>* mutants, but restoration to 100 – 150% of the wild type amount in mutants with deletions in both *jadW<sub>1</sub>* and *jadW<sub>2</sub>* indicates that *jadW<sub>1</sub>* and *jadW<sub>2</sub>* do not act independently, but participate in a regulatory cascade for *jad* cluster expression. In contrast, retention in *jadW<sub>2</sub>*-deletion mutants of the same response to ethanol treatment as shown by mutants disrupted in *jadR<sub>2</sub>* (Yang *et al.*, 1995) suggests that *jadR<sub>2</sub>* and *jadW<sub>2</sub>* act independently to exert control of JdB biosynthesis. The phenotypes of mutants in which *jadW<sub>2</sub>* had been disrupted, and also those of the wild type host transformed with a cloned copy of *jadW<sub>2</sub>* were consistent with *jadW<sub>2</sub>*, as well as *jadR<sub>2</sub>* encoding strong repressors. The observation that *jadW<sub>2</sub>*-deletion mutants produced white instead of grey spores on MYM agar, and that the wild-type strain containing an extra copy(s) of *jadW<sub>2</sub>* (VS1104) grew slowly and did not sporulate, implicated *jadW<sub>2</sub>* in the control not only of JdB production, but also of sporulation and spore pigment synthesis.



**Figure 45.** Reactions catalyzed by 3-ketosteroid dehydrogenase/isomerase during biosynthesis of steroid hormones. The 3-hydroxyl group of cholesterol is first oxidized to a keto group (step a); this is followed by an essentially irreversible migration of the double bond (step b).

## **VI. Detection of New Antibiotics Produced by *S. venezuelae***

During the efforts to isolate J-factor, two new antibiotics were found to be produced by *S. venezuelae* ISP5230. Although there was insufficient time to purify and determine their structures, the new antibiotics were produced only in Gal2I medium after ethanol treatment, and *jadW*<sub>1</sub>-disrupted mutants showed no antibacterial activity. Therefore, expression of the genes for biosynthesis of these secondary metabolites is evidently regulated by the stress response that elicits JdB production, and is mediated by the *jadW*<sub>1</sub> product.

## **VII. A Possible Regulatory Cascade involving *jadW* Genes**

The strong homology between *jadW*<sub>1</sub> and the *afsA*, *barA*, *farX*, and *scbA* family of genes implicated in positive regulation of streptomycete secondary metabolism and differentiation predicts similar functions for all of these genes. Supporting this prediction, mutants disrupted in *jadW*<sub>1</sub> were unable to produce JdB, Cm and the unidentified antibiotics, and did not sporulate, even when stressed by treatment with ethanol. By analogy with the mechanism of regulation established in *S. griseus* for control of secondary metabolism and morphological differentiation, it is suggested that *S. venezuelae* ISP5230 contains a receptor protein that normally represses these activities, but loses its repressor function after binding a specific  $\gamma$ -butyrolactone effector molecule (J-factor) at its receptor site (Fig. 46).

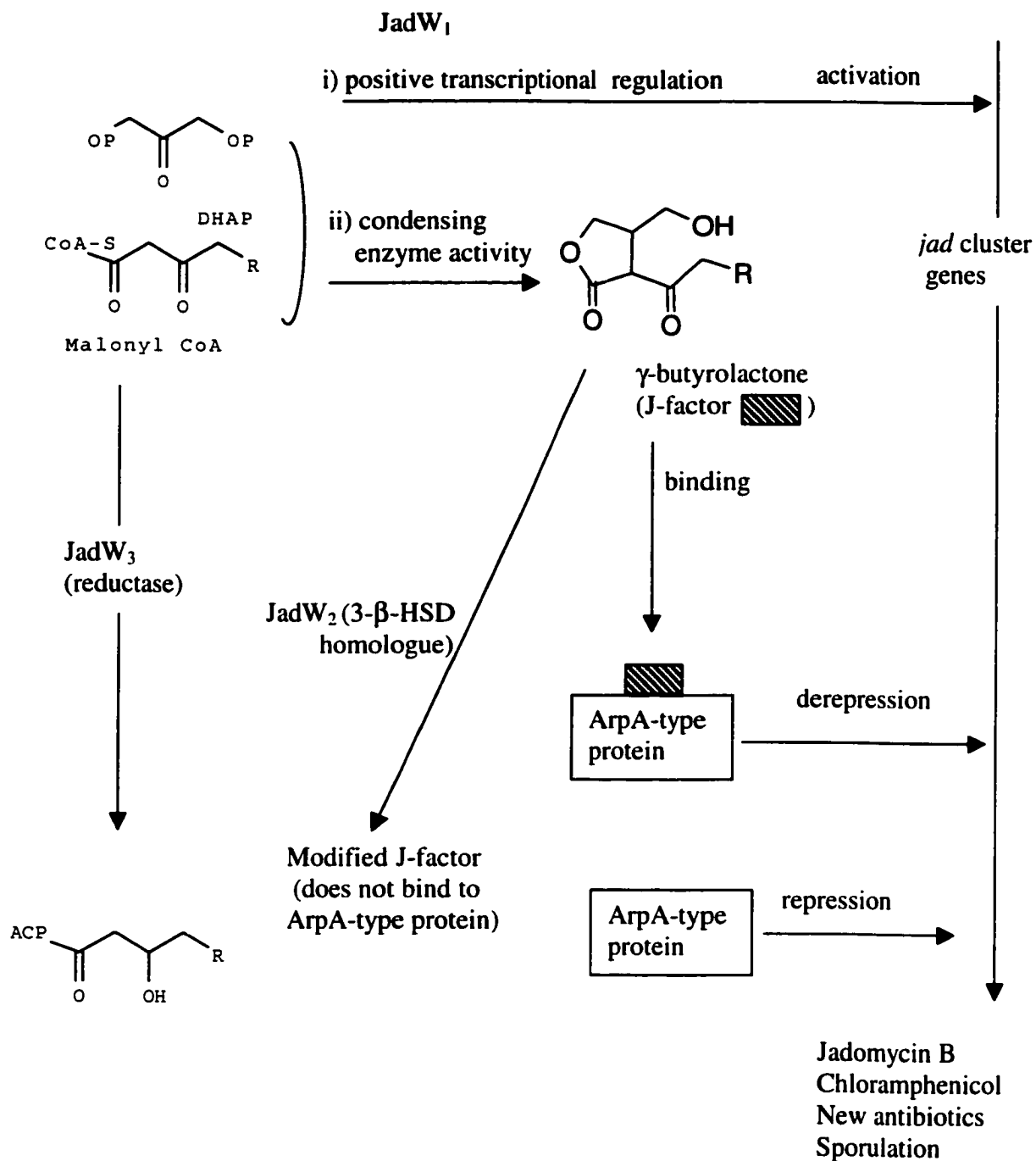


Figure 46. Possible regulatory cascade of *jadW* genes in *S. venezuelae* ISP5230.

The effector is postulated to resemble A-factor of *S. griseus*, and to be synthesized similarly in a condensation of two common intermediary metabolites (dihydroxyacetone phosphate and malonyl CoA) catalyzed by the *jadW<sub>1</sub>* product. However, unlike AfsA in *S. griseus* the *jadW<sub>1</sub>* product not only has enzyme activity but also directly activates transcription of target genes. Thus a mutant disrupted in *jadW<sub>1</sub>* cannot be complemented by culture extracts from the wild type, presumed to contain J-factor. The situation is similar to that reported for regulation by BarX and ScbA (Kawachi *et al.*, 2000; Takano *et al.*, 2000). As in these systems, although culture extracts of wild-type *S. venezuelae* were ineffective, transformation of the disrupted strain with a plasmid carrying the intact gene restored full wild-type activity. Functional similarity between J-factor and A-factor is suggested by the ability of wild-type culture extracts to stimulate the growth of *jadW<sub>1</sub>*-disrupted mutants to the same degree as an A-factor supplement, even though no spores were formed in either treatment. Also of possible significance was the 1.5 fold increase in JdB production achieved by supplementing the standard Gal2I medium with A-factor. While these results suggest that A-factor and J-factor may be structurally related, it is clear from the negative results of bioassays for A-factor activity in *S. griseus* conducted with *S. venezuelae* culture extracts that the two substances are not identical.

