Control of growth, secondary metabolism and sporulation in *Streptomyces venezuelae* ISP5230 by *jadW*1, a member of the *afsA* family of γ-butyrolactone regulatory genes

Liru Wang and Leo C. Vining

Department of Biology, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4J1

Three new genes (*jadW*1, *jadW*2 and *jadW*3) were isolated from a region of the *Streptomyces venezuelae* ISP5230 chromosome at the left-hand end of the *jad* cluster for jadomycin B (JdB) biosynthesis. The deduced amino acid sequence of *jadW*1 showed strong similarity to gene products associated in several streptomycetes with γ-butyrolactone autoregulators controlling morphological differentiation and secondary metabolism. Examination of *jadW*1 for conserved domains detected a repeat sequence characteristic of proteins in the AfsA regulatory family. Insertional inactivation of *jadW*1 reduced the growth rate of *S. venezuelae* cultures in aerated liquid media containing complex nitrogen sources and altered growth morphology in minimal medium. It also affected sporulation on agar media. Cultures of *jadW*1-disrupted mutants grown under conditions supporting biosynthesis of JdB or chloramphenicol by the wild-type strain failed to produce either of the antibiotics. Complementing the disrupted strain by transformation with pJV435, containing a cloned copy of the gene, improved sporulation and restored antibiotic biosynthesis in transformants to titres close to those of the wild-type similarly transformed with pJV435 as a control. The results are consistent with a role for *jadW*1 in regulating morphological and metabolic differentiation. Further sequence analysis of *jadR*2, which functions with *jadR*1 in stress-induced activation of JdB biosynthesis, indicated that this gene encodes a γ-butyrolactone receptor homologue. The growth-rate-sensitive phenotype of the *jadW*1-disrupted mutant, and the proximity of *jadW*1 to *jadR*2 indicate that this region of the *jad* gene cluster contains a regulatory mechanism incorporating γ-butyrolactone signalling and sensitivity to environmental stress.

**INTRODUCTION**

Gram-positive bacteria of the genus *Streptomyces* undergo complex morphological differentiation, and produce structurally diverse families of secondary metabolites that include antibiotics, enzyme inhibitors and other molecules with potent physiological activity. In the model streptomyctete *Streptomyces coelicolor* A3(2), genome sequencing has revealed not only a large number of genes dedicated to secondary metabolite biosynthesis, but also a high proportion and diversity of regulatory genes. Both presumably reflect a need in streptomycetes to access multiple nutrients and to optimize cellular morphology and metabolic differentiation for effective competition in the complex soil environment they inhabit (Bentley et al., 2002). Insights into mechanisms controlling these activities have been obtained by investigating the production in *S. coelicolor* A3(2) of two pigmented antibiotics, actinorhodin and undecylprodigiosin. Biosynthesis of these secondary metabolites is regulated by the pathway-specific transcriptional activator genes *actII*-ORF4 and *redD*, respectively, which control the expression of genes in the individual pathways. Various other regulatory genes (e.g. *afs*, *aba*, *abs*, *mia* and *bld*) that influence the biosynthesis of actinorhodin and undecylprodigiosin may exert their effects indirectly by acting on *actII*-ORF4 and *redD* (Chater & Bibb, 1997). At the upper level of the regulatory hierarchy in streptomycetes are systems controlling cellular differentiation and secondary metabolism as well as responses to environmental stimuli. Noteworthy among these global regulators is a family of repressor proteins that allow selective gene expression through specific binding of γ-butyrolactone signalling ligands (Yamada & Nihira, 1998). The role of these ligands in modulating the repressor activity of γ-butyrolactone-receptor proteins is a feature of streptomycetes

**Abbreviations:** A-factor, γ-butyrolactone in *S. griseus*; AfsA, gene product mediating regulation by A-factor in *S. griseus*; Am, apramycin; Cm, chloramphenicol; IM-2, γ-butyrolactone in *S. lavendulae* FRI-5; JdB, jadomycin B; PKS, polyketide synthase; SCB1, γ-butyrolactone in *S. coelicolor* A3(2); VB, γ-butyrolactone in *S. virginiae*.
(Kinoshita et al., 1997). In this genus the potential of the γ-butyrolactone signalling system for quorum sensing and control of biochemical reactions underlies nutrient recycling and other physiological activities that support cellular and metabolic differentiation; thus interaction between diffusible γ-butyrolactone signals and their receptors influences both sporulation and antibiotic biosynthesis.

