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Regulation of valanimycin biosynthesis in *Streptomyces viridifaciens*: characterization of VlmI as a *Streptomyces* antibiotic regulatory protein (SARP)

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Streptomyces antibiotic regulatory proteins (SARPs) have been shown to activate transcription by binding to a tandemly arrayed set of heptameric direct repeats located around the −35 region of their cognate promoters. Experimental evidence is presented here showing that *vlmI* is a regulatory gene in the valanimycin biosynthetic gene cluster of *Streptomyces viridifaciens* and encodes a protein belonging to the SARP family. The organization of the valanimycin biosynthetic gene cluster suggests that the valanimycin biosynthetic genes are located on three potential transcripts, *vlmHORBCD*, *vlmJKL* and *vlmA*. Disruption of *vlmI* abolished valanimycin biosynthesis. Western blot analyses showed that VlmR and VlmA are absent from the *vlmI* mutant and that the production of VlmK is severely diminished. These results demonstrate that the expression of these genes from the three potential transcripts is under the positive control of VlmI. The *vlmA–vlmH* and *vlmI–vlmJ* intergenic regions both exhibit a pattern of heptameric direct repeats. Gel shift assays with VlmI overproduced in *Escherichia coli* as a C-terminal FLAG-tagged protein clearly demonstrated that VlmI binds to DNA fragments from both regions that contain these heptameric repeats. When a high-copy-number *vlmI* expression plasmid was introduced into *Streptomyces coelicolor* M512, which contains mutations in the undecylprodigiosin and actinorhodin activators *redD* and *actII-orf4*, undecylprodigiosin production was restored, showing that *vlmI* can complement a *redD* mutation. Introduction of the same *vlmI* expression plasmid into an *S. viridifaciens vlmI* mutant restored valanimycin production to wild-type levels.

INTRODUCTION

Streptomyces are filamentous soil bacteria that undergo differentiation and sporulation, and produce a multitude of bioactive compounds. The production of antibiotics and other secondary metabolites in these bacteria is tightly controlled by environmental stimuli and by a complex network of regulatory proteins that function at several hierarchical levels. The highest level of regulation involves pleiotropic genes that govern differentiation and sporulation as well as secondary metabolite production (Bibb, 2005). The lowest level employs pathway-specific regulatory genes that are usually associated with individual biosynthetic gene clusters. The family of proteins known as *Streptomyces* antibiotic regulatory proteins (SARPs) were the first pathway-specific regulatory proteins to be identified (Wietzorrek & Bibb, 1997). More recent studies have shown that the SARP family contains both pathway-specific regulators and pleiotropic regulatory proteins such as AfsR (Tanaka *et al.*, 2007).

Abbreviation: SARP, *Streptomyces* antibiotic regulatory protein.

Members of the SARP family are transcriptional activators that exhibit a winged helix–turn–helix motif near their N termini that is similar to a motif found in the C terminus of the OmpR family of regulatory proteins (Wietzorrek & Bibb, 1997). Representative examples of SARPs include RedD and ActII-orf4, which control the production of undecylprodigiosin and actinorhodin, respectively, in *Streptomyces coelicolor* (Fernandez-Moreno *et al.*, 1991; Narva & Feitelson, 1990), DnrI, which controls the production of daunorubicin by *Streptomyces peucetius* (Stutzman-Engwall *et al.*, 1992), CcaR, which regulates clavulanic acid and cephamycin C biosynthesis in *Streptomyces clavuligerus* (Perez-Llarena *et al.*, 1997), and FdmR1, which controls fredericamycin production in *Streptomyces griseus* (Chen *et al.*, 2008). SARPs are postulated to activate transcription by binding to a tandemly arrayed set of heptameric repeats located around the −35 region of their cognate promoters. This hypothesis has been confirmed by gel shift mobility and DNA footprinting assays with promoter regions from the

actinorhodin and daunorubicin biosynthetic gene clusters (Arias *et al.*, 1999; Tang *et al.*, 1996). After binding to the direct repeat region, the SARP regulators are believed to initiate transcription by recruitment of RNA polymerase to the appropriate sites (Tanaka *et al.*, 2007).

The antibiotic valanimycin is a potent antitumor and antibacterial azoxy compound isolated from the fermentation broth of *Streptomyces viridifaciens* MG456-hF10 by Yamato and co-workers (Yamato *et al.*, 1986). Enzymic and genetic investigations (Parry & Li, 1997a, b; Parry *et al.*, 1997) have led to the cloning and sequencing of the valanimycin biosynthetic gene cluster, which has been found to contain 14 genes (Fig. 1a) (Garg *et al.*, 2002). The functions of the products of eight of these genes have now been established. VlmF, which is a member of the major facilitator family of transport proteins, confers valanimycin resistance (Ma & Parry, 2000). VlmD, VlmH and VlmR catalyse the conversion of valine into isobutylhydroxylamine (Garg *et al.*, 2002), while VlmL catalyses the formation of L-seryl-tRNA from L-serine (Garg *et al.*, 2006). VlmA has been shown to catalyse the transfer of L-serine from L-seryl-tRNA to isobutylhydroxylamine, to produce *O*-(L-seryl)-isobutylhydroxylamine (Garg *et al.*, 2008), while VlmJ and VlmK catalyse the phosphorylation and subsequent dehydration of the biosynthetic intermediate valanimycin hydrate (Garg *et al.*, 2009). The biosynthetic pathway for valanimycin is shown in Fig. 1(b).

The valanimycin gene cluster appears to contain two regulatory genes. The first of these is *vlmE*, which encodes a protein in the *tetR* family of repressor proteins. Evidence from other systems suggests that the *vlmE* gene product probably regulates the expression of the resistance gene *vlmF* (Garg *et al.*, 2002). The second regulatory gene is *vlmI*. The deduced translation product of *vlmI* exhibits strong similarities to a number of members of the SARP family, including DnrI, RedD and TylT (Garg *et al.*, 2002). In order to further characterize the *vlmI* gene product and to examine its role in valanimycin production, we have inactivated the *vlmI* gene, overexpressed VlmI and examined the DNA binding properties of VlmI. The results of these studies are reported here.

METHODS

General. Unless otherwise indicated, all reagents used in this study were purchased from Sigma, Roche Applied Sciences, Bio-Rad Laboratories or G.E. Healthcare. Oligonucleotides were obtained from Sigma Genosys. Restriction enzymes were obtained from either