Although there is no direct evidence that J-factor is the substrate for the product of *jadW<sub>2</sub>* (as suggested in Fig. 46), this possibility could account for the strong stimulation of JdB production when *jadW<sub>2</sub>* was disrupted. On the assumption that the 3- $\beta$ -hydroxysteroid dehydrogenase/isomerase activity encoded by *jadW<sub>2</sub>* modifies J-factor to a product unable to bind with the J-factor receptor protein, disruption of *jadW<sub>2</sub>* would prevent such

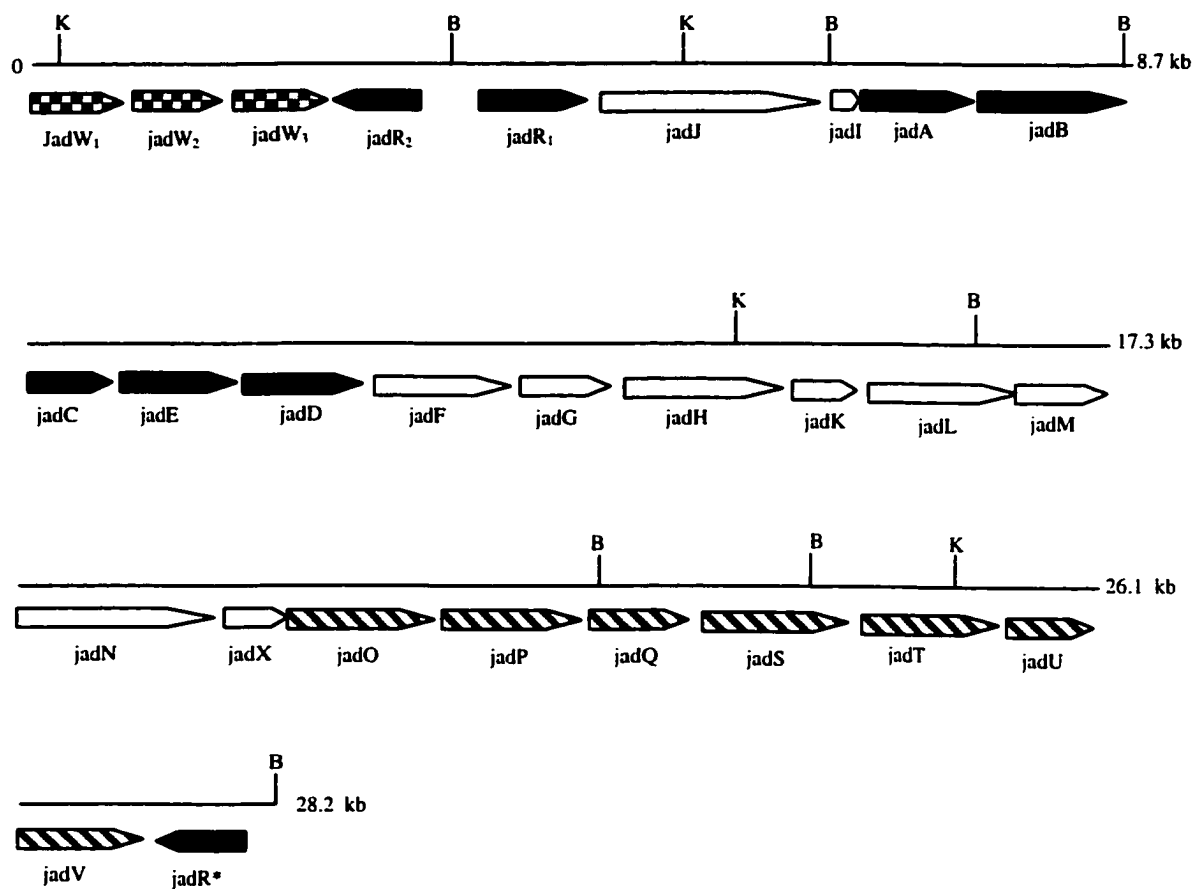
modification, thereby sustaining the derepression of  $JadW_1$  activity that stimulates JdB production. This would be consistent with the loss of JdB and Cm production as well as absence of sporulation in wild-type strains into which additional copies of *jadW<sub>2</sub>* were introduced by transformation. Alternative explanations for the results can be postulated (e.g., that inactivation of *jadW<sub>2</sub>* eliminates a repressor of JdB biosynthesis), but such explanations also lack experimental verification.

Possible participation of *jadW<sub>3</sub>* in a  $\gamma$ -butyrolactone regulatory system rests at present on the reported involvement of its *S. coelicolor* A3 (2) homologue (GenBank AJ007731) in regulation by  $\gamma$ -butyrolactones. Disruption of *jadW<sub>3</sub>* caused only a modest decrease in JdB production, and did not affect Cm production, growth of cultures or sporulation. It is conceivable that the effect on JdB production results from decreased J-factor synthesis caused by competition for malonyl CoA between the  $JadW_3$ -encoded ACP-3-ketoreductase and the  $JadW_1$  condensing enzyme. Even if this connection is confirmed, its importance in the  $\gamma$ -butyrolactone regulatory system is likely to be marginal.

## **IX. Organization of the *jad* Cluster**

Many additional enzymes are needed for the extensive post-synthase modifications of the highly reactive polyketide chains produced by PKS clusters. Their activities include amination, hydroxylation, methylation, oxidation, reduction, and synthesis and attachment of sugars to hydroxyl substituents on the polyketide-derived framework. Genes encoding such activities are usually scattered on both sides of the core PKS genes,

and may be interspersed with genes involved in regulating antibiotic production or conferring self-resistance. Chromosome walking has extended the initial *jad* cluster for JdB biosynthesis at both ends. In this thesis 16 new genes have been identified, and 14 of them were added to the *jad* cluster. The increased size of the *jad* cluster (28 kb) is within the range (22-50 kb) found for gene clusters encoding type-II PKS antibiotic biosynthesis, such as those for actinorhodin (21 kb; Hopwood *et al.*, 1995), tetracenomycin C (24 kb; Seno and Baltz, 1989), oxytetracycline (30 kb; Seno and Baltz, 1989), and landomycin (35 kb; Westrich *et al.*, 1999). Within the *jad* cluster are genes for biosynthesis of jadomycin aglycone and the dideoxyhexose, with those for the core PKS (*jadABC*) in the middle. Genes for polyketide chain cyclization and for modification of the initial aromatic structure are located on either side of *jadABC*; a sub-cluster of genes for sugar biosynthesis is to the right of the core, and genes for export, resistance and regulation are scattered on both sides of the core (Fig. 47). However, notably absent is the gene for a pathway-specific regulator, which should belong to a growing group of transcriptional activators (SARPs, *Streptomyces* antibiotic regulatory proteins; Wietzorrek and Bibb, 1997) found in antibiotic-producing *Streptomyces* spp. These include *actII-ORF4* (Arias *et al.*, 1999), *redD* (Narva and Feitelson, 1990), *cdaR* (Chong, 1998), and *dnrI* (Stutzman-Engwall *et al.*, 1992). In contrast, three repressors (*jadW*<sub>2</sub>, *jadR*<sub>2</sub>, and *jadR*\*) have been identified in the *jad* cluster. Disruption of each of them resulted in overproduction of JdB. Although *jadR*\* is probably the last gene at the right end of the *jad* cluster, *jadW*<sub>1</sub> almost certainly is not the last gene at the left end of the cluster. The sequence of *jadW*<sub>1</sub> is similar to genes associated in other species with  $\gamma$ -butyrolactone biosynthesis and regulation. If, as is likely, *jadW*<sub>1</sub> is functionally related to



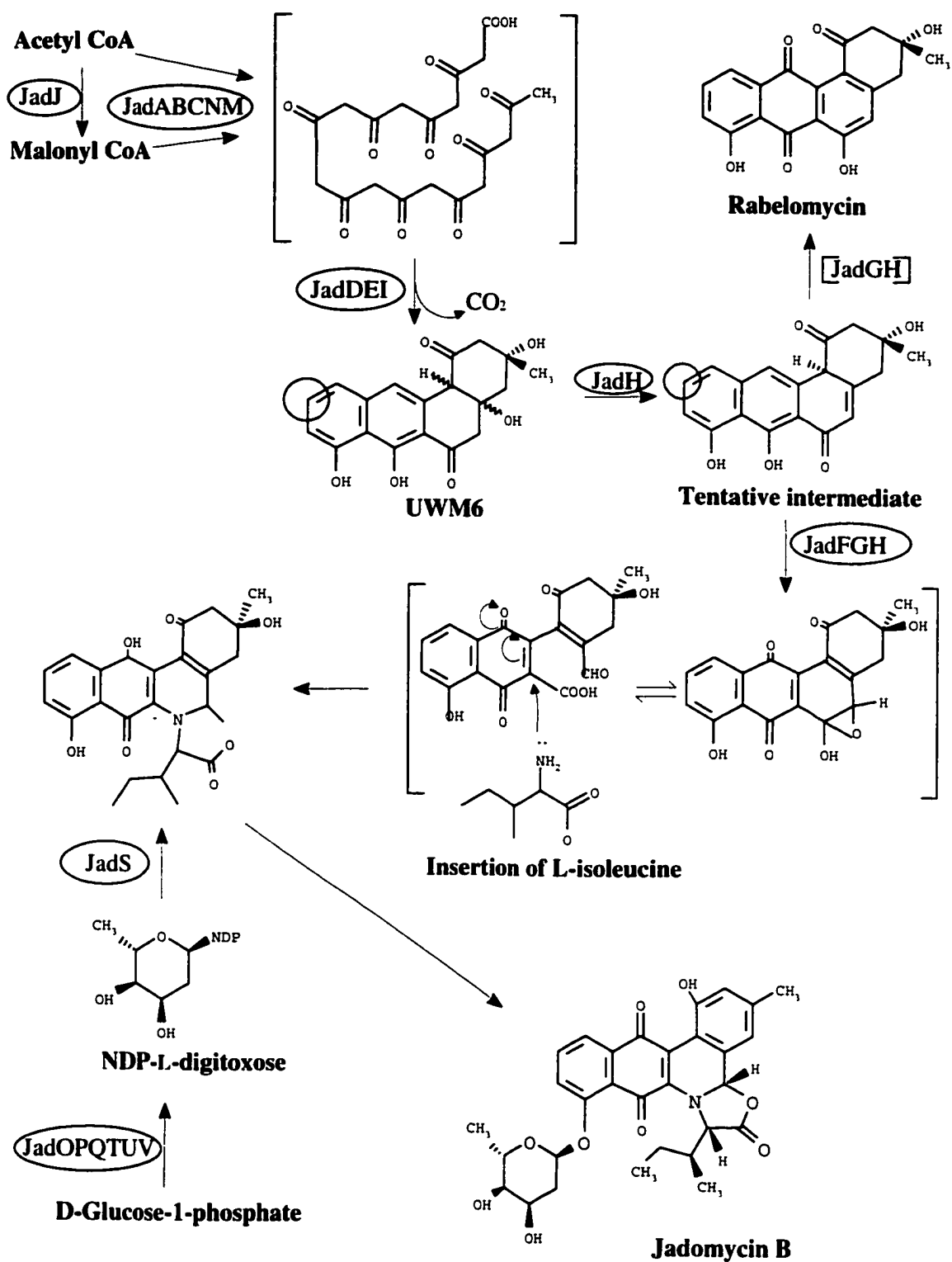
**Figure 47.** The organization of *jad* cluster. Black arrows represent the core PKS cluster; grey arrows, regulatory genes; striped arrows, genes involved in dideoxyhexose biosynthesis; chequered arrows, genes tentatively associated with  $\gamma$ -butyrolactone control; other genes are either involved in early polyketide chain elongation, cyclization or modification of the initial aromatic structure. *Bam*HI and *Kpn*I restriction enzyme sites are labeled (B and K).



these genes, it is probably accompanied further upstream by a  $\gamma$ -butyrolactone receptor gene, and perhaps also by the missing SARP.