In Streptomyces griseus the onset of streptomycin production and sporulation is associated (Khokhlov et al., 1967) with the γ-butyrolactone signalling agent identified as A-factor (2R-isocaproyl-3R-hydroxymethyl-4-butanolide; Fig. 1). An A-factor receptor protein, ArpA, binds specifically and with high affinity to the γ-butyrolactone (Miyake et al., 1990). Since ArpA in the absence of its ligand powerfully represses transcription of a gene (adpA) controlling streptomycin biosynthesis and sporulation, both of the latter activities are A-factor-dependent. γ-Butyrolactone signalling agents identified in other streptomycetes include compound 1, influencing sporulation and anthracycline biosynthesis in Streptomyces viridochromogenes (Gräfe et al., 1982), a group of virginiae butanolides (VBs), regulating production of virginiamycin antibiotics in Streptomyces virginiae (Kawachi et al., 2000), and the IM-2 group of regulatory compounds that switch secondary metabolism in Streptomyces lavendulae FRI-5 from the biosynthesis of cycloserine to the formation of nucleoside antibiotics and a blue pigment (Hashimoto et al., 1992; Waki et al., 1997). The structures of known γ-butyrolactone signalling agents differ only in detail from A-factor. In the VB and IM-2 series, the 6-keto group is reduced to a hydroxy group with the S (α) or R (β) configuration, respectively (Shikura et al., 2002). In S. coelicolor A3(2) the signalling agent SCB1 is a 6R-hydroxy-γ-butyrolactone (Takano et al., 2000), but six related structures have also been isolated from this species (Efremenkova et al., 1985). Since about 60% of streptomycetes have been estimated to use γ-butyrolactones as diffusible signalling molecules (Horinouchi & Beppu, 1992; Yamada, 1999), the structural variety may reflect a need for signal discrimination. Recognition of the signals by γ-butyrolactone receptor proteins such as ArpA (see above) has been implicated not only in pleiotropic control of differentiation in S. griseus (Onaka et al., 1995; Ohnishi et al., 1999), but also in the regulation of S. virginiiae (BarA; Kim et al., 1990; Nakano et al., 1998) and S. coelicolor A3(2) (ScbR; Takano et al., 2001). Comparable roles for the IM-2 receptor FarA in S. lavendulae FRI-5, (Waki et al., 1997) and the γ-butyrolactone receptor TylP in Streptomyces fradiae (Bate et al., 1999) are also likely.

Streptomyces venezuelae ISP5230 produces two antibiotics, chloramphenicol (Cm) and a polyketide-derived angucycline glycoside, jadomycin B (JdB). Neither of these secondary metabolites is synthesized in cultures grown in rich medium, but whereas Cm production is a response to unbalanced growth in medium containing a poorly assimilated nutrient, JdB is produced only when nutritionally unbalanced cultures are subjected to additional stress such as heat shock or ethanol toxicity (Doull et al., 1994). Many of the genes for biosynthesis of Cm and JdB have been cloned and positioned in separate clusters on the S. venezuelae chromosome. The Cm cluster encodes enzymes that convert shikimate pathway intermediates via p-aminophenylalanine to the substituted phenylpropanoid antibiotic; unexpectedly, it also includes a non-ribosomal peptide synthetase (Han et al., 1994; He et al., 2001; Fig. 2a). The cluster of genes for JdB biosynthesis encodes an iterative type-II polyketide synthase (PKS) complex that generates a decaketide intermediate (Meurer et al., 1997; Kulowski et al., 1999; Han et al., 2000; Wang et al., 2001; Fig. 2b). Downstream of the core PKS genes are tailoring genes encoding oxidoreductases that modify the initial polyketide structure (Yang et al., 1996), and a subcluster of genes for biosynthesis and attachment of the glycosidic component of JdB (Wang et al., 2002). Immediately upstream of the core PKS genes in the jad cluster is a region containing ancillary PKS genes, and beyond these is a pair of genes (jadR1 and jadR2) encoding a putative two-component regulatory system associated with the sensitivity of JdB biosynthesis to environmental stress (Yang et al., 1995, 2001). We report here three additional genes upstream of the jadR1/jadR2 pair and assign one of them (jadW1) to the family associated with production of γ-butyrolactone signalling molecules (quorum sensors). The control exerted by jadW1 affects growth as well as sporulation and antibiotic production.

**METHODS**

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

**Culture conditions.** For culturing Escherichia coli, LB medium or LB agar supplemented with appropriate antibiotics (Sambrook et al., 1989) was used. Cultures of S. venezuelae were normally maintained on MYM agar (Stuttard, 1982), but TO agar (tomato paste, 20 g; Pablum oatmeal, 20 g; agar, 20 g; distilled water to 1000 ml) was used if needed to enhance sporulation. Streptomycete strains from which genomic DNA was isolated were grown in MYM medium at 30°C for 24–48 h on a rotary shaker (220 r.p.m.). Conditions used to grow cultures of S. venezuelae for measurements of growth or antibiotic production are described below. S. griseus IFO13350 and

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**Fig. 1. Structure of A-factor (2R-isocaproyl-3R-hydroxymethyl-4-butanolide), the γ-butyrolactone autoregulator isolated from S. griseus (Khokhlov et al., 1967).**
mutant HH1 were kindly provided by Dr Suehara Horinouchi (Department of Agricultural Chemistry, University of Tokyo, Japan) and were maintained on nutrient agar (Horinouchi et al., 1984).

**Chemicals and enzymes.** Reagent grade chemicals were used; \([\text{\textsuperscript{32}P}]\text{dCTP}\) was purchased from Amersham Pharmacia Biotech. The Klenow fragment was supplied by MBI Fermentas. Dr Takuya Nihira (Department of Biotechnology, Graduate School of Engineering, Osaka University, Japan) kindly provided samples of the \(\gamma\)-butyrolactone autoregulators A-factor and IM-2.

**DNA manipulation, plasmid transformation and intergeneric transfer.** In procedures involving *E. coli*, standard methods (Sambrook et al., 1989) were used. Bacteriophage \(\lambda\) DNA was purified as described previously (Wang et al., 2001). Streptomyces plasmid and genomic DNA were isolated and streptomycetes were transformed with plasmid DNA by the methods of Hopwood et al. (1985) and Kieser et al. (2000). For conjugal transfer of plasmids from *E. coli* to streptomycetes, the protocol of Flett et al. (1997), based on procedures developed by Mazodier et al. (1989), was followed.