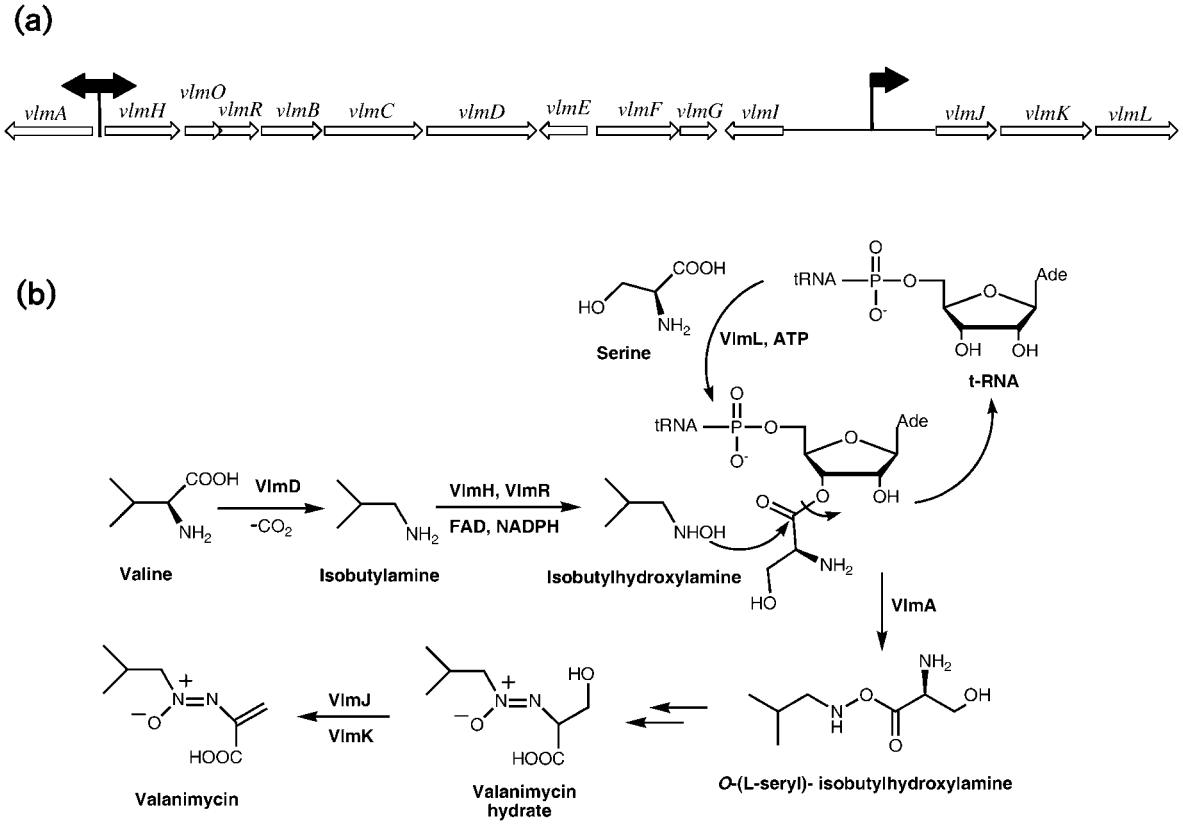

Fig. 1. (a) Valanimycin biosynthetic gene cluster of *S. viridifaciens* MG456-hF10. Black arrows indicate approximate locations of VlmI binding sites. (b) Biosynthetic pathway for valanimycin in *S. viridifaciens* MG456-hF10, showing structures of the primary precursors L-valine and L-serine and structures of known intermediates.

New England Biolabs or Promega. [α-^{32}P]dCTP was obtained from MP Biomedicals. Competent cells of *Escherichia coli* DH10B and BL21 (DE3) were purchased from Invitrogen and EMD, respectively, and were used according to the manufacturers' recommendations. The purity and molecular mass of overproduced proteins were evaluated by SDS-PAGE using broad-range protein molecular mass markers from Bio-Rad Laboratories. Prestained protein molecular mass markers from New England Biolabs were used for Western blotting. Protein concentrations were determined with the Advanced Protein Assay Reagent from Cytoskeleton or by use of the BCA reagent from Pierce. BSA was used as a standard.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this work are summarized in Table 1.

DNA manipulations. Genomic DNA was prepared from *S. viridifaciens* MG456-hF10 using DNAzol reagent (MRC) after macerating mycelium frozen with liquid nitrogen. Plasmid DNA was purified with a QIAprep Spin Plasmid kit (Qiagen). DNA fragments were isolated from agarose gels with a QIAquick Gel Extraction kit (Qiagen). PCR products were separated on agarose gels and purified from the gels. Digestion with restriction endonucleases and ligation experiments were carried out by standard procedures under conditions recommended by the manufacturers. Automated DNA sequencing was performed at Lone Star Sequencing Laboratories by using universal and synthetic oligonucleotide primers. End labelling to produce radioactive DNA fragments was carried out using [α-^{32}P]dCTP and Klenow DNA polymerase (New England Biolabs) as per the manufacturer's recommendations. The purified, end-labelled DNA was digested with various restriction enzymes in the manufacturer's recommended buffers to produce the labelled DNA fragments needed to map the VlmI binding sites.

Isolation of intergenic DNA. A 443 bp intergenic region between *vlmA* and *vlmH* in the valanimycin biosynthetic gene cluster was amplified from plasmid pVal35 (Garg *et al.*, 2002) by PCR using the following primers: 5′-CTCGTATCGCGCTCAGTAC-3′ and 5′-ATCGAGAGAACGCATTCTGG-3′. The PCR product was gel-purified and cloned into pGEM-T to produce plasmid pVlmAH. The DNA insert in pVlmAH was released as a *Not*I fragment for end labelling. Similarly, a 2.5 kb DNA fragment between *vlmI* and *vlmJ* was amplified using the primers 5′-TTCTCCTTGCCATC-GCTCATAGTT-3′ and 5′-AAGATCATTGATGGTCATGAAGA-3′, and the resulting fragment was cloned into pGEM-T. The resulting plasmid pVlmIJ was digested with *Not*I, and two DNA fragments corresponding to 1.0 and 1.5 kb in size were gel-purified and used for end labelling. A 1.2 kb *Kpn*I fragment of intergenic DNA from pVlmIJ was gel-purified and cloned into the *Kpn*I site of pMECA (Thomson & Parrott, 1998) to produce plasmid pIJMECA-1. pIJMECA-1 was then digested with *Sma*I and *Pml*I (for deletion of *Not*I–*Pml*I intergenic DNA) and religated to produce plasmid pIJMECA-2, which contains a 231 bp *Pml*I–*Kpn*I fragment of DNA from the intergenic region between *vlmI* and *vlmJ*. This fragment was end-labelled after isolation as a *Hin*dIII–*Spe*I fragment.

Cloning of VlmI. The DNA encoding VlmI was amplified by PCR from plasmid pVal35 with a mixture of *Taq* and *Vent* DNA polymerases using an N-terminal primer containing an *Nde*I site (5′-ATTAT***CATATG***GCAAGGAGAACCCGCA-3′) and a C-terminal primer with an *Eco*RI site (5′-TTTAT***GAATTC***GCGCCGGGT-GCCATG-3′) (restriction sites are indicated by bold italic type). The PCR product was gel-purified and cloned into a pCR2.1-TOPO vector (Invitrogen), and the DNA sequence was verified by sequencing using vector primers. The *vlmI* gene was subsequently cloned into the *Nde*I and *Eco*RI sites of pFLAG-CTC to produce pFLAG-CTC-VlmI-(L). The stop codon of the *vlmI* gene was removed to place the *vlmI* gene in-frame with the FLAG-tag of the vector. A smaller version of the *vlmI* gene with a start site 165 bp downstream from the start site of the larger version was also cloned by PCR. In this case, the N-terminal primer, which contained a *Hin*dIII site, had the following sequence: 5′-TATA***AAGCTT***AT-GCTGAAGTTCCAGGATTTT-3′, while the C-terminal primer was the same as that used to amplify the longer version of the gene. The resulting PCR product was purified and cloned into pGEM-T, and the DNA sequence was verified by sequencing using vector primers. The shorter version of the *vlmI* gene was subsequently subcloned into the *Hin*dIII and *Eco*RI cloning sites of pFLAG-CTC to produce pFLAG-CTC-VlmI-(S).

Overexpression, purification and characterization of VlmI. Overproduction and purification of VlmI-L and VlmI-S from pFLAG-CTC-VlmI-(L) and pFLAG-CTC-VlmI-(S) were carried out according to the protocols outlined in the FLAG vector instruction manual (Sigma). *E. coli* BL21 (DE3) cells harbouring the desired plasmid were grown overnight in Luria–Bertani (LB) broth supplemented with 100 μg ampicillin ml^{-1}, and then diluted 100-fold into fresh 2× LB broth plus 0.4 % glucose and 100 μg ampicillin ml^{-1}. The cultures were grown at 37 °C until OD_{600} ~0.7 was reached, whereupon IPTG was added to a final concentration of 0.5 mM. After 2 h, the cells were harvested and stored at −20 °C until utilized for isolation of the desired protein. The overproduced protein was purified using a column of anti-FLAG antibody resin using a previously described protocol (Garg *et al.*, 2006). The purified protein was stored at 4 °C in Tris-buffered saline buffer containing 15 % (v/v) glycerol. Western blotting with anti-GroEL antibodies (Sigma) was carried out with each protein preparation to check for the presence of GroEL contamination (Couch *et al.*, 2002). Both the smaller and larger versions of VlmI co-purified with an additional two proteins. One of these contaminants appeared to be GroEL, since it reacted with anti-GroEL antibodies.

