VisG is essential for biosynthesis of virginiamycin S, a streptogramin type B antibiotic, as a provider of the nonproteinogenic amino acid phenylglycine

Fitria Ningsih,1 Shigeru Kitani,1 Eriko Fukushima1 and Takuya Nihira1,2

1International Center for Biotechnology, Osaka University, 2-1, Yamadaoka, Suita, Osaka 565-0871, Japan
2MU-OU Collaborative Research Center for Bioscience and Biotechnology, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 10400, Thailand

A streptogramin type B antibiotic, virginiamycin S (VS), is produced by Streptomyces virginiiae, together with a streptogramin type A antibiotic, virginiamycin M1 (VM), as its synergistic counterpart. VS is a cyclic hexadepsipeptide containing a nonproteinogenic amino acid, L-phenylglycine (L-pheGly), in its core structure. We have identified, in the left-hand extremity of the virginiamycin supercluster, two genes that direct VS biosynthesis with L-pheGly incorporation. Transcriptional analysis revealed that visF, encoding a nonribosomal peptide synthetase, and visG, encoding a protein with homology to a hydroxyphenylacetyl-CoA dioxygenase, are under the transcriptional regulation of virginiae butanolide (VB), a small diffusing signalling molecule that governs virginiamycin production. Gene deletion of visG resulted in complete loss of VS production without any changes in VM production, suggesting that visG is required for VS biosynthesis. The abolished VS production in the visG disruptant was fully recovered either by the external addition of pheGly or by gene complementation, which indicates that VisG is involved in VS biosynthesis as the provider of an L-pheGly molecule. A feeding experiment with L-pheGly analogues suggested that VisF, which is responsible for the last condensation step, has high substrate specificity toward L-pheGly.

INTRODUCTION

Virginiamycin S (VS) (Fig. 1a) is a cyclic hexadepsipeptide antibiotic that belongs to the type B compounds in the streptogramin family. In Streptomyces virginiiae, VS is coproduced with a polyunsaturated macrolactone antibiotic, virginiamycin M1 (VM), which belongs to the type A compounds in the streptogramin family. Both antibiotics show strong synergistic bactericidal activity against a wide range of Gram-positive bacteria (Di Giambattista et al., 1989). Although virginiamycin has been used widely as a performance promoter in animal husbandry, human application of virginiamycin has been limited by its poor water solubility. However, Barrière et al. (1998) succeeded in creating a Streptomyces pristinaespiralis mutant that produces derivatives of pristinamycin I, another type B compound in the streptogramin family, which show greater biological activity and higher water solubility than their natural counterparts. Furthermore, chemical modification of virginiamycin has led to the design of watersoluble derivatives, such as quinupristin and dalfopristin, which are both in use as therapeutic drugs (e.g. Synercid) against vancomycin-resistant Enterococcus faecium and other Gram-positive bacteria (Manzella, 2001). Thus, the importance of the streptogramin family, including virginiamycin, has increased dramatically.

