Hierarchical control of virginiamycin production in *Streptomyces virginiae* by three pathway-specific regulators: VmsS, VmsT and VmsR

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Two regulatory genes encoding a *Streptomyces* antibiotic regulatory protein (*vmsS*) and a response regulator (*vmsT*) of a bacterial two-component signal transduction system are present in the left-hand region of the biosynthetic gene cluster of the antibiotic virginiamycin, which is composed of virginiamycin M (VM) and virginiamycin S (VS), in *Streptomyces virginiae*. Disruption of *vmsS* abolished both VM and VS biosynthesis, with drastic alteration of the transcriptional profile for virginiamycin biosynthetic genes, whereas disruption of *vmsT* resulted in only a loss of VM biosynthesis, suggesting that *vmsS* is a pathway-specific regulator for both VM and VS biosynthesis, and that *vmsT* is a pathway-specific regulator for VM biosynthesis alone. Gene expression profiles determined by semiquantitative RT-PCR on the virginiamycin biosynthetic gene cluster demonstrated that *vmsS* controls the biosynthetic genes for VM and VS, and *vmsT* controls unidentified gene(s) of VM biosynthesis located outside the biosynthetic gene cluster. In addition, transcriptional analysis of a deletion mutant of *vmsR* located in the clustered regulatory region in the virginiamycin cluster (and which also acts as a SARP-family activator for both VM and VS biosynthesis) indicated that the expression of *vmsS* and *vmsT* is under the control of *vmsR*, and *vmsR* also contributes to the expression of VM and VS biosynthetic genes, independent of *vmsS* and *vmsT*. Therefore, coordinated virginiamycin biosynthesis is controlled by three pathway-specific regulators which hierarchically control the expression of the biosynthetic gene cluster.

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INTRODUCTION

Streptomyces virginiae produces the antibiotics virginiamycin M (VM) and virginiamycin S (VS), a polyunsaturated macrolactone and a cyclic hexadepsipeptide, respectively, both of which belong to the family of streptogramin antibiotics. VM and VS show strong synergistic activity, and one of the unique features of *S. virginiae* is that both VM and VS are produced at the same time in a ratio that yields the highest synergistic activity (Di Giambattista *et al.*, 1989), although the details of the regulatory mechanism for this synchronized production remain unclear. In general, the genes involved in regulating the production of antibiotics exert their effects in a hierarchical manner at various levels to turn on antibiotic production, and these types of regulator appear to fall into two categories. The

first type consists of higher-level regulators regarded as global regulators that transmit signals from the environment. Genes that encode the higher-level regulators are most commonly situated outside of biosynthetic gene clusters. The second type of regulator consists of lower-level regulators regarded as pathway-specific regulators that control the transcription of biosynthetic structural genes associated with the synthesis of individual antibiotics, and these genes are usually found within the respective biosynthetic gene cluster. In the case of *Streptomyces coelicolor* A3(2), the factors AbsB (Adamidis & Champness, 1992) and BldA (Lawlor *et al.*, 1987) are typical higher-level regulators that sequentially control ActII-ORF4, RedD and CdaR, i.e. pathway-specific regulators that activate biosynthetic gene clusters for actinorhodin, undecylprodigiosin and calcium-dependent antibiotics, respectively (Bibb, 1996).

The biosynthesis of VM and VS in *S. virginiae* is initiated by the production of virginiae butanolide (VB), a small diffusible signalling molecule that binds to VB-specific

Abbreviations: SARP, *Streptomyces* antibiotic regulatory protein; VB, virginiae butanolide; VM, virginiamycin M; VS, virginiamycin S.

A supplementary table of primers and two supplementary figures are available with the online version of this paper.

receptor protein (BarA), a DNA-binding transcriptional repressor (Kinoshita *et al.*, 1997). The flanking regions of *barA* contain other important genes that are involved in VB biosynthesis (*barX*, *barS1* and *barS2*) and mechanisms of resistance to VM and VS (*varM*, *varR* and *varS*) (Kawachi *et al.*, 2000a; Namwat *et al.*, 2001) (Fig. 1). These genes are clustered in a 10 kb region, and thus are considered as the virginiamycin regulatory island. This 10 kb regulatory island includes one pathway-specific regulatory gene, *vmsR*, which is essential for both VM and VS production and is transcriptionally controlled by higher-level regulators such as *barA*, thus indicating that VmsR is a lower-level regulator for virginiamycin production in the VB-BarA regulatory system (Kawachi *et al.*, 2000b). Recently, we characterized a 62 kb virginiamycin biosynthesis gene cluster in the flanking region of the virginiamycin regulatory island (Pulsawat *et al.*, 2007). This gene cluster encodes six multi-functional enzymes that catalyse the formation of a hybrid peptide-polyketide and a cyclohexadepsipeptide skeleton, as well as 14 additional proteins that are responsible for the introduction of carboxylic acid and amino acid precursors into the skeleton, transporters for self-resistance, and two plausible regulators of gene expression. Expression of these genes is also under the control of higher-level factors such as BarA and BarX, both of which are involved in a number of cell functions, including virginiamycin production, VM biosynthesis and VM resistance.