**Cloning and sequencing the jadW region.** A 4-0 kb *Xho*I–*BamHI* fragment of \(\lambda\) 8 DNA was subcloned into pBluescript II SK(+) to give pJV429. Overlapping regions of the plasmid insert were sequenced by the dideoxynucleotide chain-termination method; potential ORFs were detected by sequence analysis with FRAMEPLOT 2.3 (Ishikawa & Hotta, 1999) to assess codon usage and the frequency of G+ C in codon third positions (Wright & Bibb, 1992).

**Sequence analysis.** For estimating protein isoelectric points and for routine analyses, GENERUNNER version 3.05 (Hastings Software) was used. BLAST programs (Altschul et al. 1997) were used to search nucleic acid, protein and plasmid conserved domain databases at GenBank and the Sanger Institute (http://www.sanger.ac.uk) for sequence similarities, motifs and conserved domains. Matching features and conserved regions were detected by aligning sequences with CLUSTAL W (http://www2.ebi.ac.uk/clustalw/index/html; Thompson et al., 1994).

**Subcloning and conjugal transfer of jadW.** To construct a conjugal plasmid containing only jadW, pJV429 was partially digested with BsaI. The 1-2 kb fragment was recovered and ligated into the Small site of pUC18 to give pJV434. Digestion of pJV434 with EcoRI/BamHI, and ligation of the fragments with linearized pJV326 furnished the conjugal plasmid pJV435 carrying jadW as a 1-2 kb EcoRI–BamHI insert in pBluescript II SK(+). To avoid streptomycete restriction systems (MacNeil et al., 1992), pJV435 was passaged through DNA-methylation-deficient *E. coli* ET12567 (pUZ8002) before transfer to *S. venezuelae* ISP5230 or VS1095 by methods described previously (Wang et al., 2001, 2002). Thioestrepton-resistant transconjugants were selected.

**Insertional inactivation of jadW.** Plasmid pJV429 was digested with *Xhol*/*SacI* and the 1-0 kb fragment containing jadW was retrieved from the products; ligation with *Xhol*/*SacI*-digested pBluescript SK(+) yielded pJV430. The plasmid was linearized with *Xhol*, blunt-ended with the Klenow fragment of DNA polymerase I and digested with *SacI*. The 1-0 kb *Xhol* (blunted)–*SacI* fragment was retrieved and ligated into pUC18 at corresponding sites to give pJV431. Digestion of pJV431 with *SacI* linearized the plasmid at its unique *SacI* site, located within jadW near the centre of the insert. The linear DNA was blunt-ended and ligated with a 1-6 kb *EcoRV* cassette containing the apramycin resistance (Am\(^r\)) gene retrieved from pJV225. Transformation of *E. coli* DH5\(\alpha\) with the ligation mixture yielded strains from which plasmids pJV432A/B with the Am\(^r\) cassette in alternative orientations were isolated. Excision of disrupted jadW DNA with *XbaI/EcoRI* and ligation with appropriately linearized pJV326 gave pJV433A/B. These conjugal plasmids were passaged through *E. coli* ET12567 (pUZ8002) and transferred to *S. venezuelae* ISP5230 as described above for pJV435.
### Table 1. Bacterial strains, plasmids and phages

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
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<tbody>
<tr>
<td><em>Streptomyces</em></td>
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<tr>
<td>ISP5230</td>
<td><em>S. venezuelae</em> wild-type</td>
<td>Stuttard (1982)</td>
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<td>VS1095</td>
<td>ISP5230 with <em>jadW</em> disrupted by an <em>Am</em>&lt;sup&gt;R&lt;/sup&gt; cassette</td>
<td>This study</td>
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<td>VS1096</td>
<td>VS1095 with <em>Am</em>&lt;sup&gt;R&lt;/sup&gt; oppositely oriented</td>
<td>This study</td>
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<td>VS1097</td>
<td>VS1095(pJV435) transconjugant</td>
<td>This study</td>
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<tr>
<td>VS1098</td>
<td>ISP5230(pJV435) transconjugant</td>
<td>This study</td>
</tr>
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<td>IFO13350</td>
<td><em>S. griseus</em> wild-type</td>
<td>S. Horinouchi, Tokyo</td>
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<td>HH1</td>
<td>A-factor-deficient mutant of <em>S. griseus</em></td>
<td>S. Horinouchi, Tokyo</td>
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<td><em>Escherichia coli</em></td>
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<td>DH5ΔF′IQ</td>
<td>F′&lt;sup&gt;x80d&lt;/sup&gt; lacZΔ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(r&lt;sub&gt;K&lt;/sub&gt;, m&lt;sub&gt;K&lt;/sub&gt;) phoA supE11 Δ thi-l gyrA96 relA1 F′&lt;sup&gt;proAB&lt;/sup&gt; lacUV515 zff::Tn5[Km&lt;sup&gt;R&lt;/sup&gt;]</td>
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<td>dam&lt;sup&gt;−&lt;/sup&gt; dcm&lt;sup&gt;−&lt;/sup&gt; hsdM&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>ET12567 containing pUZ8002</td>
<td>M. Paget, John Innes Centre</td>
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<td><em>Plasmids</em></td>
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<td>Stratagene</td>
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<td>pHJL400</td>
<td>tsr amp lacZ, bifunctional</td>
<td>Larson &amp; Hershberger (1986)</td>
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<td>pUC18</td>
<td>ori lacZ amp</td>
<td>Amersham</td>
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<td>pJV225</td>
<td>pBluescript II SK(+) containing <em>Am</em>&lt;sup&gt;R&lt;/sup&gt; gene flanked by multiple cloning sites</td>
<td>Chang et al. (2001)</td>
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<td>pJV326</td>
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<td>He et al. (2001)</td>
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<td>This study</td>
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<td>pJV430</td>
<td>pBluescript II SK(+) with 1.0 kb SalI–XhoI insert from pJV429 (contains <em>jadW</em>&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>This study</td>
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<tr>
<td>pJV431</td>
<td>pUC18 with 1.0 kb XhoI(BamHI)–SalI insert from pJV430 (contains <em>jadW</em>&lt;sub&gt;1&lt;/sub&gt;)</td>
<td>This study</td>
</tr>
<tr>
<td>pJV432A/B</td>
<td>pUC18 with 2.6 kb XhoI(BamHI)–SalI insert containing 1.6 kb <em>Am</em>&lt;sup&gt;R&lt;/sup&gt; gene (alternative orientations) in <em>KpnI</em> site</td>
<td>This study</td>
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<td>pJV433A/B</td>
<td>pJV326 with 2.6 kb XhoI–EcoRI insert subcloned from pJV432A/B</td>
<td>This study</td>
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<td>pUC18 with 1.2 kb <em>BsaAI</em>(SmaI) insert from pJV430 (contains <em>jadW</em>&lt;sub&gt;1&lt;/sub&gt;)</td>
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<td>pJV435</td>
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<td>This study</td>
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<td><em>Phage</em></td>
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<td>λ 8</td>
<td>λ GEM-11 with a 9.5 kb insert of <em>S. venezuelae</em> DNA</td>
<td>Ramalingam (1989)</td>
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</table>
Transconjugant colonies (VS1095/1096) resistant to apramycin but sensitive to thiostrepton were isolated and their EcoRI-digested genomic DNA was probed by Southern hybridization with the [32P]dCTP-labelled 3-35 kb EcoRI fragment of pJV429.