VS is synthesized by stepwise condensation of two proteinogenic and five nonproteinogenic amino acids: 3-hydroxypicolinic acid (derived from L-lysine) as a starter, followed by incorporation of L-threonine, D-α-aminobutyric acid (D-amBu), L-proline, N-methyl-L-phenylalanine, 4-oxo-L-pipeolic acid (derived from L-lysine) and L-phenylglycine (L-pheGly) (Fig. 1a) (Yamada et al., 1997). It has been postulated that nonribosomal peptide synthetase (NRPS) is involved in VS biosynthesis. Previously, we cloned plausible NRPS genes (visE and truncated visF) necessary for assembling the VS framework, which are located in the region downstream of vmsT (a pathway-specific regulatory
gene for VM production) in the vicinity of the VM biosynthetic gene cluster (Pulsawat et al., 2007). Based on genetic information from pristinamycin I biosynthesis, it was deduced that VisE is responsible for incorporation of the two amino acids (L-threonine and d-amBu), and that VisF is a large NRPS for condensation of the last four amino acids in the VS core structure: l-proline, N-methyl-l-phenylalanine, 4-oxo-l-pipeolic acid and l-pheGly. With respect to the supply of necessary nonproteinogenic amino acids, four VS biosynthetic genes (visA, visB, visC and visD) have been identified in the right-hand region of the virginiamycin biosynthetic gene cluster (Namwat et al., 2002). These four genes are required for creation and activation of the first amino acid moiety, 3-hydroxypicolinic acid, and the sixth amino acid moiety, 4-oxo-l-pipeolic acid, in the VS structure (Fig. 1b). However, other pathways to synthesize d-amBu and l-pheGly are still needed, and remain to be elucidated. Although p-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine are typical nonproteinogenic amino acids occasionally found in several natural products, including the family of glycopeptide antibiotics (Lamb et al., 2006), l-pheGly is only found in the type B compounds in the streptogramin family. Insights into the biosynthetic pathway of l-pheGly are important for compounds in the streptogramin family. Insights into l-phenylalanine, 4-oxo-L-pipecolic acid and L-pheGly. With gene for VM production) in the vicinity of the VM region of the supercluster, and demonstrate that visG plays an important role in the biosynthesis of L-pheGly, a nonproteinogenic amino acid, in VS production.

Here, we provide genetic and chemical data to characterize the VS biosynthetic genes that are located in a boundary region of the supercluster, and demonstrate that visG plays an important role in the biosynthesis of L-pheGly, a nonproteinogenic amino acid, in VS production.

Fig. 1. Chemical structure of virginiamycin S from S. virginiae and schematic representation of adenyl-HPA (hydroxypicolinic acid) and L-Pip (pipecolic acid) formation by VisA/B/C proteins. (a) Virginiamycin S is composed of the following seven amino acids: HPA, L-Thr, d-2-amino butyric acid (d-amBu), L-Pro, N-methyl-l-phenylalanine (L-MePhe), L-4-oxopipeolic acid (L-4-oxo-Pip) and L-phenylglycine (L-pheGly). (b) L-Lysine is enzymically converted to adenyl-HPA by VisA and VisB, and to L-Pip by VisC, respectively, in a stepwise manner.

METHODS

Bacterial strains, plasmids, media and growth conditions. Streptomyces virginiae strain MAFF 10-06014 from the National Food Research Institute (Tsukuba, Japan) was grown at 28 °C on ISP medium 2 (Becton, Dickinson and Company). Mannitol soya flour (MS) medium as described by Kieser et al. (2000) but supplemented with 10 mM MgCl2 was used for intergenic conjugation. For virginiamycin production, liquid f-medium was used as described previously (Nihira et al., 1988). Escherichia coli DH5α was used for general DNA manipulation (Kieser et al., 2000; Sambrook & Russell, 2001) and E. coli ET15267 (dam-13::Tn9 dcm-6 hsdM hsdS) containing the RP4 derivative pUZ8002 (Paget et al., 1999) was used for E. coli/Streptomyces conjugation. The plasmids used were pBluescript II SK for general cloning, pKC1132 (Bierman et al., 1992) for gene disruption and pLT101, a derivative of pSET152 (Bierman et al., 1992) containing the strong and constitutive promoter ermEp* and a ttd terminator, for gene complementation. All the primers are listed in Supplementary Table S1, available with the online version of this paper.

Transcriptional analysis by quantitative RT-PCR. Total RNAs were extracted from mycelia harvested by using an RNeasy Mini kit (Qiagen) and treated with DNase I (Takara Bio). The cDNA was synthesized using SuperScript III RNase H−, according to the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) was performed using the Applied Biosystems 7300 Real-Time PCR System and SYBR Green PCR Master Mix (Applied Biosystems) according to the supplier’s recommendations. The reaction conditions were as follows: 95 °C for 10 min followed by 40 cycles consisting of 15 s at 95 °C for denaturation and 1 min at 68 °C for annealing and extension. A final dissociation stage was performed to generate a melting curve and subsequently verify the specificity of the amplification products. rplA, encoding 50S ribosomal protein L1 in S. virginiae (Katayama et al., 1996), was used as a negative control. Gene expression was measured in triplicate and normalized to the S. virginiae 16S rDNA level of the hrdB-like gene [hrdB encodes the major sigma factor in Streptomyces coelicolor A3(2)] (Buttner et al., 1999] using the relative standard curve method, as the gene is expressed fairly constantly throughout growth.