Disruption of the *vlmI* gene. A single-crossover mutation was created to define the role of VlmI in valanimycin biosynthesis. An internal 478 bp fragment of the *vlmI* gene was amplified by PCR from pVal35 using an N-terminal primer with a *Spe*I restriction site (5′-ATTAT***ACTAGT***GGAGACACTCGTCGACGA-3′) and a C-terminal primer with a *Spe*I restriction site (5′-ATTAT***ACTAGT***ATCAGC-TGCTGATGAAGC-3′). The PCR product was cloned into pGEM-T, sequenced, and then subcloned into the *Xba*I site of the *E. coli*–*Streptomyces* shuttle vector pKC1139, which contains a temperature-sensitive *Streptomyces* replicon (Bierman *et al.*, 1992). The resulting plasmid, pKC1139Δ*vlmI*, was then introduced into *S. viridifaciens*, and a single-crossover *vlmI* mutant was recovered using previously described methods (Garg *et al.*, 2002). The genotype of the mutation was verified by Southern blot analysis of *Kpn*I-digested genomic DNA with a 478 bp internal *vlmI* fragment obtained by *Eco*RI digestion of plasmid pGEMTΔ*vlmI*. The *vlmI* mutant (*S. viridifaciens-vlmI*$^{-}$) was assayed for valanimycin production in the manner previously described (Garg *et al.*, 2002).

Complementation of *S. viridifaciens vlmI* mutant and of *S. coelicolor* M512. Three complementation plasmids were constructed. The first was derived from the integrating plasmid pSET152 (Bierman *et al.*, 1992). A portion of the vector pXY300 (Yin *et al.*, 2003) carrying ampicillin and thiostrepton resistance genes was amplified by PCR using the following forward and reverse primers: 5′-GACGTC***GAGCTC***TTACCAATGCTTAATCAGTG-3′ and 5′-GACGTC***GAGCTC***TTATCGGTTGGCCGCGAGATT-3′, where the bases marked in bold and italic type correspond to a *Sac*I site. The resulting PCR fragment was digested with *Sac*I and then ligated into pSET152 that had been digested with *Sac*I to remove most of the apramycin resistance gene. The resulting plasmid, pSET152-Tsr, was subsequently digested with *Eco*RI and *Xba*I, and then ligated to the 0.5 kb *ermE** promoter cassette isolated from plasmid pBS-VII-41F

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Amp, ampicillin; Apr, apramycin; Km, kanamycin; Spec, spectinomycin; Tet, tetracycline; Tsr, thiostrepton.

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
S. viridifaciens MG456-hF10	Valanimycin producer: wild-type	Yamato *et al.* (1986)
S. viridifaciens MG456-hF10 *vlmI*$^-$ mutant	*vlmI*::pKC1139Δ*vlmI*, *S. viridifaciens* MG456-hF10 with single-crossover disruption of *vlmI*	This work
S. coelicolor strain M512	Δ*redD*, Δ*actII-orf4*	Fernandez-Moreno (1991)
S. coelicolor strain M145	SCP1$^-$, SCP2$^-$	Kieser *et al.* (2000)
E. coli DH10B	Cloning host	BRL
E. coli BL21 (DE3)	Protein overexpression strain, Tet^R	Novagen
E. coli 12567	RP4-Tc::Mu-Km::Tn*7*, Spec^R *dam*$^-$, *dcm*$^-$, *hsdM*$^-$	MacNeil *et al.* (1992)
Plasmids		
pUZ8002	Non-transmissible, *oriT* mobilizing plasmid, Km^R	Kieser *et al.* (2000)
pKC1139	*E. coli–Streptomyces* shuttle plasmid for conjugal transfer, with temperature-sensitive *Streptomyces* replicon, Apr^R	Bierman *et al.* (1992)
pWHM3	*E. coli–Streptomyces* shuttle vector, Tsr^R, Amp^R	Vara *et al.* (1989)
pWHM3-OriT	*E. coli–Streptomyces* shuttle vector, *oriT*, Tsr^R, Amp^R	This work
pPM801	*E. coli–Streptomyces* shuttle vector, *oriT*, Tsr^R	Mazodier *et al.* (1989)
pSET152	Integrating *Streptomyces* plasmid, *lacZ*, intΦC31, repUC, Apr^R	Bierman *et al.* (1992)
pSET152-Tsr	pSET152 with Tsr^R and Amp^R genes from pXY300 inserted	This work
pSET152-ErmE*	pSET152-Tsr with *ermE** promoter inserted	This work
pBS-VII-41F	*ermE** promoter cloned into multiple cloning site of pGEM-3zf, Amp^R	B. Shen, University of Wisconsin-Madison
pVal35	pVal38 with ~3 kb *Spe*I fragment removed from the 3′ end of insert by digestion and religation	Garg *et al.* (2002)
pXY300	Derivative of pKC1139, Tsr^R	Yin *et al.* (2003)
pGEM-T	TA cloning vector, Amp^R	Promega
pCR2.1 –TOPO	TOPO cloning vector, Km^R	Invitrogen
pFLAG-CTC	FLAG-tag expression vector, *trc* promoter, Amp^R	Sigma Chemical Company
pMECA	Derivative of cloning vector pUC19 with large multiple cloning site, Amp^R	Thomson & Parrott (1998)
pFLAG-CTC-VlmI-(L)	Longer version of *vlmI* spanning nucleotides 14 748–15 735 cloned into *Nde*I and *Eco*RI sites of pFLAG-CTC	This work
pFLAG-CTC-VlmI-(S)	Shorter version of *vlmI* spanning nucleotides 14 748–15 570 cloned into *Hin*dIII and *Eco*RI sites of pFLAG-CTC	This work
pKC1139 Δ*vlmI*	Internal 478 bp fragment of *vlmI* spanning nucleotides 14 963–15 442 cloned into *Xba*I site of pKC1139	This work
pVlmI-CP2-WHM3	Complementation plasmid produced by subcloning of *ermE** promoter-VlmI-L cassette from pVlmI-CP2 into pWHM3	This work
pVlmI-CP2-WHM3-OriT	Complementation plasmid produced by subcloning of *ermE** promoter-VlmI-L cassette from pVlmI-CP2 into pWHM3-OriT	This work
pVlmK-pET28b	*vlmK* cloned into expression vector pET28b, Km^R	X. Zhang and R. J. Parry, unpublished results
pET VlmRN1RC1	*vlmR* cloned into pET28b, Km^R	Parry *et al.* (1997)
pGEMTΔvlmR	Internal 414 bp fragment of *vlmR* spanning nucleotides 7080–7493 cloned into pGEM-T	This work
pVlmR-BS	Internal 414 bp *vlmR* fragment spanning nucleotides 7080–7493 cloned into *Xba*I/*Hin*dIII-digested plasmid BS-VII-41F	This work
pKC1139Δ*vlmR*	*ermE** promoter and internal 414 bp fragment of *vlmR* spanning nucleotides 7080–7493 cloned into *Eco*RI/*Hin*dIII-digested pKC1139	This work
pVlmA-CTC	*vlmA* cloned into pFLAG-CTC, Amp^R	Garg *et al.* (2008)
pVlmAH	443 bp intergenic region between *vlmH* and *vlmA* spanning nucleotides 4947–5389 cloned into pGEM-T	This work
pVlmIJ	2.5 kb intergenic region between *vlmI* and *vlmJ* spanning nucleotides 15 723–17 754 cloned into pGEM-T	This work
pIJMECA-1	1.2 kb *Kpn*I fragment of pVlmIJ cloned into *Kpn*I site of pMECA	This work
pIJMECA-2	Plasmid derived from pIJMECA-1 by digestion with *Sma*I and *Pml*I DNA followed by religation. Contains 231 bp *vlmI–vlmJ* intergenic region	This work

(B. Shen, unpublished work) by digestion with the same restriction enzymes. The resulting plasmid was named pSET152-ErmE*. The longer version of the *vlmI* gene was amplified by PCR from pVal35 with a mixture of *Taq* and *Vent* DNA polymerases using the following forward and reverse primers: VlmI N *Bam*HI, 5′-***GGATCC***GGAGGTACGGACATGGCAAGGAGAACCGC-3′, and VlmI C *Xba*I, 5′-***TCTAGA***TCAGCGCCGGGTGCCATG-3′, where the bases marked in bold and italic type correspond to *Bam*HI and *Xba*I sites, respectively. The resulting PCR product was cloned into vector pGEM-T and the insert was sequenced to verify the absence of errors. The *vlmI* gene was released from the pGEM-T construct by digestion with *Bam*HI and *Xba*I, and then cloned into *Bam*HI–*Xba*I-digested pSET152-ErmE* to place the longer version of the *vlmI* gene downstream of the *ermE** promoter. The resulting plasmid was named pVlmI-CP2.