### **XI. Putative Biosynthesis Pathway for Jadomycin B in *S. venezuelae* ISP5230**

Most of the genes involved in the pathway for JdB biosynthesis have been located and identified. Functional analysis of many of them allows us to suggest a pathway for biosynthesis of JdB in *S. venezuelae* ISP5230 (Fig. 48). In this the products of *jadJ* catalyze the synthesis of malonyl CoA from acetyl CoA (Han *et al.*, 2000). *JadA*, *B*, *C*, *N* and *M* condense one acetyl CoA with nine malonyl CoA to form the polyketide chain (Han *et al.*, 1994; Yang *et al.*, 1996; Wang *et al.*, 2001). Cyclization of the polyketide chain is catalyzed by the products of *jadD*, *E*, and *I* (Kulowski *et al.*, 1999), and *JadI* is also involved in forming UWM6, a tentative intermediate (McVey, 1998) that requires oxidation with the gene products of *jad F*, *G*, and *H* to generate the quinone group and open the B-ring of jadomycin, allowing the insertion of L-isoleucine at the site of ring opening to form jadomycin aglycone. The *jadG* product is also needed to generate the quinonoid shunt product rabelomycin, which is not involved further in the JdB biosynthesis pathway. The products of *jadO*, *P*, *Q*, *T*, *U*, and *V* catalyze the conversion of D-glucose-1-phosphate to nucleotidyl-L-digitoxose, and the *jadS* product transfers the activated dideoxyhexose moiety to JdB aglycone.



**Figure 48.** Putative pathway for biosynthesis of JdB in *S. venezuelae* ISP5230.

## SUMMARY AND CONCLUSION

Identification of a sugar sub-cluster (*jadOPQSTUV*) provided a complete picture of the synthesis of the glycosidic dideoxyhexose moiety in JdB and its attachment to the aglycone. Characterization and identification of all the structural genes involved in JdB biosynthesis potentially allow construction of new bioactive compounds by combinatorial genetic techniques. Re-examination of the structure of JdB revealed that it is a mixture of two closely related components; the major compound is designated *cis*-JdB, and the minor component *trans*-JdB; the dideoxyhexose attached to the aglycone is L-digitoxose. Characterization of PPTase (*jadM*) and carboxylase (*jadN*) genes provided information on the early steps for jadomycin polyketide chain construction. The regulation/resistance genes controlling JdB and Cm biosynthesis are scattered in both ends of the *jad* cluster. The tetracycline resistance gene homologue (*jadR\**), at the right end of the *jad* cluster, functions as a repressor of JdB production; disrupting it resulted in overproduction of the antibiotic. Regulation of JdB and Cm biosynthesis in *S. venezuelae* ISP5230 by a  $\gamma$ -butyrolactone system resembling the group of autoregulators that control morphogenesis and sporulation in other streptomycetes was indicated by the characterization of three genes, *jadW<sub>1</sub>*, *jadW<sub>2</sub>*, and *jadW<sub>3</sub>*, upstream of the core PKS cluster. Two other secondary metabolites with antibacterial activity were detected in stressed cultures of *S. venezuelae* ISP5230. Elucidation of gene functions in the 28-kb *jad* cluster for JdB biosynthesis provided new insights into secondary metabolism.

## REFERENCES

- Abe, F., Mori, Y., Okabe, H. & Yamauchi, T. (1994).** Steroidal constituents from the roots and stems of *Aclepias fruticosa*. *Chem Pharm Bull (Tokyo)* **42**, 1777-1783.
- Abe, F. & Yamauchi, T. (2000).** Pregnane glycosides from the roots of *Asclepias tuberosa*. *Chem Pharm Bull* **48**, 1017-1022.
- Adamidis, T., Riggle, P. & Champness, W. (1990).** Mutations in a new *Streptomyces coelicolor* locus which globally block antibiotic biosynthesis but not sporulation. *J Bacteriol* **172**, 2962-2969.
- Aguirrezabalaga, I., Olano, C., Allende, N., Rodriguez, L., Brana, A. F., Mendez, C. & Salas, J.A. (2000).** Identification and expression of genes involved in biosynthesis of oleandrose and its intermediate L-olivose in the oleandomycin producer *Streptomyces antibioticus*. *Antimicrob Agents Chemother* **44**, 1266-1275.
- Alberts, A. & Vagelos, P.R. (1972).** Acyl coenzyme A carboxylases. In *The Enzymes*, pp. 17-82. Edited by P. D. Boyer. New York: Academic Press.
- Ando, N., Matsumori, N., Sakuda, S., Beppu, T. & Horinouchi, S. (1997).** Involvement of AfsA in A-factor biosynthesis as a key enzyme. *J Antibiot* **50**, 847-852.
- Andrianopoulos, K., Wang, L. & Reeves, P. R. (1998).** Identification of the fucose synthetase gene in the colanic acid gene cluster of *Escherichia coli* K-12. *J Bacteriol* **180**, 998-1001.
- Anisova, L. N., Blinova, I. N., Efremenkova, O. V., Kozmin, YuP., Onoprienko, V. V., Smimova, G. M., & Khokhlov, A. S. (1984).** Development regulators in *Streptomyces coelicolor* A3(2). *Izv Zkad Nzuk SSSR, Ser Biol* **1**, 98-109.
- Arias, P., Fernandez-Moreno, M. A. & Malpartida, F. (1999).** Characterization of the pathway-specific positive transcriptional regulator for actinorhordin biosynthesis in *Streptomyces coelicolor* A3(2) as a DNA-binding protein. *J Bacteriol* **181**, 6959-6968.
- August, P. R., Tang, L., Yoon, Y. J., Ning, S., Muller, R., Yu, T. W., Taylor, M., Hoffmann, D., Kim, C. G., Zhang, X., Hutchinson, C. R. & Floss, H. G. (1998).** Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. *Chem Biol* **5**, 69-79.
- Ayer, S. W., McInnes, A. G., Thibault, P., Walter, J. A., Doull, J. L., Parnell, T. & Vining, L. C. (1991).** Jadomycin, a novel 8H-benz[b]oxazolo[3,2-f]phenanthridine antibiotic from *Streptomyces venezuelae* ISP5230. *Tet Lett* **32**, 6301-6304.

**Baltz, R. H. & Seno, E. T. (1988).** Genetics of *Streptomyces fradiae* and tylosin biosynthesis. *Ann Rev Microbiol* **42**, 547-74.

**Bartel, P. L., Connors, N. C. & Strohl, W. R. (1990).** Biosynthesis of anthracyclines: analysis of mutants of *Streptomyces* sp. Strain C5 blocked in daunomycin biosynthesis. *J General Microbiol.* **136**, 1877

**Bechthold, A., Sohng, J. K., Smith, T. M., Chu, X. & Floss, H. G. (1995).** Identification of *Streptomyces violaceoruber* Tü22 genes involved in the biosynthesis of granaticin. *Mol Gen Genet.* **248**, 610-620.

**Berens, C., Schnappinger, D. & Hillen, W. (1997).** The role of the variable region in Tet repressor for inducibility by tetracycline. *J Biol Chem* **272**, 6936-6942.

**Bergh, S. & Uhlen, M. (1992).** Analysis of a polyketide synthesis-encoding gene cluster of *Streptomyces curacoi*. *Gene* **117**, 131-136.

**Bibb, M.J., Biro, S., Motamedi, H., Collins, J.F. & Hutchinson, C.R. (1989).** Analysis of the nucleotide sequence of the *Streptomyces glaucescens tcml* genes provides key information about the enzymology of polyketide antibiotic biosynthesis *EMBO J.* **8**, 2727-2736.

**Bibb, M.J., Sherman, D.H., Omura, S. & Hopwood, D.A. (1994).** Cloning, sequencing and deduced functions of a cluster of *Streptomyces* genes probably encoding biosynthesis of the polyketide antibiotic frenolicin. *Gene* **142**, 31-39.

**Bibb, M. J., Molle, V. & Buttner, M. J. (2000).** Sigma (BldN), an extracytoplasmic function RNA polymerase sigma factor required for aerial mycelium formation in *Streptomyces coelicolor* A3(2). *J Bacteriol* **182**, 4606-4616.