Disruption of visG. A 2.3 kb visG-upstream fragment was amplified using the primer pair visG-AX/visG-AS, and digested with XbaI and

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VisG, a phenylglycine provider for virginiamycin S

RESULTS

Cloning and in silico analysis of the region downstream of visE in the virginiamycin biosynthetic gene cluster

Previously, we demonstrated that two genes are present at the left-hand extremity of the virginiamycin biosynthetic cluster: visE, which encodes an NRPS containing two A domains for incorporation of L-Thr and d-amBu into the VS structure, and an incomplete ORF (visF), which partially encodes a protein containing one condensation (C) domain (Pulsawat et al., 2007). To isolate the complete visF gene and search for additional genes involved in virginiamycin biosynthesis, a cosmid library of S. virginiae was screened by PCR. Using a partial nucleotide sequence of the visF gene, we identified one cosmid (2B9), the insert of which covered a 28 kb region downstream of visF and contained a complete ORF of visF and seven more ORFs based on an analysis with FramePlot.

4.0beta (http://nocardia.nih.go.jp/fp4/) (Fig. 2a). The plausible function for each ORF was assigned by comparing the translated product with known proteins in public databases (Table 1).

VisF possesses NRPS modules including a total of four adenylation (A) domains, four peptidyl carrier protein (PCP) domains, four condensation (C) domains, one methyltransferase (M) domain and one thiostrepton (TE) domain. Bioinformatic analysis to predict the substrate
specificity of the four A domains using NRPSpredictor (http://ab.inf.uni-tuebingen.de/software/NRPSpredictor/welcome.html) revealed that the first A domain [VisFA1, DVQYAAHVMK] is the 10 amino acid code of the substrate recognition sequence as defined by Stachelhaus et al. (1999)] is specific for the incorporation of proline, the second A domain (VisFA2, DAWTVAVVCK) recognizes phenylalanine as a substrate, and the third and fourth A domains (VisFA3, DFQFFGAVVK; VisFA4, DIYLWGLLGK) specifically incorporate piperolic acid and L-pheGly, respectively. The M domain followed the VisFA2 domain, presumably giving methylphenylalanine as the fourth amino acid of the VS structure. In addition, VisF showed high similarity with SnaDE, a pristinamycin I synthetase from S. pristinaespiralis (de Crécy-Lagard et al., 1997), suggesting that VisF probably assembles the fourth to seventh amino acids (L-proline, phenylglycine biosynthesis, such as PgaT of Streptomyces coelicolor (Pfeifer et al., 2002)) and most likely introduces the final residue proline into pristinamycin II (Blanc et al., 1994). ORF7 possesses NRPS modules including a total of two C domains, two PCP domains, one TE domain, one A domain, and one other domain (TIGR01720) that is positioned between amino acids 1414 and 1572 and has an unknown function. However, it seems that the organization of the modules and domains in the ORF7 protein does not follow the NRPS collinearity principle, and the sole A domain (ORF7A1, DVHVTAYSK) shows no homology to the known A domain. From these analyses, it is difficult to predict the involvement of the ORF7 protein in VM biosynthesis at present.