Sporulation of mutants and transformants. Strains tested for sporulation were initially grown in TSBG medium (3 % Difco tryptone; 2 % glucose) at 28 °C for 24 h; the mycelia were pelleted, washed three times by resuspension in water and centrifugation, then resuspended in 0-3 vols water. Measured samples (10 μl) of the mycelial suspensions were placed on the surface of MYM and TO agar and the patches were examined at intervals during incubation at 28 °C for up to 2 weeks. In experiments where spores from TO agar were tested for propagation as sporulating colonies, the spores were dispersed in water with glass beads, filtered through non-absorbent cotton, pelleted by centrifugation and washed with water before suspensions were patched as above on MYM and TO agar. To detect substances in TO agar activating sporulation, aqueous extracts of the agar medium were concentrated in vacuo, absorbed on paper disks and applied to mycelial lawns of the jadW1-disrupted mutant; diffusion zones around the disks were examined for sporulation.

Growth measurements. Cultures (25 ml) in liquid media were grown at 28 °C in 125 ml Erlenmeyer flasks on a rotary shaker (220 r.p.m.; 3/8 cm eccentricity). Growth rates of test strains propagated from standardized inocula (mycelial suspensions grown as above in 25 ml TSBG medium, pelleted and washed thoroughly with water, then used at a rate of 0-2 % v/v) were calculated as follows. Samples (5 or 10 ml) of each culture were removed aseptically at 0, 3, 6, 12, 18, 24, 48, 72 h and 3 days by HPLC (Brown et al, 1995). Their JdB content was measured by HPLC (Han et al., 1994).

Bioassay and complementation procedures on agar media. Strains of S. venezuelae grown on MYM agar were assayed for antibiotic activity by comparing zones of inhibition against Micrococcus luteus with those given by Cm standards absorbed on paper disks (Wang et al., 2001). To determine whether substances diffusing from cultures of S. venezuelae ISP5230 could restore streptomycin production in an A-factor-deficient S. venezuelae mutant, the complementation procedure of Horinouchi et al. (1984) was used. Mycelium from S. griseus HH1 was patched on nutrient agar about 1 cm away from the site inoculated with 1 μl S. venezuelae ISP5230 spore suspension. Cultures were incubated for 2–3 days at 30 °C before a soft nutrient agar overlay seeded with Bacillus subtilis was added. After incubation at 37 °C overnight, the S. griseus HH1 patches were inspected for inhibition zones indicating complementation; to validate the assay, mutant HH1 was replaced with wild-type S. griseus 13350. The procedure was adapted to assay for A-factor in extracts of S. venezuelae ISP5230 by absorbing 10 μl concentrated chloroform extract (0-2 ml obtained from a filtered 25 ml culture) on a paper disk. The disk was dried and placed 1 cm away from a patch of S. griseus HH1; soft nutrient agar overlay seeded with B. subtilis was added and incubated as in the original procedure.

Tests for extracellular complementation of the jadW1 mutant phenotype. Assays were based on restoration of S. venezuelae wild-type functions in jadW1-disrupted mutants. Mutant cultures were supplemented with concentrated wild-type extracts to give γ-butyrolactone concentrations (0-1–1-0 μg ml⁻¹) comparable to those estimated in other actinomycetes (Yamada & Nihira, 1998). To detect recovery of JdB production, cultures of the mutant grown under standard conditions were supplemented 4–16 h after inoculation with 2 μl portions of chloroform extracts from wild-type cultures grown for 8, 12, 16, 20, 24, 28, 32, 36 or 48 h after ethanol treatment; each 2 μl supplement represented the total extract from 50 ml culture broth. The effect of A-factor, IM-2 and nonalactone on JdB production was assessed by adding the autoregulators to give concentrations of 2, 20 and 20 μg ml⁻¹, respectively, in cultures of the jadW1-disrupted mutant grown as above. To detect recovery of sporulation in the jadW1-disrupted mutant, paper disks were impregnated with 10 μl portions of chloroform extract from wild-type cultures grown as above (each 10 μl representing 20 ml culture broth), or with 20 μg A-factor, 20 μg IM-2 or 40 μg nonalactone. The paper disks were dried in vacuo and placed on MYM agar adjacent to patched mycelium of the mutant. The patched mycelium was examined for sporulation after incubation at 30 °C for 7 days.