Two putative regulatory genes, *vmsS* and *vmsT*, are present in the left-hand region of the virginiamycin biosynthetic gene cluster and are located approximately 50 kb from the virginiamycin regulatory island. Significant similarity between VmsS and *Streptomyces* antibiotic regulatory proteins (SARPs) (e.g. RedD, ActII-ORF4, and TylT and TylS of the tylosin pathway in *Streptomyces fradiae*; Bate *et al.*, 2002) has been demonstrated. The SARP-family proteins are known to be pathway-specific regulators that positively control the production of secondary metabolites. Meanwhile, VmsT was found to resemble the response-regulator proteins of a bacterial two-component signal

transduction system able to detect and respond to changes in the environment (Hutchings *et al.*, 2004). The phosphorylated response regulators possess the ability to positively or negatively regulate gene expression. These findings led us to investigate the potential roles played by *vmsS* and *vmsT* in the regulation of virginiamycin biosynthesis. In this study, we characterized the functions of *vmsS* and *vmsT* in the regulatory cascade of virginiamycin biosynthesis, and demonstrated that VmsS and VmsT are pathway-specific transcriptional activators of virginiamycin production. The present report also specifies the hierarchical correlation among three pathway-specific regulatory genes (*vmsS*, *vmsT* and *vmsR*) responsible for controlling the biosynthetic expression of virginiamycin.

METHODS

Bacterial strains, plasmids, media, growth conditions and DNA manipulation. All strains and plasmids used in this study are listed in Table 1. For routine cultivation on solid media, *S. virginiae* strains were grown at 28 °C on ISP medium 2 (Becton Dickinson). AS-1 medium, minimal medium with mannitol (Kieser *et al.*, 2000) and ISP medium 2 were used for observing the morphological development of gene disruptants. For virginiamycin production, liquid f-medium was used as described previously (Nihira *et al.*, 1988). For routine cloning and conjugation, *Escherichia coli* strains were grown on LB medium with the appropriate antibiotics, when necessary. Plasmid DNA was transferred from *E. coli* into *Streptomyces* by intergenic conjugation as previously described (Kitani *et al.*, 2000). PCR was performed using high-fidelity PrimeSTAR HS DNA polymerase (Takara Bio) with genomic DNA of *S. virginiae* as a template, according to the manufacturer's recommendations. Southern blot hybridization was carried out using the AlkPhos Direct Labelling and Detection System with CDP-Star (GE Healthcare Bio-Sciences). For DNA manipulation in *E. coli* and *Streptomyces*, standard procedures were performed according to the methods of Sambrook & Russell (2001) and Kieser *et al.* (2000), respectively.

Construction of *S. virginiae* mutant strains. Primer sets were designed (for sequences, see Supplementary Table S1, available with the online version of this paper) to amplify the flanking regions of the target gene for in-frame deletion as follows.

Fig. 1. The virginiamycin biosynthetic gene cluster of *S. virginiae*. The cluster of 34 genes occupies 77 kb of the genome. Eleven genes (*virA* to *virK*, starting downstream of *bkdB* and extending to the region upstream of *varL*) are VM biosynthetic genes and two genes (*virM* and *virN*) encode putative post-modification enzymes in VM biosynthesis. *vis* genes (*visA* to *visF*) are VS biosynthetic genes. Regulatory genes featured in the present study are indicated by grey arrows.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties*	Reference or source
<i>S. virginiae</i>		
MAFF10-06014	Wild-type, virginiamycin production strain	National Food Research Institute, Japan
IC102	$\Delta vmsS$	This study
IC103	$\Delta vmsT$	This study
IC104	$\Delta vmsR$	This study
IC105	Complementation strain of $\Delta vmsS$	This study
IC106	Complementation strain of $\Delta vmsT$	This study
IC107	Complementation strain of $\Delta vmsR$	This study
IC108	Constitutive expression strain of <i>vmsS</i> in $\Delta vmsR$	This study
<i>E. coli</i>		
DH5 α	General cloning strain	Hanahan (1983)
ET12567	Methylation-deficient strain for conjugation with <i>Streptomyces</i>	MacNeil <i>et al.</i> (1992)
Plasmids		
pUC19	Cloning vector	
pBluescript II SK	Cloning vector	Stratagene
pKC1132	Suicide vector containing <i>oriT</i> , and <i>aac(3)IV</i> for gene disruption	Bierman <i>et al.</i> (1992)
pSET152	Integration vector containing <i>oriT</i> , <i>attP</i> , $\phi C31$ <i>int</i> and <i>aac(3)IV</i> for complementation	Bierman <i>et al.</i> (1992)
pUZ8002	Mobilization plasmid	Paget <i>et al.</i> (1999)
pLT101	pSET152 containing <i>ermEp*</i> and <i>tfd</i> terminator	This study
pLT202	pKC1132 containing 441 bp deleted <i>vmsS</i>	This study
pLT203	pKC1132 containing 591 bp deleted <i>vmsT</i>	This study
pLT204	pKC1132 containing 717 bp deleted <i>vmsR</i>	This study
pLT205	pLT101 containing <i>vmsS</i>	This study
pLT206	pSET152 containing <i>vmsT</i>	This study
pLT207	pSET152 containing <i>vmsR</i>	This study

aac(3)IV*, apramycin resistance gene; *oriT*, origin of transfer; *ermEp, mutated constitutive promoter; *attP*, plasmid $\phi C31$ attachment site; $\phi C31$ *int*, integrase gene; *tfd* terminator, major terminator of phage ϕd .