### Table 1. Summary of ORFs in the cloned DNA

<table>
<thead>
<tr>
<th>Protein</th>
<th>Location</th>
<th>Amino acids</th>
<th>Proposed function</th>
<th>Sequence similarity (protein, origin)</th>
<th>Identity/Similarity (%)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>VisF</td>
<td>1–14763</td>
<td>4920</td>
<td>NRPS for VS</td>
<td>SnaDE, S. pristinaespiralis 67/92</td>
<td>CBH31051</td>
<td></td>
</tr>
<tr>
<td>VisG</td>
<td>14796–16190</td>
<td>464</td>
<td>Phenylacetyl-CoA dioxygenase</td>
<td>DpgC, S. toyocaensis 48/83</td>
<td>AAM80546</td>
<td></td>
</tr>
<tr>
<td>BkdC</td>
<td>16187–17290</td>
<td>367</td>
<td>BCDH E1 subunit</td>
<td>BkdA2, S. coelicolor A3(2) 54/83</td>
<td>CAD5533</td>
<td></td>
</tr>
<tr>
<td>BkdD</td>
<td>17290–18276</td>
<td>328</td>
<td>BCDH E1 subunit</td>
<td>BkdB2, S. coelicolor A3(2) 74/93</td>
<td>CAB46953</td>
<td></td>
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<tr>
<td>ORF4</td>
<td>18273–19100</td>
<td>275</td>
<td>Type II thioesterase</td>
<td>Ken1, Streptomyces violaceoruber 41/77</td>
<td>CAQ52621</td>
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<td>ORF5</td>
<td>19184–19402</td>
<td>72</td>
<td>MbtH-like protein</td>
<td>Orf46, Streptomyces fungicidicus 66/87</td>
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<td>ORF6</td>
<td>19489–20754</td>
<td>421</td>
<td>Phenylglycine aminotransferase</td>
<td>PgaT, A. balhimicina 56/88</td>
<td>CAC48367</td>
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<td>ORF7</td>
<td>21052–28320</td>
<td>2422</td>
<td>NRPS</td>
<td>SnaD, S. pristinaespiralis 69/92</td>
<td>CBW45640</td>
<td></td>
</tr>
</tbody>
</table>

Transcriptional control by virginiae butanolide of the newly identified genes

Virginiamycin production in S. virginiae is controlled by a γ-butyrolactone autoregulator, virginiae butanolide (VB), and its cognate receptor BarA as a DNA-binding transcriptional repressor (Kinoshita et al., 1997). The VB biosynthetic pathway requires enzymic function of BarX, an AfsA-family protein (Lee et al., 2010). To investigate the effect of VB on transcription of the newly identified genes, the transcriptional levels were examined by qRT-PCR analysis (Fig. 2b). The barX mutant has no ability to produce virginiamycin. On the other hand, when VB is added exogenously to the culture of the barX mutant at 8 h, virginiamycin production occurs from 10 h (Lee et al., 2010). The transcription levels of the identified genes showed a significant relative increase of 26- to 96-fold in the presence of external VB compared to those without VB addition. These results indicated that the eight identified genes downstream of visE are new members of the VB regulon, in addition to the virginiamycin biosynthetic genes.

Within the VB-responsive genes, the mRNA level of visG is highly dependent on VB (about 96-fold). Accordingly, the transcriptional level of visG was compared between the wild-type strain and the barB disruptant, which shows earlier and higher-level production of virginiamycins. BarB, whose expression is under the positive control of
VB via BarA, is a transcriptional repressor in the early process of virginiamycin biosynthesis (Matsuno et al., 2004). The results showed that after incubation for 10 h visG transcription increased up to 270 (±41.5)-fold in the barB disruptant compared with the wild-type strain (average of three independent experiments). Taken together with the homology analysis described above, these results supported the idea that VisG is one of the virginiamycin biosynthetic enzymes and supplies L-pheGly.