The additional complementation plasmids were derived from the high-copy-number plasmid pWHM3 (Vara *et al.*, 1989). To create a version of pWHM3 that can be transferred by conjugation, an *oriT* fragment was removed from plasmid pPM801(Mazodier *et al.*, 1989) as a *Pst*I fragment and cloned into *Pst*I-digested pWHM3 to give plasmid pWHM3-OriT. The *vlmI* gene and upstream *ermE** promoter were then removed from pVlmI-CP2 as an *Eco*RI–*Xba*I fragment, and cloned into the same sites in pWHM3 and pWHM3-OriT to give complementation plasmids pVlmI-CP2-WHM3 and pVlmI-CP2-WHM3-OriT.

Plasmids pVlmI-CP2, pSET152-ErmE* and pVlmI-CP2-WHM3-OriT were each introduced into methylation-deficient *E. coli* strain ET12567 (pUZ8002) (Kieser *et al.*, 2000; MacNeil *et al.*, 1992) and then transferred to *S. coelicolor* M512 (Floriano & Bibb, 1996) by conjugation, as previously described (Garg *et al.*, 2002). Plasmids pVlmI-CP2 and pVlmI-CP2-WHM3 were each passed through *E. coli* strain ET12567 (MacNeil *et al.*, 1992) and then introduced into *S. viridifaciens vlmI*$^{-}$ by transformation. Exconjugants and transformants were selected for thiostrepton resistance (10 μg thiostrepton ml^{-1}) and purified by two to three rounds of subculturing on R2YE medium (*S. coelicolor*) or ISP2 medium (*S. viridifaciens*). *S. viridifaciens vlmI*$^{-}$ containing pVlmI-CP2 or pVlmI-CP2-WHM3 was assayed for valanimycin production in several ways. TLC analysis of the ethyl acetate extract of the fermentation broth from the complemented strain exhibited a spot with an R_F value very closely matching that of the valanimycin standard (Fig. 7). Furthermore, ^{1}H NMR analysis of the compound purified from the complemented strain confirmed its identity to be valanimycin. The ^{1}H spectrum exhibited vinyl resonances for C-3 at 6.32 and 6.25 p.p.m., the isobutyl CH_2 resonance for C-4 at 4.12 p.p.m., the isobutyl CH resonance for C-5 at 2.47 p.p.m. and the isobutyl CH_3 resonance for C-6 at 1.04 p.p.m. These values are consistent with the proton chemical shifts exhibited by authentic valanimycin (Garg *et al.*, 2009; Ma & Parry, 2000). Finally the compound was shown to be valanimycin by LC-MS analysis of the crude ethyl acetate extract on a Bruker MicroTOF mass spectrometer, as described previously (Garg *et al.*, 2009). The valanimycin exhibited the expected molecular mass: high-resolution ES-MS *m/z*, 173.09208 $(M+H)^+$, calculated for $C_7H_{13}N_2O_3$, 173.09207, confirming the production of valanimycin by the complemented strain.

S. coelicolor M512 containing pVlmI-CP2, pSET152-ErmE* or pVlmI-CP2-WHM3-OriT was assayed for undecylprodigiosin or actinorhodin production by a modification (A. M. Cerdeno, personal communication) of the procedure of Scheu *et al.* (1997). Liquid R2YE medium (30 ml) was inoculated with a spore suspension (10 μl) of *S. coelicolor* M512 containing pVlmI-CP2, pSET152-ErmE* or pVlmI-CP2-WHM3-OriT, and the culture was shaken for 48 h at 30 °C and 250 r.p.m. A 5 ml aliquot of the culture broth was centrifuged and the cell pellet was resuspended in 2.5 ml methanol using a vortex mixer. The mixture was then centrifuged at 3250 ***g*** and the methanolic extract was removed. To detect the presence of undecylprodigiosin, concentrated HCl (50 μl) was added to a 1 ml aliquot of the methanol extract and the A_{530} was determined. To detect the presence of actinorhodin, 10 M NaOH (50 μl) was added to a 1 ml aliquot of the methanol extract and the A_{630} was measured. The concentration of undecylprodigiosin in a 1 ml aliquot was calculated using the reported molar absorption coefficient for the hydrochloride salt of the antibiotic at 530 nm (Wasserman *et al.*, 1976). The *redD*$^+$ strain *S. coelicolor* M145 was assayed for undecylprodigiosin in the same manner. The results are summarized in Table 2.

Gel mobility shift assay. DNA gel shift assays were performed as described previously (Garg *et al.*, 2000). Typically, 20 μl assay mixture contained 4000 c.p.m. labelled DNA, 0.2–5 μg protein in 10 mM Tris (pH 7.5), 50 mM KCl, 1 mM EDTA, 2 mM DTT, 2.6 μg BSA and 2 μg sonicated salmon sperm DNA. Unlabelled DNA was added to reactions to check the specificity of DNA–protein interactions. Incubations were carried out at 30°C for 30 min and DNA binding was then analysed by electrophoresis in a Protean II Xi cell (Bio-Rad) using a 5 % native polyacrylamide gel in 0.5× TBE (75 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 100 V for 12–24 h depending upon the size of DNA fragments. Autoradiography of the resulting gels was carried out at −80 °C using Hyperfilm (GE Healthcare).

Disruption of the *vlmR* gene. A single-crossover, non-polar mutation with an outgoing *ermE** promoter was generated to disrupt the *vlmR* gene. An internal 414 bp fragment of the *vlmR* gene was amplified by PCR from pVal35 (Garg *et al.*, 2002) using an N-terminal primer with an *Xba*I restriction site (5′-***TCTAGA***-CGAGCTGCGAGGCCTC-3′) and a C-terminal primer with an *Hin*dIII restriction site (5′-***AAGCTT***CGGCCGTCGAGAACCT-3′). The PCR product was cloned into pGEM-T, sequenced, and then subcloned into *Xba*I- and *Hin*dIII-digested plasmid pBS-VII-41F, resulting in plasmid pVlmR-BS. This plasmid was then digested with *Eco*RI and *Hin*dIII to produce a 0.9 kb DNA fragment that contained the truncated *vlmR* gene downstream of the *ermE** promoter. This DNA fragment was purified on an agarose gel and then subcloned into the *E. coli–Streptomyces* shuttle vector pKC1139 (Bierman *et al.*, 1992) digested with *Eco*RI and *Hin*dIII. The resulting plasmid, pKC1139Δ*vlmR*, was introduced into *S. viridifaciens* by conjugation, and a single-crossover *vlmR* mutant was recovered using previously described methods (Garg *et al.*, 2002). The genotype of the mutation was verified by Southern blot analysis of *Kpn*I-digested genomic DNA from the mutant using a 414 bp internal *vlmR* fragment obtained by *Eco*RI digestion of plasmid pGEMTΔ*vlmR* as the probe (data not shown). The *vlmR* mutant (*S. viridifaciens vlmR*$^{-}$) was assayed for valanimycin production in the manner previously described (Garg *et al.*, 2002) and found to be blocked in valanimycin production.

Table 2. Complementation of *redD* mutation in *S. coelicolor* M512 with *vlmI-L*

Strain	Undecylprodigiosin concn (μM)*
S. coelicolor M512 (Δ*redD*) pSETErmE*	0.1
S. coelicolor M512 (Δ*redD*) pVlmI-CP2	2.2
S. coelicolor M512 (Δ*redD*) pVlmI-CP2-WHM3-OriT	700.0
S. coelicolor M145 (*redD*$^+$)	431.9

*See Methods for details.