**Black, T. A. & Wolk, C. P. (1994).** Analysis of a HetI mutation in *Anabaena* sp. strain PCC7120 implicates a secondary metabolite in the regulation of heterocyst spacing. *J Bacteriol* **176**, 2282-2292.

**Blanco, G., Pereda, A., Mendez, C. & Salas, J. (1992).** Cloning and disruption of a fragment of *Streptomyces halstedii* DNA involved in the biosynthesis of a spore pigment. *Gene* **112**, 59-65.

**Bochkov, A. F., Zaikov, G. E. & Afanasiev, V. A. (1991).** *Carbohydrates.* <<< Publisher >>> Utrecht and Tokyo.

**Borchert, S., Stachelhaus, T. & Marahiel, M. A. (1994).** Induction of surfactin production in *Bacillus subtilis* by *gsp*, a gene located upstream of the gramicidin S operon in *Bacillus brevis*. *J Bacteriol* **176**, 2458-2462.

**Brian, P., Riggle, F. J., Santos, R. A. & Champness, W. C. (1996).** Global negative regulation of *Streptomyces coelicolor* antibiotic synthesis mediated by an *absA*-encoded putative signal transduction system. *J Bacteriol* **178**, 3221-3231.

**Brown, M. P., Aidoo, K. A., & Vining, L. C. (1996).** A role for *pabAB*, a *p*-aminobenzoate synthase gene of *Streptomyces venezuelae* ISP5230 in chloramphenicol biosynthesis. *Microbiology* **142**, 1345-1355.

**Butler, M. J., Friend, E. J., Hunter, I. S., Kaczmarek, F. S., Sudgen, D. A. & Warren, M. (1989).** Molecular cloning of resistance genes and architecture of a linked gene cluster involved in the biosynthesis of tetracycline by *Streptomyces rimosus*. *Mol Gen Genet* **215**, 231-38.

**Caballero, J. L., Malpartida, F. & Hopwood, D. A. (1991).** Transcriptional organization and regulation of an antibiotic export complex in the producing *Streptomyces* culture. *Mol Gen Genet* **228**, 372-380.

**Campbell, J. A., Davies, G. J., Bulone, V. & Henrissat, B. (1997).** A classification of nucleotide-diphospho-sugar glycosyltransferases based on amino acid sequence similarities. *Biochem J* **326**, 929-939.

**Carreras, C. W., Gehring, A. M., Walsh, C. T. & Khosla, C. (1997).** Utilization of enzymatically phosphopantetheinylated acyl carrier proteins and acetyl-acyl carrier proteins by the actinorhodin polyketide synthase. *Biochemistry* **36**, 11757-11761.

**Champness, W.C. (1988).** New loci required for *Streptomyces coelicolor* morphological and physiological differentiation. *J Bacteriol* **170**, 1168-1174.

**Chang, Z. (1999).** Genes for cysteine biosynthesis and metabolism in *Streptomyces venezuelae* ISP5230: cloning, functional analysis and relevance to chloramphenicol biosynthesis. *Ph. D. Thesis*. Dalhousie University.

**Chang, Z., Sun, Y., He, J. & Vining, L. C. (2001).** *p*-Aminobenzoic acid and chloramphenicol biosynthesis in *Streptomyces venezuelae*: gene sets for a key enzyme, 4-amino-4-deoxychorismate synthase. *Microbiology* **147**, 2113-2126.

**Chakraborty, R., White, J., Takano, E. & Bibb, M. (1996).** Cloning, characterization and disruption of a (p)ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2). *Mol Microbiol* **19**, 357-368.

**Chakraborty, R. & Bibb, M. (1997).** The ppGpp synthetase gene (*relA*) of *Streptomyces coelicolor* A3(2) plays a conditional role in antibiotic production and morphological differentiation. *J Bacteriol* **179**, 5854-61

**Chater, K. F. & Bibb, M. J. (1997).** Regulation of bacterial antibiotic production. *Biotechnology*, eds. H. Kleinkauf and H. von Dohren; VCH, Weinheim, Germany. 7: 57-105.

**Chong, P., Podmore S. M., Kieser, H. M., Redenbach, M., Turgay, K., Marahiel, M., Hopwood, D. A. & Smith, C. P. (1998).** Physical identification of a chromosomal locus encoding biosynthetic genes for the lipopeptide calcium-dependent antibiotic (CDA) of *Streptomyces coelicolor* A3(2). *Microbiol* **144**, 193-199.

**Christopher, T. W., Gehring, A. M., Weinreb, P. H., Quadri, L. E. & Flugel, R. S. (1997).** Post-translational modification of polyketide and nonribosomal peptide synthases. *Current Opinion in Chemical Biology* **1**, 309-315.

**Cox, R. J., Hitchman, T. S., Byrom, K. J., Findlow, I. S., Tanner, J. A., Crosby, J. & Simpson, T. J. (1997).** Post-translational modification of heterologously expressed *Streptomyces* type-II polyketide synthase acyl carrier proteins. *FEBS Letters* **405**, 267-272.

**Crowell, K.G. (1993).** Biosynthesis of jadomycin. *B.Sc. Honors Thesis*. Acadia University, Canada.

**Decker, H., Gaisser, S., Pelzer, S., Schneider, P., Westrich, L., Wohlleben, W. & Bechthold, A. (1996).** A general approach for cloning and characterization dNDP-glucose dehydratase genes from actinomycetes. *FEMS Microbiol Lett* **141**, 195-201.

**Doull, J., Ahmed, Z., Stuttard, C. & Vining, L. C. (1985).** Isolation and characterization of *Streptomyces venezuelae* mutants blocked in chloramphenicol biosynthesis. *J Gen Microbiol* **131**, 97-104.

**Doull, J. L., Vats, S., Chaliciopoulos, M., Stuttard, C., Wong, K. & Vining, L. C. (1986).** Conjugational fertility and location of chloramphenicol biosynthesis genes on the chromosomal linkage map of *Streptomyces venezuelae*. *J Gen Microbiol* **132**, 1327-1338.

**Doull, J. L., Ayer, S. W., Singh, A. K. & Thibault, P. (1993).** Production of a novel polyketide antibiotic, jadomycin B, by *Streptomyces venezuelae* following heat shock. *J Antibiot* **46**, 849-871.

**Doull, J. L., Singh, A. K., Hoare, M. & Ayer, S. W. (1994).** Conditions for the production of jadomycin B by *Streptomyces venezuelae* ISP5230: effects of heat shock, ethanol treatment and phage infection. *J Ind Microbiol* **13**, 120-125.

**Doull, J. L. & Vining, L. C. (1995).** Global physiological controls. In *Genetics and Biochemistry of Antibiotic Production*. p 9-64. edited by L. C. Vining and C. Stuttard, Butterworth-Heinemann.

**Draeger, G., Park, S-H. & Floss, H. G. (1999).** Mechanism of the 2-deoxygenation step in the biosynthesis of the deoxyhexose moieties of the antibiotics granaticin and oleandomycin. *J Am Chem Soc* **121**, 2611-2612.

**Efremenkova, O. V., Anisova, L. N. & Bartoshevich I. E. (1983).** Regulators of actinomycete differentiation. *Antibiot Med Biotekhnol* **30**, 687-707.

**Ehmann, E. E., Gehring, A. M. & Walsh, C. T. (1999).** Lysine biosynthesis in *Saccharomyces cerevisiae*: mechanism of  $\alpha$ -aminoacidate reductase (Lys 2) involved in post-translational phosphopantetheinylation by Lys 5. *Biochemistry* **38**, 6171-6177.

**Elliot, M., Damji, F., Passantino, R., Vhater, K. & Leskiw, B. (1996).** The *bldD* gene of *Streptomyces coelicolor* A3(2): a regulatory gene involved in morphogenesis and antibiotic production. *J Bacteriol.* **180**, 1549-1555.

**Elliot, M. A, Bibb, M. J., Buttner, M. J. & Leskiw, B. K. (2001).** BldD is a direct regulator of key developmental genes in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **40**, 257-269.

**Facey, S. (1994).** Cloning, sequencing and expression of a bromoperoxidase-catalase gene from *Streptomyces venezuelae*. *Ph.D. Thesis*. Hohenheim University.

**Faust, B., Hoffmeister, D., Weitnauer, G., Westrich, L., Haag, S., Schneider, P., Decker, H., Kunzel, E., Rohr, J. & Bechthold, A. (2000).** Two new tailoring enzymes, a glycosyltransferase and an oxygenase, involved in biosynthesis of the angucycline antibiotic urdamycin A in *Streptomyces fradiae* Tü2717. *Microbiology.* **146**, 147-54.

**Fernandez-Moreno, M. A., Cabellero, J. L., Hopwood, D. A. & Malpartida, F. (1991).** The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by the *bldA* tRNA gene of *Streptomyces*. *Cell* **66**, 769-780.

**Flett, F., Mersinias, V. & Smith, C. P. (1997).** High efficiency intergeneric conjugal transfer of plasmid DNA from *Escherichia coli* to methyl DNA-restricting streptomycetes. *FEMS Microbiol Lett* **155**, 223-229.

**Folcher, M., Morris, R. P., Dale, G., Salah-Bey-Hocini, K., Viollier, P. H. & Thompson, C. J. (2001).** A transcriptional regulator of a pristinomycin resistance gene in *Streptomyces coelicolor*. *J Biol Chem* **276**, 1479-1485.

**Gaisser, S., Bohm, G. A., Doumith, M., Raynal, M. C., Dhillon, N., Corres, J. & Leadlay, P. F. (1998).** Analysis of *eryBI*, *eryBIII* and *eryBVII* from the erythromycin biosynthetic gene cluster in *Saccharopolyspora erythraea*. *Mol Gen Genet* **258**, 78-88.