RESULTS

Nucleotide sequence of the region downstream of jadR2

In earlier studies, genes encoding JdB biosynthesis were identified by subcloning, sequencing and analysis of genomic DNA fragments cloned from S. venezuelae ISP5230 in the recombinant phages λ 8 and λ LH7 (Han et al., 1994; Yang et al., 1995; Wang et al., 2001, 2002). A 4-3 kb SacI fragment present in both phages included a pair of regulatory genes (jadR1/jadR3; Fig. 3a) and an incompletely characterized ORF encoding a protein with sequence similarity to sugar dehydrogenases. The nucleotide sequence of this chromosomal region (GenBank accession no. U24659) was subsequently revised to correct sequencing errors and a 4-0 kb XhoI–BamHI fragment was subcloned in pBluescript II SK(+) to give pJV429. Sequence analysis of the insert in pJV429 detected jadR1 and three additional ORFs jadW1, jadW2 and jadW3. The latter were oriented in the same direction as the core PKS gene cluster and the response regulator component of the jadR1/jadR2 pair, but oppositely oriented to the repressor component jadR2 (see Fig. 3a).

Role of jadW1 in γ-butyrolactone regulation

The ORF jadW1, located at the left-hand end of the cloned jad cluster genes (see Fig. 2b) has the G+C content (mean 71·8 mol% ; codon third letter bias 95-3 mol%) expected for a streptomycete gene (Wright & Bibb, 1992) and encodes a protein of 309 aa. The deduced amino acid sequence of jadW1 closely resembled that of AfsA from S. griseus; in a BLASTP 2.2.23 two-sequence alignment, the overall identity of JdB1 and AfsA was 43 ‰. A CLUSTAL W alignment (Fig. 4) of the JadW1 sequence with matching protein sequences in GenBank detected by BLASTP showed similarities to the products of barX from S. virginiæ (42 ‰ identity), scbA and...
mmfL from S. coelicolor A3(2) (42 and 30% identity, respectively), afsA from S. griseus (41% identity) and farX from S. lavendulae FRI-5 (40% identity). Each of these matching gene products is associated with pleiotropic regulation of cellular and metabolic differentiation in streptomycetes by γ-butyrolactones (Yamada & Nihira, 1998; Yamada, 1999). In the deduced amino acid sequences of jadW1 and each of its putative homologues, searches with RPS-BLAST as well as searches of the pfam database (http://www.sanger.ac.uk) detected the conserved AfsA-repeat domain (gnl|CDD|8712, pfam03756) associated with the γ-butyrolactone regulatory mechanism. A cladogram generated from the CLUSTAL W alignment of six sequences containing the AfsA repeat domain indicated that JadW1 is most closely related to the AfsA/ScbA pair (Fig. 5).

**Insertional inactivation of jadW1**

Plasmids pJV432A and pJV432B, constructed by inserting an apramycin resistance gene into the KpnI site within jadW1 (see Fig. 3a), were shown by restriction analysis to contain the disruption cassette in different transcriptional

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**Fig. 3.** (a) Restriction map of the 4.0 kb fragment subcloned in pJV429 from the insert of S. venezuelae ISP5230 genomic DNA cloned in λ phage (Ramalingam, 1989). The large filled arrows below the map locate and orient the ORFs identified in this study (jadW1, jadW2, jadW3). The unfilled arrows locate ORFs (jadR1 and jadR2) reported by Yang et al. (1995). The bold lines represent chromosomal fragments cloned in pJV429, pJV430, pJV431, pJV432, pJV433, pJV434 and pJV435. They and the restriction sites defining them are aligned below the map. The double-line arrows inserted into pJV432 and pJV433 to disrupt jadW1 represent the apramycin resistance cassette in each orientation. Abbreviations: B, BamHI; Bs, BsaAI; E, EcoRI; K, KpnI; S, SacI; X, XhoI. (b) Southern hybridization of S. venezuelae genomic DNA digests with the 3.35 kb EcoRI fragment from pJV429. Lanes: 1, VS1095; 2, VS1096; 3, ISP5230 (wild-type); M, DNA size markers.
orientations. In pJV432A the Am\textsuperscript{R} and JadW\textsubscript{1} genes were oppositely oriented, whereas in pJV432B the genes had the same orientation. Each plasmid was then recloned into the conjugal vector pJV326 and passaged through the DNA-non-methylating \textit{E. coli} strain ET12567(pUZ8002) to avoid restriction in a streptomycte host. The resulting plasmids, pJV433A and pJV433B, respectively, were transferred into \textit{S. venezuelae} ISP5230 by intergeneric conjugation. Selection for an Am\textsuperscript{R} Ts\textsuperscript{S} phenotype ensured that the transconjugants recovered (VS1095 and VS1096, respectively) had undergone double crossovers eliminating vector DNA and exchanging the native \textit{jadW\textsubscript{1}} with a disrupted allele containing the resistance marker. Allele exchange in each transconjugant was confirmed by the sizes (3 - 35 and 4 - 9 kb) of hybridizing fragments detected in \textit{EcoRI}-digested wild-type and transconjugant genomic DNA, respectively, with a \textit{jadW\textsubscript{1}} probe (Fig. 3b).