(i) ***vmsS* deletion.** A 1.8 kb *vmsS*-upstream DNA fragment was amplified by the primer pair *vmsSA-E/vmsSA-S*, and digested by *EcoRI* and *SpeI*. Similarly, a 1.8 kb *vmsS*-downstream DNA fragment was amplified by the primer pair *vmsSB-S/vmsSB-H*, and digested by *SpeI* and *HindIII*. The two resultant fragments were cloned together into the *EcoRI* and *HindIII* sites of pUC19 and were recovered as a 3.7 kb *EcoRI/HindIII* fragment, which was then transferred into pKC1132 at the *EcoRI* and *HindIII* sites, thereby yielding the *vmsS*-disruption plasmid pLT202.

(ii) ***vmsT* deletion.** A 2.1 kb *vmsT*-upstream DNA fragment was amplified by the primer pair *vmsTA-X/vmsTA-K* and was digested by *XbaI* and *KpnI*. Similarly, a 2.2 kb *vmsT*-downstream DNA fragment was amplified by the primer pair *vmsTB-K/vmsTB-H* and was digested by *KpnI* and *HindIII*. The two resultant fragments were cloned together into the *XbaI* and *HindIII* sites of pUC19 and were recovered as a 4.3 kb *XbaI/HindIII* fragment, which was then transferred into pKC1132 at the *XbaI* and *HindIII* sites, thereby yielding the *vmsT*-disruption plasmid pLT203.

(iii) ***vmsR* disruption.** A 2.0 kb *vmsR*-upstream DNA fragment was amplified by the primer pair *vmsRA-H/vmsRA-S* and was digested by *HindIII* and *SpeI*, while a 2.1 kb *vmsR*-downstream DNA fragment was amplified by the primer pair *vmsRB-S/vmsRB-X* and was digested by *SpeI* and *XbaI*. The two resultant fragments were cloned

together into the *HindIII* and *XbaI* sites of pUC19 and were recovered as a 4.1 kb *HindIII/XbaI* fragment, which was then transferred into pKC1132 at the *HindIII* and *XbaI* sites, thereby yielding the *vmsR*-disruption plasmid pLT204.

Each of the disruption plasmids was introduced into *S. virginiae* via conjugation, and the wild-type gene was replaced with the disrupted allele by homologous recombination. The genotype of the corresponding mutants was confirmed by PCR and Southern blot hybridization (Supplementary Fig. S1), and representatives of the *vmsS*, *vmsT* and *vmsR* mutants were designated *S. virginiae* strains IC102, IC103 and IC104, respectively.

Complementation of the *S. virginiae* mutant strains. A 1.0 kb *vmsS* gene was PCR-amplified by the primer pair *vmsS-N2/vmsS-C2*, and inserted into the *EcoRV* site of pBluescript II SK. The resulting plasmid was digested by *BamHI*, and was then cloned into the *BamHI* site of pLT101, yielding the *vmsS*-complementing plasmid pLT205 to place *vmsS* under the control of *ermEp**. A 1.5 kb DNA fragment containing the entire *vmsT* gene and the *vmsT*-upstream region was PCR-amplified by the primer pair *vmsT-CF/vmsT-CR*, digested by *EcoRV* and *XbaI*, and cloned into the *EcoRV* and *XbaI* sites of pSET152 to obtain pLT206. A 1.3 kb DNA fragment containing the entire *vmsR* gene and the *vmsR*-upstream region was PCR-amplified by the primer pair *vmsR-CF/vmsR-CF*, digested by *EcoRV* and *BamHI*, and inserted into the *EcoRV* and *BamHI* sites of pSET152 to

obtain pLT207. The fidelity of the amplified fragments was confirmed by DNA sequencing. By intergenic conjugation, pLT205, pLT206 and pLT207 were introduced into *S. virginiae* strains IC102 ($\Delta vmsS$), IC103 ($\Delta vmsT$) and IC104 ($\Delta vmsR$), respectively. The correct integration in the exconjugants was confirmed by PCR analysis and Southern blot hybridization to yield the $\Delta vmsS$ -, $\Delta vmsT$ - and $\Delta vmsR$ -complemented strains IC105, IC106 and IC107, respectively.

Constitutive expression of *vmsS* in the mutant IC104. By intergenic conjugation, pLT205 was introduced into *S. virginiae* strain IC104 ($\Delta vmsR$). The correct integration in the exconjugants was confirmed by PCR analysis to yield a strain in which *vmsS* is constitutively expressed under the genetic background of $\Delta vmsR$; this strain was designated *S. virginiae* strain IC108.