Preparation of VlmA, VlmK and VlmR antibodies. VlmA, VlmK and VlmR were overproduced from the expression plasmids pVlmA-FLAG-CTC for VlmA (Garg *et al.*, 2008), pVlmK-pET28b for VlmK (X. Zhang and R. J. Parry, unpublished data), and pET-VlmRN1RC1 for VlmR (Parry & Li, 1997a), respectively. Each protein was then purified by affinity chromatography as per the manufacturer's recommendations. Although the purified VlmK and VlmR needed no additional purification, VlmA was contaminated with GroEL and required further purification. This was accomplished by SDS-PAGE. The VlmA band was excised from a developed SDS-PAGE gel using UV light for visualization. The gel slice was then macerated with elution buffer (25 mM Tris, 150 mM NaCl, 0.1 %SDS), and the resulting protein solution was concentrated and the SDS removed by repeated filtration through a 10 kDa molecular-mass-cutoff filter. The purified proteins were each suspended in PBS (50 mM Na_2HPO_4, 0.85 % NaCl, pH 7.4) at a concentration of 1 mg ml^{-1}. Antibodies to VlmK and VlmA were raised by Alpha Diagnostics, while antibodies to VlmR were prepared by Bethyl Laboratories. Rabbits served as the host animals. Serum samples from the second bleed after booster doses were used for Western analysis.

Western blot analyses. *S. viridifaciens* spore suspensions (5 µl) derived from the wild-type and mutant strains were each used to inoculate 50 ml valanimycin production medium contained in a 250 ml conical flask and the cultures were then shaken at 37 °C and 250 r.p.m. for 15 h. The cell pellet from 3 ml of each culture was lysed in the manner previously described (Garg *et al.*, 2008). A 100 µl aliquot of each lysate was mixed with an equal amount of 2× Laemmli buffer (Laemmli, 1970) and heated at 100 °C for 5 min. The mixtures were then centrifuged at 13 200 ***g***, and 10–20 µl of each supernatant was loaded onto a 10 % SDS-PAGE gel along with prestained molecular mass markers and purified VlmK, VlmA and VlmR proteins as a positive control. Western blots were then performed as described previously (Garg *et al.*, 1994) and detected with anti-rabbit IgG conjugated to alkaline phosphatase using the Sigma Fast BCIP-NBT reagent following the manufacturer's instructions.

RESULTS

Disruption of *vlmI*

To confirm that VlmI regulates the expression of the valanimycin biosynthetic gene cluster, a single-crossover disruption was created in the *vlmI* gene. The disruption plasmid pKC1139Δ*vlmI* was introduced into *S. viridifaciens* by conjugation and a single-crossover *vlmI*$^-$ mutant was recovered using previously described methods (Garg *et al.*, 2002). The genotype expected for the mutation was verified by Southern blot analysis of *Kpn*I-digested genomic DNA with a 478 bp internal *vlmI* fragment obtained by *Eco*RI digestion of plasmid pGEMTΔ*vlmI*. Two hybridizing bands were observed at the expected positions (Fig. 2). When the *vlmI*-disrupted mutant was grown in valanimycin production medium and assayed for valanimycin production, no valanimycin production was observed. These results indicate that *vlmI* is essential for valanimycin biosynthesis.

Influence of VlmI on expression of valanimycin biosynthetic proteins

The organization of the valanimycin gene cluster suggests that the valanimycin biosynthetic genes are located on three potential transcripts: *vlmHORBCD*, *vlmJKL* and *vlmA*. In order to determine whether VlmI regulates the

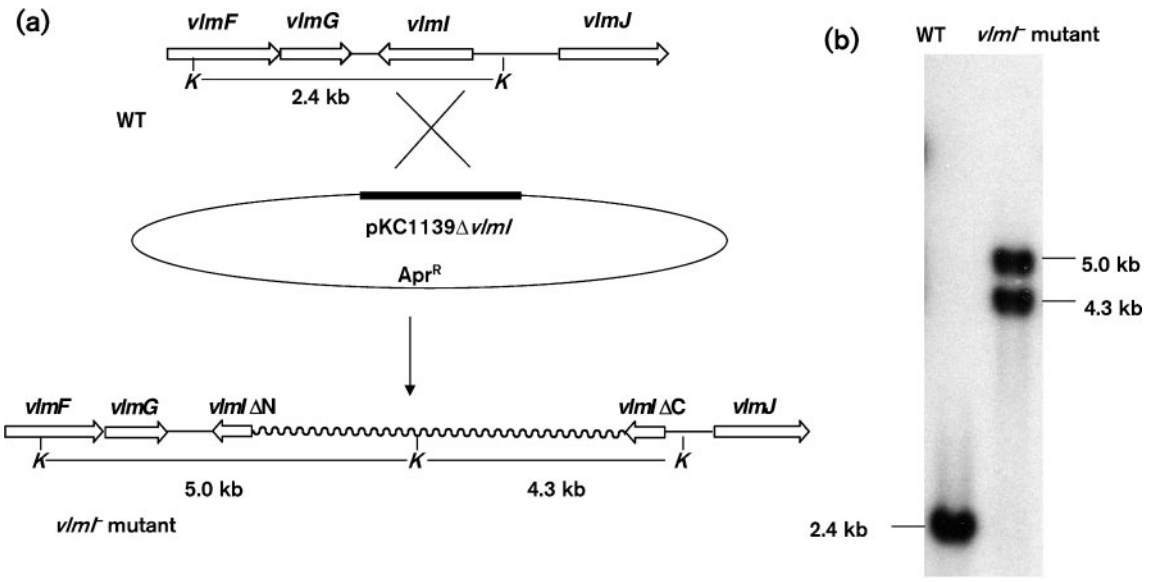

Fig. 2. (a) Diagrammatic illustration of single-crossover disruption in the *vlmI* gene created by plasmid pKC1139Δ*vlmI*, showing expected restriction fragments. (b) Southern blot analysis of *Kpn*I-digested genomic DNA from wild-type *S. viridifaciens* MG456-hF10 (WT) and *S. viridifaciens* MG456-hF10 (*vlmI*::pKC1139Δ*vlmI*). A 478 bp internal *vlmI* fragment obtained by *Eco*RI digestion of plasmid pGEMTΔ*vlmI* was labelled with [α-^{32}P]dCTP and used as a probe. *Kpn*I sites are indicated by *K*.

expression of all three of these transcripts, Western blotting experiments were carried out using polyclonal antibodies to proteins encoded by each potential transcript. Anti-VlmR antibodies were used to detect a translation product of the *vlmHORBCD* transcript, while anti-VlmK and -VlmA antibodies were used to detect a translation product of the *vlmJKL* and *vlmA* transcripts, respectively. The Western blot analyses utilized cell-free extracts from wild-type and mutant strains as well as the purified VlmR, VlmK and VlmA proteins as positive controls (Fig. 3). The results showed that extracts prepared from the *vlmI* mutant contained no cross-reacting material corresponding to the VlmR and VlmA proteins and a very low level of the VlmK protein. Extracts of wild-type *S. viridifaciens* used as positive control showed the presence of all these proteins, ruling out the possible instability of these proteins in the cell-free extracts. When single-crossover *vlmA* and *vlmR* mutants were used as negative controls, Western analysis showed that these proteins were absent, as expected. However, a single-crossover *vlmK* mutant used as a negative control appeared to show some cross-reacting material. This may result from the tendency of the *vlmK* mutant to revert (Garg *et al.*, 2002) or from the presence of an antigenic, truncated version of VlmK produced by translational fusion of *vlmK* with the vector and termination at a vector stop codon. The results from the Western analyses suggest that VlmI regulates the expression of all of the transcripts that encode valanimycin biosynthetic enzymes.