Characterization of \textit{jadW\textsubscript{1}}-disrupted mutants

Because the stop codon of 
\textit{jadW\textsubscript{1}} is separated from the start codon of \textit{jadW\textsubscript{2}} by only 15 bp of non-coding DNA, the phenotype of \textit{jadW\textsubscript{1}}-disrupted mutants could be due to a polar effect of the disruption on expression of downstream genes. However, the Am\textsuperscript{R} disruption cassette used here has been shown (Wang et al., 2001) to contain a promoter that, if appropriately oriented, reinitiates transcription of DNA following an insertion; where both orientations can be tested, only one elicits a polar effect.

Comparisons of VS1095, VS1096 and the wild-type grown as surface and submerged cultures on a variety of media did not distinguish between the two mutants, but showed significant differences between the mutant strains and the wild-type. The results implied that \textit{jadW\textsubscript{1}} disruption per se

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**Fig. 4.** CLUSTAL W alignment of the amino acid sequence of \textit{JadW\textsubscript{1}} (GenBank U24659), BarX (AB001608), FarX (AB001683), ScbA (A007731) and AfsA (Horinouchi et al., 1989). Highly conserved amino acids are in bold letters and are marked below the alignment as identical (asterisk), strongly conserved (colon) or partially conserved (period).
caused the altered phenotype. Whereas the wild-type produced abundant aerial mycelium and spores on MYM agar, the mutants rapidly colonized the agar surface with substrate mycelium, producing sparse aerial mycelium and spores only near the growth perimeter (Fig. 6a). Inoculating small (< 2 cm diam.) regions of the agar surface with either VS1095 or VS1096 gave patches of mycelium carrying some spores, but distributing the inoculum over MYM agar in a Petri plate to produce a confluent lawn gave only bald mycelium. The effect of jadW1 disruption on sporulation depended not only on growth conditions but also on the composition of the nutrient medium used. The most robust sporulation medium tested was TO agar, on which the wild-type strain produced aerial mycelium within 24 h and abundant blue-green spores after 2–3 days (Fig. 6b). Mutants VS1095 and VS1096 sporulated more slowly than the wild-type, but spores were present after 3–4 days. These spores gave sporulating colonies when plated on MYM agar, even after several serial transfers or washes with water, but the proportion of bald colonies increased with the number of washes, implying that the stimulatory agent was strongly but reversibly bound to the spores. Aqueous extracts of uninoculated TO agar did not induce sporulation of the disrupted mutants on MYM agar, suggesting that the active agent was formed during sporulation.

In MYM medium submerged cultures of mutants VS1095 and VS1096 grew more slowly than the wild-type; the rate of biomass increase in VS1096 closely matched that in VS1095 and was consistently below that of the wild-type (Fig. 7). Replacing maltose in MYM medium with glucose markedly decreased growth rates and yields in both the wild-type and mutant cultures (GYM-ISP5230 and GYM-VS1095 in Fig. 7). In media with tryptone as a nitrogen source, glucose selectively reduced the growth rate and formation of melanoid pigment in mutant relative to wild-type cultures (GT-ISP5230 and GT-VS1095 in Fig. 7). This contrasted with responses in the glucose-asparagine minimal medium of Hopwood (1967), where the mutant grew faster than the wild-type (GAsn-ISP5230 and GAsn-VS1095 in Fig. 7). The growth rate differences in GAsn medium correlated with striking differences in growth morphology (Fig. 8). Submerged cultures of the jadW1-disrupted mutants grew as uniform slurries of short, unbranched filaments (Fig. 8a), resembling in this respect the wild type and mutant cultures grown in media containing complex nitrogen sources. In GAsn medium the growth of the wild-type was distinctly different, generating discrete aggregates of entangled mycelium (Fig. 8b). Submerged cultures of VS1095 and VS1096 inoculated with bald mycelium from MYM agar plates and incubated in glucose-isoleucine or galactose-isoleucine media under conditions optimized for Cm or JdB biosynthesis, respectively (Doull et al., 1985, 1994), resembled those of the wild-type in growth characteristics, but HPLC analyses showed that they failed to produce the antibiotics. In contrast, cultures in

![Fig. 6. Cultures of S. venezuelae strains incubated for 3 days on (a) MYM agar or (b) TO agar. The strains were: 1. ISP5230 (wild-type); 2. VS1095 (jadW1-disrupted mutant); 3. VS1097 (VS1095 transformed with pJV435).](image-url)
galactose-isoleucine medium inoculated with spores of the mutants obtained directly from TO agar produced 24–27 μg JdB ml⁻¹. The JdB titre in wild-type control cultures was 43–49 μg ml⁻¹.