**Gaitatzis, N., Han, A., Muller, R. & Beyer, S. (2001).** The *mtaA* gene of the myxothiazol biosynthetic gene cluster from *Stigmatella aurantiaca* DW4/3-1 encodes a



phosphopantetheinyl transferase that activates polyketide synthases and polypeptide synthetases. *J Biochem (Tokyo)* **129**, 119-124.

**Gehring, A. M., Lambalot, R. H., Vogel, K. W., Drucehammer, D. G. & Walsh, C. T. (1996).** Ability of *Streptomyces* spp. acyl carrier proteins and coenzyme A analogs to serve as substrates *in vitro* for *E. coli* holo-ACP synthase. *Chem Biol* **4**, 17-24.

**Gehring, A. M., Bradley, K. A. & Walsh, C. T. (1997).** Enterobactin biosynthesis in *Escherichia coli*; isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. *Biochemistry* **36**, 8495-8503.

**Gehring, A. M., Yoo, N. J. & Losick, R. (2001).** RNA polymerase sigma factor that blocks morphological differentiation by *Streptomyces coelicolor*. *J Bacteriol* **183**, 5991-5996.

**Gokhale, R. S., Tsuji, S. Y., Cane, D. E. & Khosla, C. (1999).** Dissection and exploiting intermodular communication in polyketide synthases. *Science* **284**, 482-485.

**Grimm, A., Madduri, K., Ali, A. & Hutchinson, C. R. (1994).** Characterization of the *Streptomyces peucetius* ATCC 29050 genes encoding doxorubicin polyketide synthase. *Gene* **151**, 1

**Gross, F., Lewis, A. L., Pirae, M., Van Pée, K.-H., Vining, L. C. & White, R. L. (2002).** Isolation of 3'-O-Acetylchloramphenicol: a possible intermediate in chloramphenicol biosynthesis. *Bioorg & Med Chem Lett* **12**, 283-286.

**Guilfoile, P. G. & Hutchinson, C. R. (1992).** The *Streptomyces glaucescens* TcmR protein represses transcription of the divergently oriented *tcmR* and *tcmA* genes by binding to an intergenic operator region *J Bacteriol* **174**, 3659-3666.

**Han, L., Yang, K., Kulowski, K., Wendt-Pienkowski, E., Hutchinson, C. R. & Vining, L. C. (2000).** An acyl-coenzyme A carboxylase-encoding gene associated with jadomycin biosynthesis in *Streptomyces venezuelae* ISP 5230. *Microbiology* **146**, 903-910.

**Han, L., Yang, K., Ramalingam, E. Mosher, R. H. & Vining, L. C. (1994).** Cloning and characterization of polyketide synthase genes for jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230. *Microbiology* **140**, 3379-3389.

**Hara, O., Horinouch, S., Uozumi, T. & Beppu, T. (1983).** Genetic analysis of A-factor synthesis in *Streptomyces coelicolor* A3(2) and *Streptomyces griseus*. *J Gen Microbiol* **129**, 2939-2944.

**Hashimoto, K., Nihira, T., Sakuda, S. & Yamada, Y. (1992).** IM-2, a butyrolactone autoregulator, induces production of several nucleoside antibiotics in *Streptomyces* sp. FRI-5. *J Fermentation and Bioengineering* **73**, 449-455.

**He, J., Magarvey, N., Pirae, M. & Vining, L. C. (2001).** The gene cluster for chloramphenicol biosynthesis in *Streptomyces venezuelae* ISP5230 includes novel shikimate pathway homologues and a monomodular nonribosomal peptide synthetase gene. *Microbiology* **147**, 2817-2829.

**Hernandez, C., Olano, C., Mendez, C. & Salas, J. A. (1993).** Characterization of a *Streptomyces antibioticus* gene cluster encoding a glycosyltransferase involved in oleandomycin inactivation. *Gene*. **134**, 139-140.

**Hoffmeister, D., Ichinose, K., Domann, S., Faust, B., Trefzer, A., Drager, G., Kirschning, A., Fischer, C., Kunzel, E., Bearden, D.W., Rohr, J. & Bechthold, A. (2000).** The NDP-sugar co-substrate concentration and the enzyme expression level influence the substrate specificity of glycosyltransferases: cloning and characterization of deoxysugar biosynthetic genes of the urdamycin biosynthetic gene cluster. *Chem & Biol* **7**, 821-831.

**Hopwood, D. A. (1988).** Towards an understanding of gene switching in *Streptomyces*, the basis of sporulation and antibiotic production. *Proc R Soc Lond* **235**, 121-138.

**Hopwood, D. A. (1997).** Genetic contributions to understanding polyketide synthases. *Chem Rev* **97**, 2465-2497.

**Hopwood, D. A. & Sherman, D. H. (1990).** Molecular genetics of polyketides and its comparison to fatty acid biosynthesis. *Annu Rev Genet* **24**, 37-66.

**Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P., Ward, J. M. & Schrempf, H. (1985).** *Genetic Manipulation of Streptomyces: a Laboratory Manual*. The John Innes Foundation, Norwich.

**Hopwood, D.A., Chater, K.F. & Bibb, M.J. (1995).** Genetics of antibiotic production in *Streptomyces coelicolor* A3(2), a model streptomycete. In *Genetics and Biochemistry of Antibiotic Production*, edited by Vining, L.C. & Stuttard, C. Butterworth-Heinemann, Newton, MA. p 65-102.

**Horinouchi, S. & Beppu, T. (1992).** Autoregulatory factors and communication in actinomycetes. *Annu Rev Microbiol* **46**, 377-98.

**Horinouchi, S., Hara, O. & Beppu, T. (1983).** Cloning of a pleiotropic gene that positively controls biosynthesis of A-factor, actinorhodin, and prodigiosin in *Streptomyces coelicolor* A3(2) and *Streptomyces lividans*. *J Bacteriol* **155**, 1238-1248.

**Horinouchi, S., Kumada, Y. & Beppu, T. (1984).** Unstable genetic determinant of A-factor biosynthesis in streptomycin-producing organisms: cloning and characterization. *J Bacteriol* **158**, 481-487.

**Horinouchi, S., Suzuki, H., Nishiyama, M & Beppu, T. (1989).** Nucleotide sequence and transcriptional analysis of the *Streptomyces griseus* gene (*afsA*) responsible for A-factor biosynthesis. *J Bacteriol* **171**, 1206-1210.

**Horinouchi, S., Kito, M., Nishiyama, M., Furuya, K., Hong S., Miyake, K. & Beppu, T. (1990).** Primary structure of AfsR, a global regulatory protein for secondary metabolite formation in *Streptomyces coelicolor* A3(2). *Gene* **95**, 49-56.

**Huan, V. D., Ohtani, K., Kasai, R., Yamasaki, K. & Tuu, N. V. (2001).** Sweet pregnane glycosides from *Telosma procumbens*. *Chem Pharm Bull* **49**, 453-460.

**Hutchinson, C. R. & Fujii, I. (1995).** Polyketide synthase gene manipulation: A structure-function approach in engineering novel antibiotics. *Annu Rev Microbiol* **49**, 201-238.

**Hutchinson, C. R. (1999).** Microbial polyketide synthases: more and more prolific. *Proc. Natl. Acad. Sci. USA* **96**, 3336-3338.

**Ikeda, H., Nonomiya, M. U., Ohta, T. & Omura, S. (1999).** Organization of the biosynthetic gene cluster for the polyketide antihelminthic macrolide avermectin in *Streptomyces avermitilis*. *Proc. Natl Acad Sci USA* **96**, 9509-9514.

**Ingram, C., Brawner, M., Youngman, P. & Westpheling, J. (1989).** *xylE* functions as an efficient reporter gene in *Streptomyces* spp.: use for the study of *galP1*, a catabolite-controlled promoter *J Bacteriol* **171**, 6617-6624.

**Ishikawa, J. & Hotta, K. (1999).** FramePlot: a new implementation of the Frame analysis for predicting protein-coding regions in bacterial DNA with a high G+C content. *FEMS Microbiol Lett* **174**, 252-253.

**Ishizuka, H., Horinouchi, S., Kieser, H. M., Hopwood, D. A. & Bepp, T. (1992).** A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. *J Bacteriol* **174**, 7585-7594.

**Kaster, K. R., Burgett, S. G., Nagarajah, R. R. & Ingolia, T. D. (1983).** Analysis of a bacterial hygromycin B resistance gene by transcriptional and translational fusions and by DNA sequencing. *Nucleic Acids Res* **11**, 6895-6911.

**Katz, L. & Donadio, S. (1993).** Polyketide synthesis: prospects for hybrid antibiotics. *Annu Rev Microbiol* **47**, 875-912.

**Kawachi, R., Akashi, T., Kamitani, Y., Sy, A., Wangchaisoonthorn, U., Nihira, T. & Yamada, Y. (2000).** Identification of an AfsA homologue (BarX) from *Streptomyces virginiae* as a pleiotropic regulator controlling autoregulator biosynthesis, virginiamycin biosynthesis and virginiamycin M1 resistance. *Mol Microbiol* **36**, 302-313.

**Kelemen, G. H., Viollier, P. H., Tenor, J., Marri, L., Buttner, M. J. & Thompson, C. J. (2001).** A connection between stress and development in the multicellular prokaryote *Streptomyces coelicolor* A3(2). *Mol Microbiol* **40**, 804-814.

**Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F. & Hopwood, D. A. (2000).** *Practical Streptomyces Genetics*. The John Innes Foundation, Norwich.

**Kim, E-S., Bibb, M.J., Butler, M.J., Hopwood, D.A. & Sherman, D.H. (1994).** Nucleotide sequence of the oxytetracycline (*otc*) polyketide synthase genes from *Streptomyces rimosus*. *Gene* **141**, 141-142.

**Kinoshita, H., Ipposhi, H., Okamoto, S., Nakano, H., Nihira, T. & Yamada, Y. (1997).** Butyrolactone autoregulator receptor protein (BarA) as a transcriptional regulator in *Streptomyces virginiae*. *J Bacteriol* **179**: 6986-6993.

**Kinashi, H., Shimaji, M. & Sakai, A. (1987).** Giant linear plasmids in *Streptomyces* which code for antibiotic biosynthesis genes. *Nature* **328**, 454-456.

**Kirschning, A., Bechthold, A. & Rohr, J. (1997).** Chemical and biochemical aspects of deoxysugars and deoxysugar oligosaccharides. *Topics Curr. Chem.* **188**, 1-84.

**Kitani, S., Kinoshita, H., Nihira, T. & Yamada, Y. (1999).** In vitro analysis of the butyrolactone autoregulator receptor protein (FarA) of *Streptomyces lavendulae* FRI-5 reveals that FarA acts as a DNA-binding transcriptional regulator that controls its own synthesis. *J Bacteriol* **181**, 5081-5084.

**Krohn, K. & Rohr, J. (1997).** Angucyclines: total syntheses, new structures, and biosynthetic studies of an emerging new class of antibiotics. *Topics Curr Chem* **188**, 127-195.

**Krugel, H., Schumann, G., Hanel, F. & Fiedler, G. (1993).** Nucleotide sequence analysis of five putative *Streptomyces griseus* genes, one of which complements an early function in daunosamin biosynthesis that is linked to a putative gene cluster involved in TDP-daunosamine formation. *Mol Gen Genet* **241**, 193-202.

**Kulowski, K., Wendt-Pienkowski, E., Han, L., Yang, K., Vining, L. C. & Hutchinson, C. R. (1999).** Functional characterization of the *jadI* gene as a cyclase forming angucyclinones. *J. Am. Chem. Soc.* **121**, 1786-1794.

**Kunzel, E., Faust, B., Oelkers, C., Weissbach, U., Bearden, D. W., Weitnauer, G., Westrich, L., Bechthold, A. & Rohr, J. (1999).** Inactivation of the *urdGT2* gene, which

encodes a glycosyltransferase responsible for the C-glycosyltransfer of activated D-olivose, leads to formation of the novel urdamycins I, J, and K. *J Am Chem Soc* **121**, 11058-11062.

**Laemmli, U. K. (1970).** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

**Lambalot, R. H., Gehring, A. M., Flugel, R. S., Zuber, P., LaCelle, M., Marahiel, M. A., Reid, R., Khosla, C., & Walsh, C. T. (1996).** A new enzyme superfamily - the phosphopantetheinyl transferases. *Chem Biol* **3**, 923-936.

**Larson, J. L. & Hershberger, C. L. (1986).** The minimal replicon of a streptomycete plasmid produces an ultrahigh level of plasmid DNA. *Plasmid* **15**, 199-209.

**Leder, P., Tiemeier, D., & Enquist, L. (1977).** EK2 derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms: The  $\lambda$ gtWES system. *Science* **196**, 175-177.

**Leskiw, B. K., Lawlor, E. J., Fernandez-Abalos, J. M. & Chater, K. F. (1991).** TTA codons in some genes prevent their expression in a class of developmental, antibiotic-negative, *Streptomyces* mutants *Proc Natl Acad Sci USA* **88**, 2461-2465.

**Lezhava, A., Kameoka, D., Sugino, H., Goshi, K., Shinkawa, H. & Nimi, O. (1997).** Chromosomal deletions in *Streptomyces griseus* that remove the *afsA* locus. *Mol Gen Genet* **253**, 478-483.

**Liu, H-w. & Thorson, J. S. (1994).** Pathways and mechanisms in the biosynthesis of novel deoxysugars by bacteria. *Annu. Rev. Microbiol.* **48**, 223-56.

**MacNeil, D. J., Gewain, K. M., Rudy, C. L., Dezeny, G., Gibbons, P. H. & MacNeil, T. (1992).** Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61-68.

**Madduri, K., Kennedy, J., Rivola, G., Inventi-Solari, A., Filippini, S., Zanuso, G., Colombo, A. L., Gewain, K. M., Occi, J. L., MacNeil, D. J. & Hutchinson, C. R. (1998).** Production of the antitumor drug epirubicin (4'-epidoxorubicin) and its precursor by a genetically engineered strain of *Streptomyces peucetius*. *Nature Biotechnology* **16**, 69-74.

**Malik, V. S. & Vining, L. C. (1970).** Metabolism of chloramphenicol by the producing organism. *Can J Microbiol* **16**, 173-179.

**Mallams, A. K., Puar, M. S. & Rossman, R. R. (1981).** Kijanamicin. 1. Structure of the individual sugar components. *J Am Chem Soc* **103**, 3938-3940.

**Malpartida, F. & Hopwood, D. A. (1986).** Physical and genetic characterisation of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). *Mol Gen Genet* **205**, 66-73.

**Malpartida, F., Hallam, S. E., Kieser, H. M., Motamedi, H., Hutchinson, C. R., Butler, M. J., Sugden, D. A., Warren, M., McKillop, C., Bailey, C. R., Humphreys, G. O. & Hopwood, D. A. (1987).** Homology between *Streptomyces* genes coding for synthesis of different polyketides used to clone antibiotic biosynthesis genes. *Nature* **325**, 818-821

**Maniatis, T., Fritsh, E. F. & Sambrook, J. (1982).** *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratories, Cold Spring Harbor, N. Y.

**Mann, J. (1987).** *Secondary metabolism*. Clarendon Press, Oxford.

**Martinez-Costa, O. H., Arias, P., Romero, N. M., Parro, V., Mellado, R. P. & Malpartida, F. (1996).** A *relA/spoT* homologous gene from *Streptomyces coelicolor* A3(2) controls antibiotic biosynthetic genes. *J Biol Chem* **271**, 10627-10634.

**Matsumoto, A., Ishizuka, H., Bepp, T & Horinouch, S. (1995).** Involvement of a small ORF downstream of the *afsR* gene in the regulation of secondary metabolism in *Streptomyces coelicolor* A3(2). *Actinomycetologica* **9**, 37-43.

**Mazodier P., Peter, R. & Thompson, C. (1989).** Intergeneric conjugation between *Escherichia coli* and *Streptomyces* species. *J Bacteriol* **171**, 3583-3585.

**McDaniel, R., Ebert-Khosla, S., Hopwood, D. A. & Khosla, C. (1995).** Rational design of aromatic polyketide natural products by recombinant assembly of enzymatic subunits. *Nature*. **375**, 549-554.

**McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M. & Ashley, G. (1999).** Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. *Proc. Natl. Acad. Sci. USA* **96**, 1846-1851.

**Merrick, M. J. (1976).** A morphological and genetic study of bald colony mutants of *Streptomyces coelicolor*. *J Gen Microbiol* **96**, 299-315.

**Merson-Davies, L. A. & Cundliffe, E. (1994).** Analysis of five tylosin biosynthetic genes from the *tyllBA* region of the *Streptomyces fradiae* genome. *Mol Microbiol* **13**, 349-55.

**Meurer, G., Gerlitz, M., Wendt-Pienkowski, E., Vining, L. C., Rohr, J. & Hutchinson, C. R. (1997).** Iterative type II polyketide synthases, cyclases and keto-reductases exhibit context-dependent behavior in the biosynthesis of linear and angular decapolyketides. *Chem Biol* **4**, 433-443.

**McVey, J. (1998).** Characterization of the downstream genes for jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230. *M.Sc. thesis*. Dalhousie University.

**Miyake, K., Horinouchi S., Yoshida, M., Chiba, N., Mori, K., Nogawa, N., Morikawa, N. & Beppu, T. (1989).** Detection and properties of A-factor-binding protein from *Streptomyces griseus* *J Bacteriol* **171**, 4298-4302.

**Miyake, K., Kuzuyama, T., Horinouch, S. & Beppu. T. (1990).** The A-factor-binding protein of *Streptomyces griseus* negatively controls streptomycin production and sporulation. *J Bacteriol* **172**, 3002-3008.

**Murakami, R., Tomikawa, T., Shin-ya, K., Shinozaki, J., Kajiura, T., Seto, H. & Hayakawa, Y. (2001).** Ammocidin, a new apoptosis inducer in *ras*-dependent cells from *Saccharothrix* sp. II. Physico-chemical properties and structure elucidation. *J Antibiot* **54**, 714-717.

**Narva, K. E. & Feitelson, J. S. (1990).** Nucleotide sequence and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). *J Bacteriol* **172**, 326-333.

**Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M. & Umberger, H. E. (1987).** *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology. American Society for Microbiology. Washinton, D. C.

**Nodwell, J. R., McGovern, K. & Losick, R. (1996).** An oligopeptide permease responsible for the import of an extracellular signal governing aerial mycelium formation in *Streptomyces coelicolor*. *Mol Microbiol.* **22**, 881-893.