Transcriptional analysis by RT-PCR. RT-PCR was conducted as described by Pulsawat *et al.* (2007). Total RNAs were extracted from fresh mycelia harvested after 8, 10, 12 and 14 h cultivation for the wild-type strain, after 8 and 14 h cultivation for strains IC102, IC103 and IC104, and after 14 h cultivation for strain IC108. The cDNAs were amplified from 34 gene transcripts in the virginiamycin biosynthetic gene cluster using the previously described primers. The primers used for the co-transcription analysis of *virN-virM-vmsS* genes were *virN*-F, *virM*-R and *vmsS*-R as previously described (Pulsawat *et al.*, 2007), and *vmsS*-F1, *vmsS*-F2 and *vmsS*-F3 as listed in Table S1. For PCR, the amplification conditions were a single round of denaturation at 94 °C for 5 min and 25 cycles of denaturation (98 °C for 10 s), annealing (58 °C for 5 s) and extension (72 °C for 1 min), followed by a single extension at 72 °C for 7 min.

Detection of virginiamycin production. VM and VS in the culture broth were detected by a bioassay against *Bacillus subtilis* PCI219 (Yanagimoto, 1983) and reverse-phase C_{18} -HPLC analysis as described by Pulsawat *et al.* (2007).

RESULTS

Features of two regulatory genes, *vmsS* and *vmsT*

VmsS, a SARP-family protein, showed high similarity to VmsR, which acts as a positive regulator of virginiamycin biosynthesis in *S. virginiae*. Transcriptional analysis by semiquantitative RT-PCR (Fig. 2a) clearly demonstrated the presence of *vmsR* mRNA after 12 h and 14 h of cultivation, whereas *vmsS* mRNA was detected only after 14 h of cultivation, when all of the virginiamycin biosynthetic genes were actively transcribed along with the maximal virginiamycin production. This result suggested that the transcription of *vmsS* is preceded by that of *vmsR*. As regards gene organization, *vmsS* runs in the same direction as the upstream *virM*, which encodes a homologue of sarcosine oxidase, and *virN* encodes flavin-dependent oxidoreductase, both of which seem to participate in the post-modification steps of virginiamycin biosynthesis (Pulsawat *et al.*, 2007). Because *virN* and *virM* overlap in a 47 bp region, and there is only a narrow (50 bp) intergenic region between *virM* and *vmsS*, *vmsS* may form a tricistronic operon with *virN* and *virM*. To examine this possibility, primers were designed to detect the tricistronic (*virN-virM-vmsS*) as well as bicistronic transcripts (*virN-virM* and *virM-vmsS*) (Fig. 2b). RT-PCR

analysis using RNA from the 14 h mycelia gave the expected band for each primer set, implying that *vmsS* forms a tricistronic operon, *virN-virM-vmsS*.

The deduced amino acids of the *vmsT* product showed moderate similarity to response regulators of a bacterial two-component signal transduction system, many of which are accompanied by sensor histidine kinases. Unlike the genes of typical two-component response regulators, *vmsT* appears to lack a cognate sensor kinase gene in the 77 kb identified region and in the left-hand extremity extending for at least 30 kb (our unpublished data). Response regulators possess two distinctive domains, namely a signal receiver domain that typically interacts with the sensor kinase to induce the phosphorylation of the response regulator itself, and the helix–turn–helix DNA-binding domain for gene activation or repression. Sequence comparison with well-characterized response regulators demonstrated that VmsT lacks all of the highly conserved amino acid residues for the formation of the phosphorylation pocket by the substitution of D80P, D125H, T149V and K167A (Fig. 3) (O'Connor & Nodwell, 2005) although the DNA-binding domain is significantly conserved. Furthermore, the N-terminus of VmsT is approximately 70 amino acids longer than that of other response regulators. These results, taken together, suggest that VmsT might exert different regulatory functions other than those typically carried out by response regulators. Similar to the case of the *vmsS* transcript, the *vmsT* transcript was barely detectable until 14 h cultivation (Fig. 2a), indicating that the active expression of *vmsT* occurred only at the stage of virginiamycin production.

vmsS and *vmsT* differentially control virginiamycin production

To examine the requirement of VmsS and VmsT in the regulation of virginiamycin biosynthesis, each gene was inactivated by in-frame deletion as described in Methods. The correct disruption of *vmsS* or *vmsT* was confirmed by Southern blot analysis using the appropriate probes (Fig. S1). The resulting strains, mutants IC102 (*vmsS*-disrupted strain) and IC103 (*vmsT*-disrupted strain), showed growth in liquid culture and morphological characteristics on solid medium (formation of aerial mycelium and spores) identical to those of the wild-type strain (data not shown). These results suggested that the two regulatory genes, *vmsS* and *vmsT*, are not involved in primary metabolism or morphological differentiation. We next assessed the effect of mutations on virginiamycin production by a bioassay involving *B. subtilis* (Fig. 4a). The culture filtrate of the wild-type strain showed an inhibition zone of virginiamycin, whereas no clear zone was observed in the case of the culture filtrate of mutant IC102. On the other hand, a culture filtrate of the mutant IC103 exhibited an inhibition zone, albeit smaller than that of the wild-type strain. Because this phenomenon might have been due to the