Overexpression and characterization of VlmI

Analysis of the *vlmI* gene reveals the presence of two potential start sites. Translation from the downstream start site yields a shorter version of VlmI that differs from the longer version by the absence of 55 amino acids at the N terminus. The longer (L) and shorter (S) versions of VlmI were overproduced in *E. coli* as C-terminal FLAG-tagged proteins using a pFLAG-CTC vector in which expression is driven from the inducible *trc* promoter. Both proteins were overproduced mainly in soluble form with yields of approximately 2 mg protein l^{-1} of culture broth. The maximum amounts of protein were observed after 2 h induction at 37 °C. Longer incubation at 37 °C or the use of lower temperatures did not improve the yield or quality of the overproduced proteins. On SDS-PAGE analysis, both the longer and shorter versions of VlmI exhibited the molecular masses expected for the denatured proteins, but the proteins contained contaminating proteins (data not shown). Western blot analysis of VlmI-L using anti-FLAG antibodies detected almost equal quantities of two proteins with molecular masses of about 38 and 32 kDa. These values correspond to the predicted molecular masses for VlmI-L and VlmI-S. The protein with the smaller molecular mass might be produced from an internal promoter that is present in the longer *vlmI* sequence or by proteolytic degradation of VlmI-L. As expected, over-produced VlmI-S was not contaminated with VlmI-L. Two additional proteins appeared as contaminants in over-produced, affinity-purified VlmI-L and VlmI-S. One protein, with a mass of 59 kDa, appeared to be GroEL, since it reacted with anti-GroEL antibodies, while the identity of the second protein, with a mass of 75 kDa, was not established.

DNA gel shift mobility assays with overproduced VlmI protein

As previously reported (Garg *et al.*, 2002), the intergenic region between *vlmA* and *vlmH* exhibits three imperfect heptameric repeats with a conserved spacing found in other SARP binding sites (TCACGTG-15x-TCAGGTG-4x-TCAGGGA). A similar group of three imperfect heptameric repeats with a conserved spacing occurs upstream of *vlmJ* (TCACAGG-15x-TCAGAAA-4x-TCAGGAA). A sequence alignment of these heptameric repeats with verified and putative SARP binding sites present in other antibiotic biosynthetic gene clusters is shown in Fig. 4. DNA gel shift assays were conducted to evaluate the putative VlmI binding sites. A 443 bp intergenic region between *vlmA* and *vlmH* was amplified by PCR and labelled by end filling with Klenow DNA polymerase and [α-^{32}P]dCTP after restriction enzyme digestion. DNA binding assays were carried out using purified, C-terminal

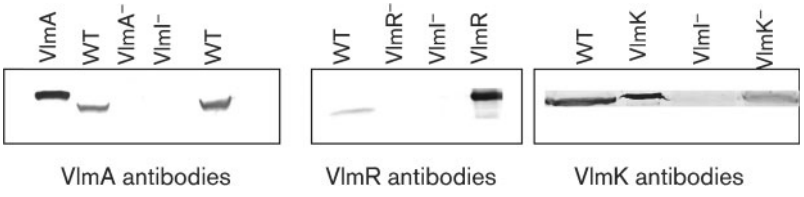

Fig. 3. Western blot analyses of cell-free extracts of *S. viridifaciens* MG456-hF10 strains using anti-VlmA, -VlmR and -VlmK antibodies. The strains used include the wild-type (WT), the single-crossover *vlmI* mutant *vlmI*::pKC1139Δ*vlmI* (VlmI⁻), a single-crossover *vlmA* mutant (VlmA⁻), a single-crossover *vlmR* mutant (VlmR⁻) and a single-crossover *vlmK* mutant (VlmK⁻). Overproduced VlmA, VlmR and VlmK were used as positive controls. Proteins were detected with anti-rabbit IgG conjugated to alkaline phosphatase using the Sigma-Fast BCIP NBT reagent.

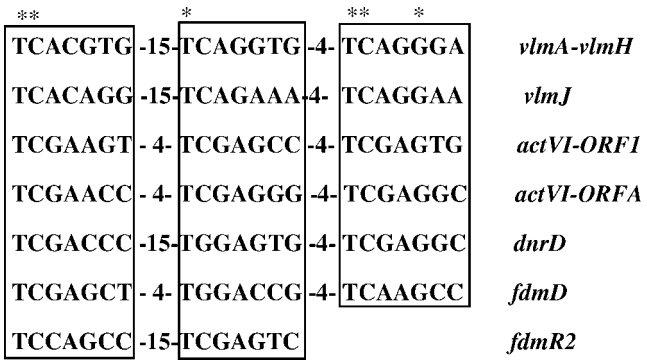

Fig. 4. Sequence alignment of verified and putative SARP binding sites present in the valanimycin (*vlm*), actinorhodin (*act*), daunorubicin (*dnr*) and fredericamycin (*fdm*) biosynthetic gene clusters (Wietzorrek & Bibb, 1997; Chen *et al.*, 2008). Conserved residues are indicated by asterisks, while spacings between the binding sites are indicated numerically.

FLAG-tagged forms of VlmI-L and VlmI-S. VlmI-L exhibited tight binding to the 443 bp DNA fragment, with nearly 100 % of the DNA being shifted when 3.2 μg of protein was added. Addition of a 50-fold excess of the unlabelled form of the 443 bp DNA fragment to the binding assay resulted in reversal of VlmI binding to the radiolabelled DNA, thereby showing that the binding is specific (Fig. 5a). In contrast to the behaviour of VlmI-L, VlmI-S did not produce any gel shift with the 443 bp DNA fragment (Fig. 5b). The lack of DNA binding activity displayed by VlmI-S also shows that the DNA binding activity exhibited by overproduced VlmI-L, which is contaminated with VlmI-S, is entirely due to VlmI-L.

Localization of the VlmI binding site within the *vlmA–vlmH* intergenic region

The binding of VlmI to the *vlmA–vlmH* intergenic region having been established, experiments were carried out to localize the binding site within this DNA region. The labelled 443 bp DNA fragment was digested with several restriction enzymes and gel shift assays were carried out with the labelled fragments to determine which fragments bound to VlmI. The clearest results were obtained when the end-labelled 443 bp fragment was digested with *Pml*I. Since two *Pml*I sites are present within the 443 bp fragment, digestion resulted in the formation of two end-labelled fragments 258 and 128 bp in size as well as an unlabelled 57 bp fragment. Both of the labelled *Pml*I DNA fragments failed to show any gel mobility shift (Fig. 5a), thereby suggesting that the 57 bp fragment was most probably essential for VlmI binding. Since the DNA sequence of this 57 bp fragment contains the tandem heptameric repeat sequences TCACGTG-15-TCAGGTG-4-TCAGGGA, these results support the hypothesis that these repeats constitute the VlmI binding site. One *Sma*I site is present in the 443 bp labelled DNA fragment. Partial digestion of the labelled fragment with *Sma*I produced two end-labelled fragments of 250 and 193 bp in size.

The 250 bp fragment contains the 57 bp DNA sequence with the potential VlmI binding site. As expected, the undigested 443 bp fragment remaining after partial digestion and the 250 bp fragment both exhibited gel shifts, while the 193 bp fragment exhibited no gel shift (Fig. 5a). This behaviour is consistent with the hypothesis that the tandem heptameric repeat sequences TCACGTG-15x-TCAGGTG-4x-TCAGGGA are responsible for VlmI binding, since this sequence resides on the 250 bp fragment produced by *Sma*I digestion.