**Ochi, K. (1990).** A relaxed (*rel*) mutant of *Streptomyces coelicolor* A3(2) with a missing ribosomal protein lacks the ability to accumulate ppGpp, A-factor and prodigiosin. *J Gen Microbiol* **136**, 2405-2412.

**Ohnishi, Y., Kameyama, S., Onaka, H. & Horinouchi S. (1999).** The A-factor regulator leading to streptomycin biosynthesis in *Streptomyces griseus*: identification of a target gene of the A-factor receptor. *Mol Microbiol* **34**, 102-111.

**Olano, C., Lomovskaya, N., Fonstein, L., Roll, J. T. & Hutchinson, C. R. (1999).** A two-plasmid system for the glycosylation of polyketide antibiotics: bioconversion of epsilon-rhodomyconone to rhodomycon D. *Chem Biol* **6**, 845-55.

**Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y. & Hattori, M. (2001).** Genome sequence of an industrial microorganism *Streptomyces avermitilis*: Deducing the ability of producing secondary metabolites. *PNAS* **98**, 12215-12220.

**Onaka, H., Nakagawa, T. & Horinouchi, S. (1998).** Involvement of two A-factor receptor homologues in *Streptomyces coelicolor* A3(2) in the regulation of secondary metabolism and morphogenesis. *Mol Microbiol* **34**, 102-111.

**O'Rourke, S. J., & Chater, K. F. (2001).** A pathway-specific extracellular factor for methylenomycin biosynthesis. In *12<sup>th</sup> International Symposium on the Biology of Actinomycetes*, Vancouver, Canada. O. 56.

**Orth, P., Saenger, W. & Hinrichs, W. (1999).** Tetracycline-chelated Mg<sup>2+</sup> ion initiates helix unwinding in Tet repressor induction. *Biochemistry* **38**, 191-198.

**Otten, S. L., Liu, X., Ferguson, J. & Hutchinson, C. R. (1995).** Cloning and characterization of the *Streptomyces peucetius* *dnrQS* genes encoding a daunosamine biosynthesis enzyme and glycosyl transferase involved in daunorubicin biosynthesis. *J Bacteriol* **177**, 6688-6692.

**Parris, K. D., Lin, L., Tam, A., Mathew, R., Hixon, J., Stahl, M., Fritz, C. C., Seehra, J. & Somers, W. S. (2000).** Crystal structures of substrate binding to *Bacillus subtilis* *holo*-(acyl carrier protein) synthase reveal a novel trimeric arrangement of molecules resulting in three active sites. *Structure Fold Des* **8**, 883-895.

**Perez-Llarena, F J., Liras, P., Garcia, A. R. & Martin, J. F. (1997).** A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both beta-lactam compounds. *J Bacteriol* **179**, 2053-2059.

**Pope, M. K., Green, B. D. & Westpheling, J. (1996).** The *bld* mutants of *Streptomyces coelicolor* are defective in the regulation of carbon utilization, morphogenesis and cell-cell signaling. *Mol Microbiol.* **19**, 747-756.

**Pope, M. K., Green, B., & Westpheling, J. (1998).** The *bldB* gene encodes a small protein required for morphogenesis, antibiotic production, and catabolite control in *Streptomyces coelicolor*. *J Bacteriol.* **180**, 1556-1562.

**Quadri, L. E., Weinreb, P. H., Lei, M., Nakano, M. M., Zuber, P. & Walsh, C. T. (1998a).** Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry* **37**, 1585-1595.

**Quadri, L. E., Sello, J., Keating, T. A., Weinreb, P. H. & Walsh, C. T. (1998b).** Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chem Biol* **5**, 631-645.

**Quiros, L. M., Aguirrezabalaga, I., Olano, C., Mendez, C. & Salas, J. A. (1998).** Two glycosyltransferases and a glycosidase are involved in oleandomycin modification during its biosynthesis by *Streptomyces antibioticus*. *Molecular Microbiology* **28**, 1177-1185.



**Rao, R. N., Allen, N. E., Hobbs, Jr., J. N., Alborn, Jr., W. E., Kirst, H. A., & Paschal, J. W. (1983).** Genetic and enzymatic basis for hygromycin B resistance in *E. coli*. *Antimicrob Agents Chemother* **24**, 689-695.

**Ramalingam, E. (1989).** Polyketide synthase genes in *Streptomyces venezuelae* ISP5230. *M. Sc. Thesis*, Dalhousie University.

**Reichstein, T. & Weiss, E (1962).** The sugars of the cardiac glycosides. *Adv Carbohydr Chem* **17**, 65-120.

**Reuter, K., Mofid, M. R., Marahiel, M. A. & Ficner, R. (1999).** Crystal structure of the surfactin synthetase-activating enzyme Sfp: a prototype of the 4'-phosphopantetheinyl transferase superfamily. *EMBO J* **18**, 6823-6831.

**Rohr, J., Schonewolf, M., Udvarnoki, G., Eckardt, K., Schumann, G., Wagner, C., Beale, J. M. & Sorey, S. D. (1993).** Investigation on the biosynthesis of the angucycline group antibiotics aquayamycin and the urdamycins A and B. Results from the structural analysis of novel blocked mutant products. *J Org Chem.* **58**, 2547-2551

**Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

**Samols, D., Thornton, C. G., Murtif, V. L., Kumar, G. K., Hasse, F. C. & Wood, H. G. (1988).** Evolutionary conservation among biotin enzymes. *J Biol Chem* **263**, 6461-6464.

**Sanger, F., Nicklen, S. & Coulson, A. R. (1977).** DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463-5467.

**Seno, E. T. & Baltz, R. H. (1989).** Structural organization and regulation of antibiotic biosynthesis and resistance genes in actinomycetes. In *Regulation of Secondary Metabolism in Actinomycetes*. p 2-41. Edited, S. Shapiro. CRC Press. Inc. Boca Raton, Florida.

**Sergey, Z., Haugan, K., Sekurova, O., Sletta, H., Ellingsen, T. E. & Valla, S. (2000).** Identification of a gene cluster for an antibacterial polyketide-derived antibiotic in *Streptomyces noursei* ATCC 11455. *Microbiology* **146**, 611-619.

**Shapiro, S. & Vining, L. C. (1983).** Nitrogen metabolism and chloramphenicol production in *Streptomyces venezuelae*. *Can J Microbiol* **29**, 1706-1714.

**Shen, Y., Yoon, P., Yu, T-w., Floss, H. G., Hopwood, D. A. & Moore, B. S. (1999).** Ectopic expression of the minimal *whiE* polyketide synthase generates a library of aromatic polyketids of diverse sizes and shapes. *Proc. Natl. Acad. Sci. USA* **96**, 3622-3627.

**Shenin, I.D. (1986).** Physicochemical properties and structure of the nonmacrolide polyene antibiotic altamycin A. *Antibiot Med Biotekhnol* **31**, 835-41.

**Sherman, D. H., Malpartida, F., Bibb, M. J., Kieser, H. M. & Hopwood, D. A. (1989).** Structure and deduced function of the granaticin producing polyketide synthase gene cluster of *Streptomyces violaceoruber* Tü22. *EMBO J.* **8**, 2717-2725.

**Silakowski, B., Schairer, H. U., Ehret, H., Kunze, B., Weinig, S., Nordsiek, G., Brandt, P., Bloecker, H., Hoefle, G., Beyer, S. & Mueller, R. (1999).** New lessons for combinatorial biosynthesis from myxobacteria: the myxothiazol biosynthetic gene cluster of *Stigmatella aurantiaca* DW4/3-1. *J Biol Chem* **274**, 37391-37399.

**Simard, J., Melner, M. H., Breton, N., Low, K. G., Zhao, H., Periman, L. M. & Labrie, F. (1991).** Characterization of macaque 3- $\beta$ -hydroxy-5-ene steroid dehydrogenase/  $\Delta$ -5  $\Delta$ -4 isomerase: structure and expression in steroidogenic and peripheral tissues in primates *Mol Cell Endocrinol* **75**, 101-110.

**Singh, A. K. (1992).** Production of the antibiotic, jadomycin B by *Streptomyces venezuelae* ISP5230: effect of heat and ethanol. M.Sc. Thesis. Dalhousie University.

**Snipes, C. E., Chang, C-j. & Floss, H. G. (1978).** Biosynthesis of the antibiotic granaticin. *J Am Chem Soc* **101**, 701-706.

**Steffensky, M., Muhlenweg, A., Wang, Z., Li, A. & Heide, L. (2000).** Identification of the novobiocin biosynthesis gene cluster of *Streptomyces spheroides* NCIB 11891. *Antimicrob Agents & Chemother* **44**, 1214-1222.

**Stevens, C. L., Blumbergs, P. & Wood, D. L. (1964).** Stereochemical identification and synthesis of amicitose and the stereochemical identification of rhodinose, a sugar from streptolydigin. *J Am Chem Soc* **86**, 3592

**Stuible, H-P., Meier, S. & Schweizer, E. (1997).** Identification, isolation and biochemical characterization of a phosphopantetheine: protein transferase that activates the two type-I fatty acid synthases of *Brevibacterium ammoniagenes*. *Eur J Biochem* **248**, 481-487.

**Stuible, H-P., Meier, S., Wager, C., Hannappel, E. & Schweizer, E. (1998).** A novel phosphopantetheine: protein transferase activating yeast mitochondrial acyl carrier protein. *J Biol Chem* **273**, 22334-22339.

**Stuttard, C. (1982).** Temperate phages of *Streptomyces venezuelae*: lysogeny and host specificity shown by SV1 and SV2. *J Gen Microbiol* **128**, 115-121.