Fig. 2. Transcriptional analysis of genes involved in virginiamycin production by semiquantitative RT-PCR. (a) Transcription profile of the wild-type strain. Total RNAs were extracted from mycelia harvested after 8 h, 10 h, 12 h and 14 h cultivation. Virginiamycin production was detected in the culture broth after 14 h of cultivation. Data for RT-PCR by using RNA after 8 h and 14 h cultivation are reproduced from Fig. 7 in Pulsawat *et al.* (2007) with permission from Elsevier (license no. 2098180242774). (b) Gene expression analysis of the *virN-virM-vmsS* operon. Total RNA used as a template was prepared from mycelium after 14 h of cultivation. The lengths and positions of the expected amplified cDNA bands are indicated by bars (1 to 5) below each gene.

composition or change in the quantity of virginiamycin, HPLC analysis was performed in order to investigate in more detail virginiamycin production in mutants IC102 and IC103. As shown in Fig. 4(b), both VM and VS peaks were missing in the culture filtrate of the mutant IC102, whereas only the VS peak was present in the culture filtrate of the mutant IC103, indicating that VmsS is necessary for

both VM and VS biosynthesis, but VmsT is only necessary for VM biosynthesis.

Trans complementation of *vmsS*- and *vmsT*-disrupted strains

To ensure that the inactivation of *vmsS* and *vmsT* was the sole reason for the observed changes in virginiamycin

Fig. 3. Sequence alignment of the N-terminal receiver domains of VmsT and other *Streptomyces* response regulators. Active-site residues in the phosphorylation-dependent receiver domain are indicated by ▼. The numbers indicate the amino acid positions within each sequence. Black boxes indicate positions in the alignment at which the same amino acid is found in at least four of the seven sequences. RedZ (CAA69209), AbsA2 (AAB08053), ChiR (CAB94548) and RamR (NP_733711) are from *Streptomyces coelicolor* A3(2); CinR (CAD60529) is from *Streptomyces cinnamoneus*; DnrN (AAD15247) is from *Streptomyces peucetius*. RedZ is also a member of the pseudo-response regulators, which are not regulated by phosphorylation.

Fig. 4. Virginiamycin production analysed by gene disruption of *vmsS* and *vmsT*. From left to right: WT, the wild-type strain; IC102, *vmsS* disruptant ($\Delta vmsS$); IC103, *vmsT* disruptant ($\Delta vmsT$); IC102 + pLT205, complemented $\Delta vmsS$ strain; IC103 + pLT206, complemented $\Delta vmsT$ strain. (a) Bioassays of 24 h culture broths against *B. subtilis*. (b) HPLC analysis of virginiamycin production. The peaks of VM and VS are indicated.

production in mutants IC102 and IC103, respectively, intact *vmsS* and *vmsT* were reintroduced into each disrupted strain together with the respective upstream regions. The introduction of an intact copy of *vmsS* with a 0.5 kb upstream region into mutant IC102 did not restore virginiamycin production (data not shown). Taken together with the results of co-transcription of *vmsS* with *virM*, and/or *virN* as described above, this failure of complementation appeared to indicate that the region immediately upstream of *vmsS* does not possess promoter activity or that its activity is insufficient for the transcription of *vmsS*. Thus, we constructed the plasmid pLT205, which contains *vmsS* under the control of the strong constitutive *ermEp** promoter on pSET152. The mutant IC102 harbouring pLT205 produced both VM and VS in amounts comparable to those produced by the wild-type strain (Fig. 4), thus confirming that VmsS is indeed involved in both VM and VS biosynthesis. Meanwhile, the clear inhibition zone formation by mutant IC103 harbouring pLT206, which contains the entire *vmsT* gene and the upstream region, was greater than that of the parent strain (mutant IC103), and was identical to that of the wild-type strain (Fig. 4a). HPLC analysis revealed that the reintroduction of *vmsT* into the *vmsT* mutant restored VM production (Fig. 4b), whereas VS production was retained in the mutant IC106 (Table 1); these results clearly indicated that *vmsT* is essential only for VM biosynthesis.

Gene expression analysis in *vmsS*- and *vmsT*-disrupted strains

To examine whether VmsS is involved in regulation at the transcriptional level, the total RNA was extracted from the mycelia of the mutant IC102 after 8 h and 14 h cultivation,

i.e. the time points before and after the onset of virginiamycin production in the wild-type strain. Semiquantitative RT-PCR analysis of the transcripts of the virginiamycin biosynthetic gene cluster (Fig. 5a) indicated that, at 8 h of cultivation, all of the virginiamycin biosynthetic genes in the mutant IC102 were transcribed at a low level; these results were similar to those observed with the wild-type strain (Fig. 2a). Moreover, no differences were observed between the mutant IC102 and the wild-type strain in terms of the expression profiles of genes in the regulatory island (e.g. *barA* and *varR*). After 14 h cultivation in the case of the mutant IC102, eight VM structural genes (*virD* to *virK*) showed markedly reduced transcription compared to that of other VM structural genes such as *virA* and *virB*. In addition, no transcripts of the three VS structural genes (*visB*, *visC* and *visD*) (Namwat *et al.*, 2002) were detected in the case of mutant IC102, which would account for the lack of VS production in mutant IC102. These results indicated that *vmsS* regulates, either directly or indirectly, the expression of VM and VS structural genes without affecting the transcription level of genes in the regulatory island.