DNA gel shift assay for the intergenic region between *vlmI* and *vlmJ*

The 2.5 kb intergenic DNA between *vlmI* and *vlmJ* was also evaluated for VlmI binding properties. The 2.5 kb fragment was cleaved into 1.0 and 1.5 kb fragments by digestion with *Not*I and the fragments were then end-labelled after gel purification. Gel shift assays showed that VlmI-L did not bind to the 1 kb fragment of DNA located immediately upstream of the *vlmI* start site. However, VlmI-L did exhibit binding to the 1.5 kb segment of DNA that is located immediately upstream from *vlmJ*. The 1.5 kb fragment was further digested with several restriction enzymes, and gel shift assays were carried out with the labelled fragments to determine which fragments bound to VlmI. The results are summarized in Fig. 6(a). When the 1.5 kb segment was digested with *Pml*I, two fragments with sizes of 1.2 and 0.33 kb were produced. Of these two, only the 1.2 kb fragment exhibited a gel mobility shift (Fig. 6b). When the 1.5 kb fragment was digested with *Kpn*I, a 0.57 kb fragment was produced that showed a positive gel shift with VlmI, while the remaining *Kpn*I fragments showed no affinity to VlmI (Fig. 6b). A 231 bp *Pml*I–*Kpn*I fragment common to the 1.2 and 0.57 kb fragments was then subcloned and used for gel shift assays. This fragment exhibited a strong gel shift in the presence of VlmI-L (Fig. 6b). This result is consistent with the fact that the 231 bp fragment contains a set of heptameric repeats predicted to be a VlmI binding site (TCACAGG-15x-TCAGAAA-4x-TCAGGAA). Additional evidence to support the hypothesis that these heptameric repeats constitute a VlmI binding site was obtained by digestion of the 231 bp fragment with *Bsa*HI, which cleaves the DNA between the TCAGAAA and TCAGGAA repeats. Neither of the two DNA fragments produced by *Bsa*HI digestion exhibited a gel mobility shift (Fig. 6b).

Complementation of *S. viridifaciens vlmI* mutant and of *S. coelicolor redD* mutation

A copy of the longer version of *vlmI* was cloned into the integrating vector pSET152-ErmE* and into the high-copy-number, non-integrating vectors pWHM3 and pWHM3-OriT to produce the complementation plasmids pVlmI-CP2, pVlmI-CP2-WHM3 and pVlmI-CP2-WHM3-OriT,

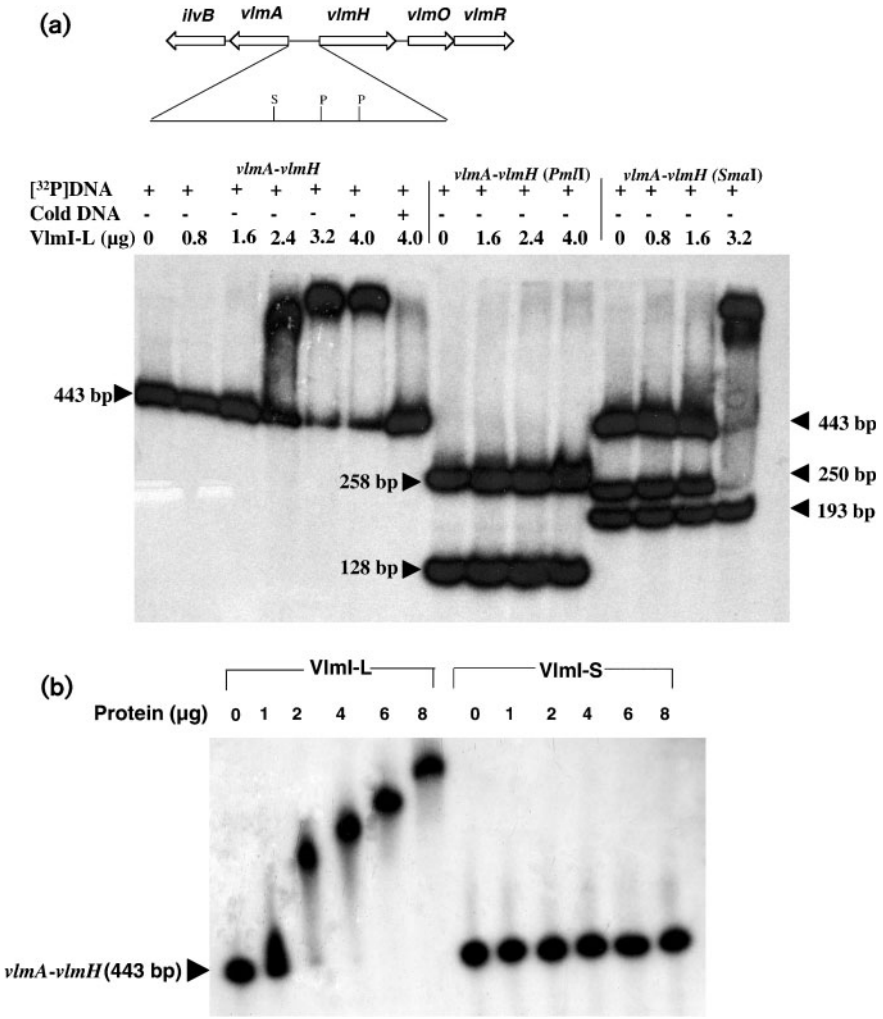

Fig. 5. (a) DNA gel shift assays showing binding of VlmI-L to a 443 bp *vlmA–vlmH* intergenic DNA fragment. The 443 bp DNA fragment was labelled at both ends with [α-^{32}P]dCTP. The assays in lanes 1–6 show the behaviour when the complete 443 bp fragment was mixed with increasing amounts of VlmI-L. Lane 7 shows the behaviour when the labelled DNA fragment plus 4 μg VlmI-L was mixed with a 50-fold excess of the unlabelled 443 bp DNA fragment. Lanes 8–11 show the results of gel shift assays after the labelled 443 bp fragment was digested with *Pml*I, and lanes 12–15 show the results of gel shift assays after digestion of the same fragment with *Sma*I. The quantity of VlmI used in each assay is shown at the top of each lane. S and P show the positions of the *Sma*I and *Pml*I sites, respectively. (b) Comparison of DNA gel shifts produced by VlmI-L and VlmI-S with a radiolabelled 443 bp *vlmA–vlmH* intergenic DNA fragment.

respectively. In order to determine whether VlmI can complement mutations in the SARP genes *redD* and *actII-orf4*, which respectively control undecylprodigiosin and actinorhodin production in *S. coelicolor*, the complementation plasmids were introduced into *S. coelicolor* M512 (Δ*redD*, Δ*actII-orf4*) (Floriano & Bibb, 1996). Spectrophotometric analysis was used to measure the concentration of undecylprodigiosin and actinorhodin in methanol extracts of the mycelium, as described in Methods. When pVlmI-CP2 was introduced into *S. coelicolor* M512, a low level of undecylprodigiosin production was detectable (Table 2). When pVlmI-CP2-WHM3-OriT was used for complementation, the amount of undecylprodigiosin produced was about 300 times greater than that observed with the integrating plasmid pVlmI-CP2, and it was about 1.5 times higher than the level of undecylprodigiosin produced by the *redD*$^{+}$ strain *S. coelicolor* M145 (Table 2). Production of actinorhodin was not detected with either complementation plasmid. These observations indicate that *vlmI* can complement the *redD* mutation, but it is unable to complement the *actII-orf4* mutation.

When plasmid pVlmI-CP2 was introduced into the *S. viridifaciens vlmI*$^{-}$ mutant, it failed to restore valanimycin production. However, the introduction of the high-copy-number plasmid pVlmI-CP2-WHM3 successfully comple-