- Stutzman-Engwall, K. J., Otten, S. L. & Hutchinson, C. R. (1992).** Regulation of secondary metabolism in *Streptomyces* spp. and overproduction of daunorubicin in *Streptomyces peucetius*. *J Bacteriol* **174**, 144-154.
- Takano, E., Nihira, T., Hara, Y., Jones, J. J., Gershater, C. J., Yamada, Y. & Bibb, M. (2000).** Purification and structure determination of SCB1, a gamma-butyrolactone that elicits antibiotic production in *S. coelicolor* A3(2). *J Biol Chem* **275**, 11010-11016.
- Takano, E., Chakraborty, R., Nihira, T., Yamada, Y. & Bibb, M. J. (2001).** A complex role for the gamma-butyrolactone SCB1 in regulating antibiotic production in *Streptomyces coelicolor* A3(2). *Mol Microbiol* **41**, 1015-28
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994).** ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673-4680.
- Thorson, J. S., Lo, S. F. & Liu, H-w. (1993).** Biosynthesis of 3,6-dideoxyhexoses: new mechanistic reflections upon 2,6-dideoxy, 4,6-dideoxy, and amino sugar construction. *J Am Chem Soc* **115**, 6993-6994.
- Torkkell, S., Ylihonko, K., Hakala, J. & Shurnik, M. (1997).** Characterization of *Streptomyces nogalater* genes encoding enzymes involved in glycosylation steps in nogalamycin biosynthesis. *Mol Gen Genet* **256**, 203-209.
- Thorson, J. S. & Liu, H-w. (1993).** Characterization of the first PMP-dependent iron-sulfur-containing enzyme which is essential for the biosynthesis of 3,6-dideoxyhexoses. *J Am Chem Soc* **115**, 7539-7540.
- Thorson, J. S., Kelly, T. M. & Liu, H-w. (1994).** Cloning, sequencing, and overexpression in *Escherichia coli* of the  $\alpha$ -D-glucose-1-phosphate cytidyltransferase gene isolated from *Yersinia pseudotuberculosis*. *J Bacteriol.* **176**, 1840-1849.
- Tomita, F., Tamaoki, T., Shirahata, K., Kasai, M., Morimoto, M., Ohkubo, S., Mineura, K. & Ishii, S. (1980).** Novel antitumor antibiotics, tetrocarcins. *J Antibiot* **33**, 668-670.
- Trefzer, A., Salas, J. A. & Bechthold, A. (1999).** Genes and enzymes involved in deoxysugar biosynthesis in bacteria. *Nat Prod Rep* **16**, 283-299.
- Umeyama, T., Tanabe, Y., Aigle, B. D. & Horinouchi, S. (1996).** Expression of the *Streptomyces coelicolor* A3(2) *ptpA* gene encoding a phosphotyrosine protein phosphatase leads to overproduction of secondary metabolites in *Streptomyces lividans*. *FEMS Microbiol Lett* **144**, 177-184.
- Vining, L.C. (1990).** Functions of secondary metabolites. *Annu Rev Microbiol* **44**, 395-427.

**Waki, M., Nihira, T. & Yamada, Y. (1997).** Cloning and characterization of the gene (*farA*) encoding the receptor for an extracellular regulatory factor (IM-2) from *Streptomyces* sp. strain FRI-5. *J Bacteriol* **179** 5131-5137.

**Walker, S., Valentine, KG. & Kahne, D. (1990).** Sugars as DNA binders: a comment on the calicheamicin oligosaccharide. *J Am Chem Soc* **112**, 6428-29.

**Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E. & Flugel, R. S. (1997).** Post-translational modification of polyketide and nonribosomal peptide synthases. *Current Opinion in Chemical Biology* **1**, 309-319.

**Wang, L., McVey, J. & Vining, L. C. (2001).** Cloning and functional analysis of a phosphopantetheinyl transferase superfamily gene associated with jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230. *Microbiology* **147**, 1535-1545.

**Wang, L., White, R. L. & Vining, L. C. (2001).** Biosynthesis of the L-digitoxose component in jadomycin B: identification of genes for dideoxysugar formation and attachment in the *jad* cluster of *Streptomyces venezuelae* ISP5230. *Microbiology* **148**, 1091-1103.

**Wang, Z., Li, S. & Heide, L. (2000).** Identification of the coumermycin A1 biosynthesis gene cluster of *Streptomyces rishiriensis* DSM 40489. *Antimicrob. Agents Chemother* **44**, 3040-3048.

**Warashina, T. & Noro, T. (2000a).** Steroidal glycosides from the aerial part of *Asclepias incarnata* L. II. *Chem Pharm Bull* **48**, 99-107.

**Warashina, T. & Noro, T. (2000b).** Cardenolide and oxypregnane glycosides from the root of *Asclepias incarnata* L. *Chem Pharm Bull* **48**, 516-524.

**Weber, J. M., Leung, J. O., Maine, G. T., Potenz, R. B., Paulus, T. J. & DeWitt, J. P. (1990).** Organization of a cluster of erythromycin genes in *Saccharopolyspora erythraea*. *J Bacteriol* **172**, 2372-2383.

**Westrich, L., Domann, S., Faust, B., Bedford, D., Hopwood, A. & Bechthold, A. (1999).** Cloning and characterization of a gene cluster from *Streptomyces cyanogenus* S136 probably involved in landomycin biosynthesis. *FEMS Microbiol Lett* **170**, 381-387.

**Wietzorrek, A. & Bibb, M. (1997).** A novel family of proteins that regulates antibiotic production in streptomycetes appears to contain an OmpR-like DNA-binding fold. *Mol Microbiol* **25**, 1181-1184.

**Willey, J., Schwedock, J & Losick, R. (1993).** Multiple extracellular signals govern the production of a morphogenetic protein involved in aerial mycelium formation by *Streptomyces coelicolor*. *Genes Dev.* **7**, 895-903.

- Wright, F. & Bibb, M. J. (1992).** Codon usage in the G+C-rich *Streptomyces* genome *Gene* **113**: 55-65.
- Xue, Y., Ahao, L., Liu, H-w & Sherman, D. (1998).** A gene cluster for macrolide antibiotic biosynthesis in *Streptomyces venezuelae*: Architecture of metabolic diversity. *Proc Natl Acad Sci USA* **95**, 12111-12116.
- Yamamoto, K. R., Alberts, B. M., Benzinger, R., Lawhorne, L. & Treiber, G. (1970).** Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. *Virology* **40**, 734-744.
- Yamada Y., Sugamura, K., Konda, K., Yanagimoto, M. & Okada, H. (1987).** The structure of inducing factors for virginiamycin production in *Streptomyces virginiae*. *J Antibiot* **40**, 496-504.
- Yamada, Y. & Nihira, T. (1998).** Microbial hormones and microbial chemical ecology. In *Comprehensive Natural Products Chemistry*. Vol. 8. Barton, D. H. R., Nakanishi, K., and Meth-Cohn, O. (eds). Oxford: Elsevier Science Ltd, pp. 377-413.
- Yamada, Y. (1999).** Autoregulatory factors and regulation of antibiotic production in *Streptomyces*. In *Microbial Signalling and Communication*. England, R., Hobbs, G., Bainton, N., and Roberts, D. M. (eds). Cambridge: Society for General Microbiology, pp. 177-196.
- Yang, K., Han, L., He, J., Wang, L. & Vining, L. C. (2001).** A repressor-response regulator gene pair controlling jadomycin B production in *Streptomyces venezuelae* ISP5230. *Gene* **279**, 165-173.
- Yang, K., Han, L. & Vining, L. C. (1995).** Regulation of jadomycin B biosynthesis in *Streptomyces venezuelae* ISP5230: involvement of a repressor gene, *jadR*<sub>2</sub>. *J Bacteriol* **177**, 6111-6117.
- Yang, K., Han, L., Ayer, S. W. & Vining, L. C. (1996).** Accumulation of the angucycline antibiotic rabelomycin after disruption of an oxygenase gene in the jadomycin B biosynthetic gene cluster of *Streptomyces venezuelae*. *Microbiology* **142**, 123-132.
- Yoshimoto, A., Ogasawara, A., Kitamura, T., Oki, I., Inui, T., Takeuchi, T., & Umezawa, H. (1979).** Enzymatic conversion of aclacinomycin A to Y by a specific oxidoreductase in *Streptomyces*. *J Antibiot* **32**, 472.
- Yu, T-W., Bibb, M. J., Revill, W. P. & Hopwood, D. A. (1994).** Cloning, sequencing and analysis of the griseusin polyketide synthase gene cluster from *Streptomyces griseus*. *J Bacteriol* **176**, 2627-2634.

**Zaslavskaya, P. L., Zhukov, V. G., Kornitskaya, E. Y., Tovarova, II & Khokhlov, A. S. (1979).** Influence of A-factor on the ultrastructure of the A-factor deficient mutant of *Streptomyces griseus*. *Microbios* **25**, 145-153.

**Zhao, L., Sherman, D. H. & Liu, H-w. (1998).** Biosynthesis of desosamine: Construction of a new methymycin/neomethymycin analogue by deletion of a desosamine biosynthetic gene. *J Am Chem Soc* **120**, 10256-10257.

**Zielinski, J., Jereczek E., Sowinski, P., Falkowski, L., Rudowski, A. & Borowski, E. (1979).** The structure of a novel sugar component of polyene macrolide antibiotics: 2,6-dideoxy-L-ribohexopyranose. *J Antibiot* **32**, 565-568.