After 8 h cultivation, the transcription levels of genes in the regulatory island in the mutant IC103 (Fig. 5a) were similar to those of the corresponding genes in the wild-type strain. Surprisingly, after 14 h cultivation, all 15 genes designated as VM biosynthetic genes (*bkdA*, *bkdB* and *virA* to *virN*) were transcribed in a manner similar that of the corresponding genes in the wild-type strain, albeit with deficient VM production in the case of mutant IC103. This result indicated that the inactivation of *vmsT* does not exert a major influence on the transcription of *vir* genes in the 77 kb region; moreover, these findings suggested the possibility that *vmsT* may regulate unidentified gene(s)

Fig. 5. Transcriptional analysis of genes involved in virginiamycin production in (a) mutants IC102 ($\Delta vmsS$) and IC103 ($\Delta vmsT$), (b) mutant IC104 ($\Delta vmsR$), and (c) mutant IC108 (constitutive expression of *vmsS* in $\Delta vmsR$). Total RNAs (a) and (b) were extracted from mycelia harvested after 8 h and 14 h cultivation, which corresponded to time points prior to and after the onset of virginiamycin production in the wild-type strain. Total RNA (c) was extracted from mycelia harvested after 14 h cultivation.

outside of this 77 kb region. In addition, the amount of transcripts associated with VS biosynthesis (*visA* to *visD*) remained unaffected by *vmsT* mutation, which is a finding consistent with observed VS production in mutant IC103 after a 14 h cultivation period.

To investigate whether an autoregulatory mechanism operates on *vmsS* or *vmsT*, transcriptional analysis was performed with primer sets designed to detect transcript of the region downstream of the mutation (Supplementary Fig. S2). Although some reduction occurred, especially for *vmsT*, transcription of *vmsS* or *vmsT* was observed in the corresponding mutant, indicating that neither of the genes required intact protein product for their transcription.

Transcription of *vmsS* and *vmsT* is under the control of *vmsR*, a SARP-family gene

Neither the *vmsS* nor the *vmsT* mutation affected the expression of *vmsR*, which is an activator for the control of virginiamycin production. This finding suggested the

possibility that *vmsR* regulates virginiamycin production by controlling the expression of *vmsS* and/or *vmsT* genes. To examine this possibility, we investigated the transcription profile of a *vmsR*-disrupted strain. As the previously created *vmsR*-disrupted strain (DR1 strain) contained a deletion of a 145 bp 5'-untranslated region (UTR) in addition to the deletion of a 698 bp region of *vmsR* (Kawachi *et al.*, 2000b), we reconstructed an in-frame deletion mutant for *vmsR* (mutant IC104), as described in Methods, to completely avoid any indirect effects due to the deletion of the 5'-UTR. The behaviour of mutant IC104 was similar to that of the DR1 strain: it did not produce any virginiamycin throughout the cultivation period. Transcriptional analysis to examine the possibility of autoregulation by *vmsR* suggested that *vmsR*, similar to the cases of *vmsS* and *vmsT*, does not require intact VmsR for its expression (Fig. S2). At 14 h cultivation, only scant amounts of *vmsS* and *vmsT* transcripts were detected in the case of mutant IC104 (Fig. 5b), in clear contrast to the results obtained with the wild-type strain (Fig. 2a). These findings suggested that *vmsR* upregulates the expression of

both *vmsS* and *vmsT*. In addition, similar to the case of the *vmsS* mutant (IC102), a remarkable decrease in the amounts of the transcripts of three VS structural genes (*visB* to *visD*) was observed, implying that the expression of these three genes is controlled by *vmsR* via *vmsS*. Moreover, it was of note that the levels of transcripts of four other genes (*visE* and *visF* as VS structural genes, and *virM* and *virN* with putative involvement in VM biosynthesis) decreased dramatically, in clear contrast to the unaltered transcriptional profiles of these four genes among mutants IC102, IC103 and the wild-type strain. To further examine which genes are controlled by *vmsS*, we constructed mutant IC108, in which *ermEp**-driven *vmsS* was integrated in the genome of the *vmsR* mutant (strain IC104), and analysed the transcriptional profile of the virginiamycin biosynthetic genes. As shown in Fig. 5(c), six VS biosynthetic genes (*visA* to *visF*) that showed reduced (*visA*) or missing (*visB* to *visF*) transcription in the *vmsR* mutant became clearly transcribed by the introduction of *vmsS*, which was considered to be transcribed constitutively in the mutant IC108, but the amount of the *vmsT* transcript was not affected. These results suggested that these VS biosynthetic genes are plausible direct targets of VmsS (see Discussion). Intriguingly, transcription of five VM biosynthetic genes (*bkdA/bkdB* and *virA/virB/virC*) was not influenced by the *vms* mutations. This result suggested the involvement of another regulator for their expression, which is coordinated with expression of other virginiamycin biosynthetic genes.