### Complementation of jadW₁-disrupted strains

Conjugal transfer of pJV435 (containing jadW₁) into jadW₁-disrupted and wild-type strains of S. venezuelae, and selection for thiostrepton resistance, yielded transconjugants VS1097 and VS1098, respectively. Submerged cultures of the transconjugants in MYM medium resembled ISP5230 in growth morphology. The growth rate of VS1097, the strain in which introduction of pJV435 was expected to complement disruption of jadW₁, was faster than that of VS1095, but not fully restored to the wild-type rate (see Fig. 7). On MYM agar VS1097 produced more spores than VS1095 (see Fig. 6a, b) but again did not entirely recover the wild-type pattern. Inoculation of galactose-isoleucine medium from vegetative MYM cultures of VS1097 and VS1098 and incubation under standard conditions gave cultures producing JdB in titres 1.8- to 2.5-fold higher than those of wild-type. The titre was not influenced by supplementing galactose-isoleucine production medium with up to 50 μg thiostrepton ml⁻¹, favouring multiple chromosomal integration of pJV435 over selection for an increase in cytoplasmic copies of the plasmid as the explanation for a high JdB titre. Multiple insertion into the chromosome of plasmids carrying cloned homologous DNA has been encountered previously in S. venezuelae ISP5230 (Paradkar et al., 1993). Whereas JdB production increased when jadW₁ was introduced into VS1095 and ISP5230 to give transconjugants VS1097 and VS1098, respectively, production of Cm in MYM agar or glucose-isoleucine broth cultures showed a 30–60% decrease. Accompanying Cm at 7 days in VS1098, but not in the VS1097 or wild-type cultures, were small amounts of rabelomycin and 3-O-acetyl-Cm. The first product is a shunt metabolite previously identified in an S. venezuelae mutant unable to complete JdB biosynthesis (Yang et al., 1996). The 3-O-acetyl derivative of Cm is an inactive shielded intermediate formed during Cm biosynthesis to avoid intracellular accumulation of the antibiotic (Groß et al., 2002). The presence of these substances in a strain containing extra copies of jadW₁ suggests excessive activation of pathways for antibiotic biosynthesis.

### Assays for γ-butyrolactones in S. venezuelae ISP5230

Sequence analysis of the jadW₁ product indicates that it is an AfsA-family protein associated with the biosynthesis or function of a butyrolactone regulatory agent in S. venezuelae ISP5230. A role for A-factor itself as the ligand in this species was excluded by failure to detect the metabolite in culture broths and extracts with the sensitive bioassay complementing the A-factor-deficient S. griseus mutant HH1 (Horinouchi et al., 1984), but the possibility that an indigenous γ-butyrolactone participated in the regulatory system remained. In support of this was the evidence that introduction in trans of a cloned copy of jadW₁ into the jadW₁-disrupted S. venezuelae mutant VS1095 complemented some of the phenotypic effects of the mutation. To further test for the presence of an indigenous γ-butyrolactone ligand, the A-factor bioassay procedure was modified to detect extracellular metabolites promoting sporulation or JdB production in cultures of VS1095. No stimulatory products were found in culture filtrates or extracts of wild-type S. venezuelae, despite adjustments of the procedure to accommodate potential

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**Fig. 7.** Growth of S. venezuelae strains as submerged cultures in various culture media: ISP5230 (wild-type), VS1095 (jadW₁-disrupted mutant) and VS1097 (VS1095 transformed with pJV435). The culture media used were: MYM (maltose-yeast extract-malt extract), GYM (glucose-yeast extract-malt extract), GT (glucose-tryptone) and GAsn (glucose-asparagine).
differences in the timing of γ-butyrolactone synthesis and target strain response, and VS1095 was not induced to sporulate by proximity to paper disks impregnated with A-factor, IM-2 or nonalactone. In addition, assays for JdB and Cm biosynthesis in cultures of VS1095 showed no response to supplementation with extracts from wild-type cultures, or with γ-butyrolactones active in other streptomycetes.

Fig. 8. Growth morphology of wild-type (ISP5230) and jadW1-disrupted (VS1095) strains of S. venezuelae grown in aerated GAsn liquid medium. Mycelium from cultures sampled at 24 h was photographed under phase-contrast. (a) VS1095; (b) ISP5230.
DISCUSSION

Role of jadW₁ in S. venezuelae

The overall sequence similarity between JadW₁ and proteins associated in other streptomycetes with γ-butyrolactone control of gene expression implicates the gene product in regulation by this mechanism in S. venezuelae ISP5230. The presence of conserved AfsA-repeat domains in JadW₁ supports such a role. Investigations of the function of AfsA in S. griseus have shown that streptomycetes normally unable to make γ-butyrolactones can be transformed with the AfsA gene to biosynthesize A-factor (Horinouchi & Beppu, 1992). These, and similar results from transforming E. coli with afsA cloned from S. griseus, suggest (Ando et al., 1997) that AfsA is an enzyme that can synthesize A-factor from common intermediates of cellular metabolism. However, biochemical evidence for such activity is not yet available, and phenotypic differences in streptomycetes containing the AfsA domain in their translated genome have suggested a more complex function. Whereas mutants of S. griseus disrupted in afsA are bald, strains of S. virginiae disrupted in barX are unaffected in sporulation; they also retain the capacity for antibiotic production, although the output of products is disturbed (Kawachi et al., 2000). Since both antibiotic production and sporulation in S. venezuelae are affected by inactivation of jadW₁, this gene resembles afsA more closely than barX in function. However, jadW₁-disrupted mutants differ from the comparable afsA mutants in not responding to extracellular complementation, and in this respect resemble barX mutants of S. virginiae, which are not restored to normal virginiamycin production by supplementation with VB. Kawachi et al. (2000) indicated that BarX is a regulatory protein controlling VB biosynthesis rather than a catalytic enzyme in the VB biosynthesis pathway, and evidence from transcriptional analyses suggest that the regulatory role of barX involves adjacent genes. Among these are genes encoding a VB receptor protein (BarA) and homologues. Acting alone, BarX is a positive regulator facilitating VB biosynthesis, but in concert with BarA it functions as a co-repressor, reinforcing the negative regulation of target enzymes exerted by the unliganded VB receptor (Kawachi et al., 2000). In S. coelicolor A3(2), the BarX equivalent (ScbA) controlling biosynthesis of the γ-butyrolactone SCB1 also appears to function as a transcriptional regulator rather than as a biosynthetic enzyme, and as in S. virginiae, transcriptional analyses implicated the SCB1 receptor protein (ScbR) in the regulatory mechanism. Acting alone ScbR inhibited expression of scbA, but with ScbA present it bound to the scbA promoter, activating transcription and enhancing SCB1 biosynthesis (Takano et al., 2001).