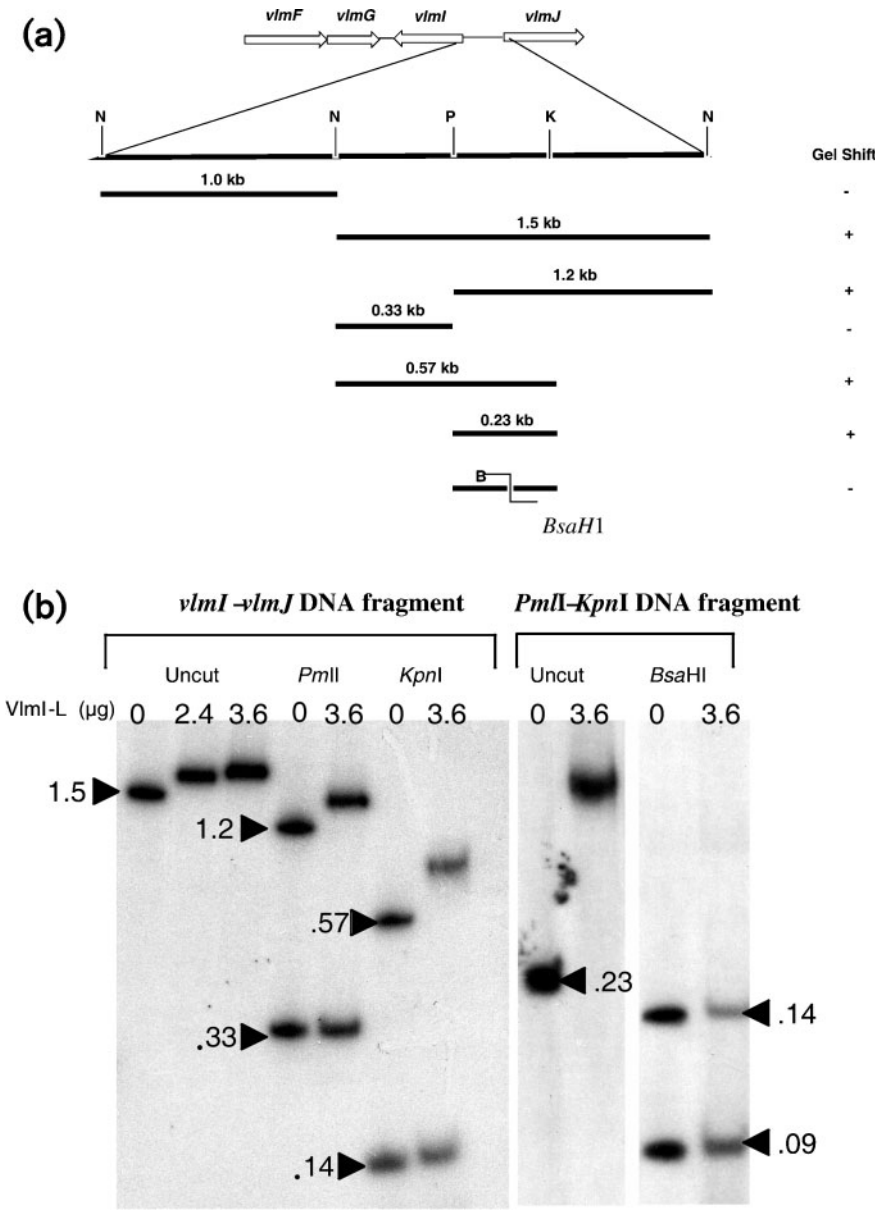

Fig. 6. (a) Summary of DNA gel shifts produced by VlmI-L with the 2.5 kb *vlmI–vlmJ* intergenic region and its digestion products. B, N, P and K indicate results of digestion with *Bsa*HI, *Not*I, *Pml*I and *Kpn*I, respectively. (b) DNA gel shifts produced by Vlm-L with a radiolabelled 1.5 kb *Not*I fragment from the *vlmI–vlmJ* intergenic region and fragments derived from it by digestion with *Pml*I, *Kpn*I and *Bsa*HI. Fragment sizes are given in kb. The main text provides additional details.

mented the *vlmI* mutation. TLC analysis of the ethyl acetate extract of the fermentation broth from the complemented strain exhibited a spot with an R_F value very closely matching that of the valanimycin standard (Fig. 7). This compound was confirmed to be valanimycin by NMR spectroscopy and high-resolution electrospray ionization-MS analysis.

DISCUSSION

Genes that encode SARPs have been found in a number of antibiotic biosynthetic gene clusters, including those for actinorhodin, undecylprodigiosin, daunorubicin and cephamycin. Sequence analysis of the *vlmI* gene from the valanimycin biosynthetic gene cluster indicates that VlmI is a member of the SARP family of transcriptional activators. The *vlmI* gene contains two potential start sites. Translation from the downstream start site yields a version of VlmI that differs from the longer version by the absence of 55 amino acids at the N terminus. In this study, we have demonstrated by gel shift mobility assays that the longer version of VlmI (VlmI-L) binds to a bidirectional promoter in the intergenic region between *vlmA* and *vlmH* as well as

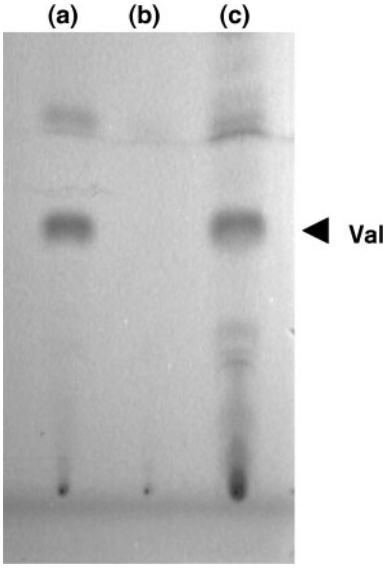

Fig. 7. Thin-layer chromatogram (SiO_2, $CHCl_3$: methanol : acetic acid, 5 : 1 : 0.05) of crude ethyl acetate extracts derived from fermentations of (a) wild-type *S. viridifaciens*, (b) *S. viridifaciens vlmI*$^-$ and (c) *S. viridifaciens vlmI*$^-$ (pVlmI-CP2-WHM3). Valanimycin was visualized with UV light. Val, valanimycin.

to a promoter region upstream of *vlmJ*. Both of these regions contain SARP boxes that are similar to those that have been shown to be the binding sites for ActII-Orf4 and DnrI (Fig. 4). VlmI therefore controls the expression of most, if not all, of the valanimycin biosynthetic genes. The studies also show that the shorter version of VlmI does not bind to these promoter regions. This suggests that the additional 55 amino acids present in the N terminus region of VlmI-L are important for the proper folding of the protein. The failure of the shorter version of VlmI to bind DNA was unexpected, since the N-terminal region of VlmI-L does not contain any conserved motifs and it does not appear to be present in most of the other SARPs whose sequences have been reported. However, the transcriptional activator for the undecylprodigiosin biosynthetic pathway, RedD, is an exception to this pattern, since it contains an extended N terminus that exhibits some sequence similarities to the N terminus of VlmI-L (Garg *et al.*, 2002). The presence of an extended N terminus in both VlmI-L and RedD may explain our finding that VlmI-L is able to complement a *redD* mutation in *S. coelicolor*, but not an *actII-orf4* mutation in the same organism. Complementation of the *redD* mutation was much more efficient when *vlmI-L* was introduced into the M512 strain using a high-copy-number plasmid instead of an insertional plasmid. This behaviour is presumably due to the higher level of VlmI expression achieved with the high-copy-number vector. Attempts to complement a *vlmI* mutation in *S. viridifaciens* with the insertional plasmid bearing *vlmI-L* were unsuccessful, while the use of a high-copy-number plasmid resulted in wild-type levels of valanimycin production (Fig. 7). These results may also be the consequence of the higher level of VlmI expression produced by the multicopy plasmid. Gel shift studies and Western blot analyses indicate that VlmI-L binds to the *vlmA–vlmH* intergenic region and activates transcription of both the *vlmA* and the *vlmHORBCD* transcripts from the divergently arranged promoters in this region. A precedent for this type of behaviour is provided by DnrI and ActII-Orf4, which have also been shown to activate bidirectional transcription (Arias *et al.*, 1999; Tang *et al.*, 1996). Gel shift studies indicate that VlmI-L binds to a region of heptameric repeats upstream from *vlmJ*. The location of this VlmI binding site is unusual since it lies about 0.9 kb upstream of the predicted *vlmJ* translational start site. Analysis of the sequence between the VlmI binding site and the *vlmJ* start site reveals the presence of seven direct 122 bp repeats. These repeats do not represent an N-terminal extension of VlmJ, since they are not in-frame with VlmJ. An analysis of the repeat sequence with the MFOLD program (http://mfold.bioinfo.rpi.edu/) suggests that each repeat forms a complex hairpin structure. The location of the VlmI binding site at a relatively large distance from the translational start of *vlmJ* and the presence of these repeats may indicate that another regulatory protein is involved in the regulation of the *vlmJKL* transcript. This might explain the weaker binding affinity of VlmI to this region and the leaky VlmK phenotype observed in the *vlmI* disruptant.

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