DISCUSSION

In the left-hand extremity of the 77 kb virginiamycin biosynthetic gene cluster of *S. virginiae*, we have characterized in this study two pathway-specific regulatory genes, *vmsS* and *vmsT*, in addition to the previously identified *vmsR* gene (Kawachi *et al.*, 2000b). VmsS is among the SARP-family proteins that are frequently present in biosynthetic gene clusters of *Streptomyces* origin and that regulate the production of corresponding secondary metabolites. VmsT resembles response regulators of two-component systems, but belongs to a minor group of response regulators that lack the conserved amino acid residues necessary for phosphorylation and do not possess a neighbouring cognate sensor kinase gene. Gene disruption analysis clearly demonstrated that VmsS is necessary for the production of both VM and VS, whereas VmsT is only necessary for the production of VM. These findings, taken together with demonstration of the presence of another SARP-family gene, *vmsR*, in the right-hand region of the virginiamycin biosynthetic gene cluster and its involvement in the production of both VM and VS, suggest that the three regulators form a hierarchical network to ensure the synchronized production of virginiamycin.

The loss of *vmsT* expression in the *vmsR* disruptant indicated that *vmsT* is under the control of VmsR. Surprisingly, no transcriptional changes were observed in the *vmsT* disruptant among the 33 genes in the 77 kb

region examined, although VM production was lost completely; these observations suggested that VmsT controls VM biosynthetic gene(s) located outside the 77 kb region of interest, and these genes might be associated with the last condensation step of VM biosynthesis (proline condensation), as the only missing module for VM biosynthesis in this 77 kb region is that for proline condensation (Pulsawat *et al.*, 2007). Defective VM production in the *vmsR* disruptant appears to have been primarily due to the lack of *vmsT* transcription, because almost all of the VM structural genes in the 77 kb region, with the exception of *virN* and *virM*, showed similar levels of expression among the *vmsR* and *vmsT* disruptants, and the wild-type strain. The reduced expression of *virN* and *virM*, encoding putative post-polyketide modification enzymes for VM biosynthesis, in the *vmsR* disruptant may additionally have been responsible for the loss of VM production in the *vmsR* disruptant.

To date, only a few response regulators, referred to as 'pseudo-response regulators', have been examined in terms of their regulatory functions in the control of secondary metabolism; these regulators possess DNA-binding ability for the regulation of gene expression, but are incapable of phosphorylation. Representative proteins are RedZ of the undecylprodigiosin pathway from *S. coelicolor* A3(2) (Guthrie *et al.*, 1998) and DnrN of the daunorubicin pathway from *S. peucetius* (Furuya & Hutchinson, 1996). RedZ lacks an Asp residue for phosphorylation, a Tyr residue as a rotameric residue, and a Lys residue for formation of the 'acid pocket' binding site. Similarly, DnrN lacks Asp and Thr residues, which are key amino acid residues for phosphorylation. In both cases, the pseudo-response regulators are upper-level regulators in the signalling cascade, and they activate the transcription of the SARP-family genes *redD* and *dnrI*, which in turn directly activate the transcription of biosynthetic genes for undecylprodigiosin and daunorubicin, respectively. However, in the case of virginiamycin biosynthesis, the SARP-family gene *vmsR* controls the expression of *vmsT*, the pseudo-response regulator gene for VM production in *S. virginiae*. To the best of our knowledge, this hierarchical direction from SARP-family protein to pseudo-response regulator is the first case of such directionality in the *Streptomyces* regulatory cascade of secondary metabolism.

In the *vmsR* disruptant, *vmsS* expression was severely reduced, although the basal level expression of *vmsS* appeared to be maintained in the absence of *vmsR* (Fig. 5b). These findings were in clear contrast to the requirement of *vmsR* for the expression of *vmsT*. In the *vmsS* disruptant, defective in terms of both VM and VS biosynthesis, levels of transcription of eight VM biosynthetic genes (*virD* to *virK*) were markedly lower than in the wild-type strain, and the transcription of three VS biosynthetic genes (*visB*, *visC* and *visD*) was abolished, suggesting that VmsS acts as a major transcriptional activator of these 11 genes. Similar loss of *visB/visC/visD* transcription was observed in the *vmsR* disruptant, which

may indicate that the baseline level of VmsS production is insufficient to activate the expression of these three genes. The SARP-family proteins activate the transcription of their target genes by binding to heptameric direct repeats in the cognate promoters (Arias *et al.*, 1999; Sheldon *et al.*, 2002; Tanaka *et al.*, 2007). As regards the target sequence of VmsS for the expression of *visB/visC/visD*, it was of note that two direct repeats composed of a heptameric sequence (GTGTCAG or GTGTCAA, with intervals of 4 bp) were present in the -35 region of *visC*, and an identical sequence (GTGTCAA) was present around the -35 region of *visB*. Because these genes formed two bicistronic operons in the opposite direction (i.e. the *visB-visA* operon and the *visC-visD* operon), the intergenic region between *visB* and *visC* is the most plausible candidate to serve as the VmsS target. The constitutive expression of *vmsS* in the *vmsR*-disruptant conferred the increased transcription of six VS structural genes, including *visA*, *visB*, *visC* and *visD*. This result strongly supported that the transcription of the four VS biosynthetic genes (*visA* to *visD*) is directly activated by VmsS, expression of which is under the control of VmsR, although *in vitro* experiments will still be needed to clarify the regulatory mechanism.