Phenotypic effects of jadW₁ disruption

Pioneering studies of the γ-butyrolactone regulatory mechanism in streptomycetes have demonstrated the importance of this system in controlling morphological differentiation and secondary metabolism (Horinouchi & Beppu, 1992; Yamada, 1999), and recent investigations have explored its participation in developmental programmes (Folcher et al., 2001; Kato et al. 2002). Our results demonstrate that the γ-butyrolactone regulatory mechanism in S. venezuelae ISP5230 has a role in mediating the influence of nutrition over growth and developmental activities leading to cellular differentiation and secondary metabolism. The consequences of disrupting the quorum-sensing jadW₁ component of the control system indicate that a relatively small decrease of the growth rate in a complex medium can be correlated with failure of surface cultures to sporulate, as well as with loss of antibiotic production in normally supportive broth media. Kato et al. (2002) have shown that in S. griseus the A-factor-responsive γ-butyrolactone system controls production of an extracellular protease postulated to be involved in recycling the proteins of substrate mycelium for synthesis of aerial mycelium. Submerged aerated cultures of S. venezuelae in media with complex nitrogen sources grow as slurry of relatively short unbranched filaments that disperse uniformly in shaken cultures. The effect of disrupting jadW₁ on the growth rate of such cultures is plausibly explained by a decrease in the supply of amino acids from proteolysis. Supporting a role for the product of jadW₁ in proteolysis is the marked decrease in growth rate of cultures with glucose in place of a carbon source such as maltose, causing less repression of proteolysis. In glucose-asparagine medium, the much higher initial growth rate of the mutant relative to the wild-type correlated with hyphal fragmentation, giving a growth morphology similar to that in media with complex nitrogen sources. The wild-type cultures were strikingly different and consisted of discrete wefts of tangled elongated hyphae. The growth morphology of the wild-type mycelium was unaffected by replacing the carbon source in glucose-asparagine medium with maltose and was consistent with the activity of a developmental gene that was not expressed in the mutant. The results imply that the jadW₁ component of the γ-butyrolactone control system acts as a positive regulator of cellular differentiation.

Role of jadR₂: potential involvement in the γ-butyrolactone regulatory system

A BLASTP search re-examining the jad cluster for genes encoding components of a γ-butyrolactone regulatory system revealed that the deduced amino acid sequence of jadR₂ (Yang et al., 1995) is 27–31% identical to sequences of the γ-butyrolactone receptors ArpA of S. griseus (Miyake et al., 1989), BarA of S. virginiae (Kim et al., 1990), FarA of S. lavendulae FRI-5 (Waki et al., 1997) and ScbR of S. coelicolor A3(2) (Takano et al., 2001). The sequence shows even closer resemblance (41–42% identity) to the receptor homologues BarB of S. virginiae (Kinoshita et al., 1997) and TylQ of S. fradiae (Bate et al., 1999). Contributing to the similarity of these products is the presence in JadR₂⁺, the receptor proteins and their homologues of a TetR helix-turn-helix DNA-binding domain (Yang et al., 1995; Kisker...
et al., 1995). In *S. virginiae*, where genes for the receptor and homologue are clustered with a transporter gene, BarB modulates the γ-butyrolactone-controlled signal from BarA to influence the resistance of the producer to its antibiotic metabolite, virginiamycin S (Kawachi et al., 2000). The functions of other receptor homologues are uncertain, but a correlation between the acidic character (pI ~ 5) of proteins proven to bind a cognate γ-butyrolactone, and the more basic character (pI ~ 10) of homologues for which such binding has not so far been demonstrated has been noted (Kawachi et al., 2000). The isoelectric point (pI 6·14) of JadR2 estimated from the deduced amino acid sequence favours assignment to the receptor group.

In *S. venezuelae* ISP5230, jadR2 is located 1184 bp away from jadW1, a relatively small distance compared with the separation of afsA and arpA on the *S. griseus* chromosome (Ohnishi et al., 1999). However, even shorter distances are reported for the separation of γ-butyrolactone biosynthesis/receptor gene pairs in *S. virginiae* (barX/barA, 259 bp; Kawachi et al., 2000), *S. lavendulae* FRI-5 (farX/farA, 184 bp; Kitani et al., 1999) and *S. coelicolor* A3(2) (scbAscbR, 118 bp; Takano et al., 2001). Like scbA, the AfS-family gene *mmfL* involved with methylomycin production in *S. coelicolor* A3(2) is located near genes for candidate γ-butyrolactone receptors (O’Rourke & Chater, 2001). Takano et al. (2001) noted that distances between genes for γ-butyrolactone receptors and those for γ-butyrolactone biosynthesis or regulation affect opportunities for regulatory DNA interactions and may account for species-specific differences in the operation of the γ-butyrolactone regulatory system. The species-specific features determined by the different gene organizations of *S. venezuelae* and *S. griseus* may include ligand-receptor binding or other factors influencing extracellular complementation of jadW1 and afsA mutants.

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