On the other hand, the loss of VM production in the *vmsS* disruptant could be attributed to the reduced levels of transcription of eight VM biosynthetic genes (*virD* to *virK*). Although *vmsS* transcription was found to be under the control of VmsR, no reduction in the transcription of eight VM biosynthetic genes was observed in the *vmsR* disruptant, suggesting that the basal level of expression of *vmsS* is sufficient to maintain the expression of the eight VM biosynthetic genes, and the loss of VM production in the *vmsR*-disruptant could be attributed primarily to the loss of VmsT (see discussion above).

The results of the present extensive transcriptional analysis strongly suggested that *vmsR* has two signalling pathways for the regulation of virginiamycin production, namely (i) a *vmsS/vmsT*-dependent pathway, and (ii) a *vmsS/vmsT*-independent pathway. Initiated by VB production starting at 10–11 h cultivation, the *vmsR/barZ* bicistronic transcript became readily detected at 12 h cultivation. At 14 h, most genes in the biosynthetic cluster, including *vmsS* and *vmsT*, were expressed to yield virginiamycin, while the transcription of *barS2* and *barX* appears to have slightly decreased by a mechanism or physiological factor that remains unknown at the present time. The disruption of either *vmsS* or *vmsT* did not appear to affect the expression of *vmsR* or genes in the regulatory island, suggesting that *vmsS* and *vmsT* are positioned downstream of *vmsR* in the signalling cascade. This possibility was supported by the finding that *vmsS* transcription was severely reduced and *vmsT* transcription abolished in the *vmsR* disruptant. Thus, *vmsR* appears to act as the major transcriptional activator of the two regulatory genes, although further investigation will be needed to clarify whether the mechanism is direct or indirect. No heptameric direct repeats were found in the region immediately upstream of

vmsS, although such repeats are frequently recognized by SARP-family proteins. However, in the *vmsT-virN* intergenic region, which would be expected to contain the promoter(s) responsible for controlling the expression of *vmsT* and the divergently transcribed *virN-virM-vmsS* tricistronic operon, at least three kinds of direct repeat were found, suggesting that VmsR might regulate the expression of *vmsS* and *vmsT* in a coordinated manner via binding to these direct repeats.

In addition to its involvement in the *vmsS/vmsT*-dependent pathway for the regulation of virginiamycin production, VmsR appears to regulate the expression of genes (e.g. *visE*, *visF*, *virN* and *virM*) by a pathway that differs from the *vmsS/vmsT*-dependent pathway, as a loss of transcription of *visE* and *visF*, together with a loss of *virN* and *virM* (as part of the putative tricistronic *virN-virM-vmsS* operon) transcription, was observed in the *vmsR* disruptant, whereas levels of transcription of these genes in the *vmsS* and *vmsT* disruptants were similar to those of the wild-type strain. However, as shown in mutant IC108, when constitutively expressing *vmsS* was introduced into the *vmsR* disruptant, *visE* and *visF* were transcribed at levels higher than those of the parent strain, but the amounts of the transcripts of *vmsT*, *virN* and *virM* were unchanged, indicating that the expression of the three genes (*vmsT*, *virN* and *virM*) was dominated by a *vmsS*-independent pathway, most likely by *vmsR* itself. Regarding the transcription of *visE* and *visF*, combined data indicated that either VmsR or a sufficient amount of VmsS could drive their transcription. One possibility is that VmsR drives the transcription of a *vmsT-visE-visF* tricistronic operon (together with the transcription of the *virN-virM-vmsS* operon in the opposite direction), while VmsS might drive that of a *visE-visF* bicistronic operon in the absence of intact VmsR, which should be clarified in the future by detailed *in vitro* analysis. Detailed transcriptional analysis of these three genes would provide information on *vmsR*- and *vmsS*-regulatory mechanisms of virginiamycin production.

In general, the biosynthetic gene cluster for a secondary metabolite in streptomycetes possesses either one or two pathway-specific regulators for controlling production. It is of note that three pathway-specific regulators were found to be present in the virginiamycin biosynthetic gene cluster, which is a compressed-interspersing gene cluster (i.e. a so-called 'supercluster'). These regulators are responsible for the coordinated synthesis of two structurally different compounds of the streptogramin family. In our previous study (Pulsawat *et al.*, 2007), the expression of *vmsR*, and hence that of *vmsS* and *vmsT*, was shown to be under the control of BarA, which is a pivotal regulator for sensing the presence of VB in the environment. These previous observations, taken together with those of the present study, indicate that *vms* regulatory genes are lower-level regulators in the regulatory cascade of the VB-BarA system responsible for controlling virginiamycin production.

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