The visG disruptant lacks the ability to produce VS, not VM

To provide direct evidence that visG is involved in virginiamycin production, we disrupted visG by in-frame deletion of 353 amino acids. The resulting mutants were analysed by PCR in order to detect the size of the replaced allelic visG gene (Fig. 3a). The mutant IC109 grew normally in liquid cultivation and showed morphological characteristics on solid medium identical to those of the wild-type strain (results not shown). These results indicated that visG is not involved in either primary metabolism or morphological differentiation. To assess virginiamycin production, the visG disruptant was cultivated in liquid medium and the culture supernatant was analysed by a bioassay against B. subtilis (Fig. 3b). The results showed that the visG disruptant (strain IC109) exhibited a smaller clear zone than that of the wild-type strain. Because this phenomenon could have been due either to a compositional or quantity change of virginiamycin, the culture supernatant was analysed by HPLC. As shown in Fig. 3(c), a peak corresponding to VS (25.7 min) was missing in strain IC109, whereas the peak corresponding to VM remained intact. The peak at 25.9 min was eluted later than VS and thus was not VS. Thus, inactivation of visG completely abolished VS production, but not VM production, indicating that VisG is necessary only for VS biosynthesis.

To ensure that inactivation of visG was the sole reason for the observed loss of the VS production, the intact visG gene was reintroduced into the mutant strain. FramePlot analysis and sequence alignment analysis with DpgC proteins predicted two plausible initiation codons for the VisG protein (Fig. 2c, d): one is a TTG codon with a putative ribosome-binding site (RBS) located 7 bp upstream and the other is a CTG codon with no distinct RBS. Furthermore, because there is only a narrow intergenic region (about 40 bp) between visF and visG, visG may form a bicistronic operon with visF, with no definite promoter-like sequences in the region immediately upstream of visG. Thus, we constructed plasmids that each contained different visG genes driven by the strong constitutive ermEp* promoter. Introduction of pLT209, containing a shorter visG gene, into the mutant IC109 did not restore VS production (results not shown). On the other hand, complementation of the mutant IC109 with pLT210, containing a longer visG gene, restored the VS production to a level similar to that of the wild-type strain (Fig. 3b, c).

Fig. 3. Inactivation of visG by gene replacement (a), and bioassay (b) and HPLC analysis (c) of virginiamycin production. (a) Schematic representation of visG disruption. The black triangles indicate the deleted visG gene (ΔvisG). PCR analysis of chromosomal DNA from the wild-type strain (WT) and ΔvisG disruptants (IC109-1 and IC109-2) using the primer pair visG-DF/visG-DR for detection of the ΔvisG mutation. (b) Bioassay of 24 h culture broths against B. subtilis. S. virginiae strain IC111 is a visG-complemented visG disruptant in which pLT210 is integrated into the chromosome. (c) HPLC analysis of virginiamycin production. Production profiles corresponding to the retention time (19–30 min) with detection at 305 nm are shown. The peaks of virginiamycin M₁ (VM) and virginiamycin S (VS) are indicated.
These results demonstrated that functional visG should start from the TTG codon to encode a 464 amino acid protein and should be essential for the VS biosynthesis.

**Addition of L-pheGly restores the deficiency of VS production**

Due to the similarity of VisG to a hydroxyphenylacetyl-CoA dioxygenase, VisG is predicted to be involved in the biosynthesis of L-pheGly. To confirm this possibility, L-pheGly (Fig. 4a) was added to the production medium of the visG disruptant and the biological activity of the culture supernatant was assessed. Bioassay analysis (Fig. 4b) as well as HPLC analysis (Fig. 4c) demonstrated that the addition of L-pheGly resulted in recovery of the VS production in the visG disruptant, indicating that the loss of VS production was due to a lack of L-pheGly and implying that VisG would be an L-pheGly-producing enzyme in the L-pheGly biosynthetic pathway.

In contrast to the case of L-pheGly addition, addition of L-pheGly analogues, such as p-hydroxyphenylglycine (Fig. 4a), L-phenylalanine, or L-3,4-dihydroxyphenylalanine (L-DOPA), did not result in any difference in the size of the clear zone (Fig. 4b) or in the HPLC profiles (Fig. 4c), suggesting that these analogues are not incorporated into the elongating VS backbone structure, probably due to the strict recognition of the L-pheGly structure by the enzyme responsible, VisF.

**DISCUSSION**

Hydroxylated phenylglycines are found in various natural products of peptidic nature, such as glycopeptide antibiotics (vancomycin and teicoplanin) and other antimicrobial compounds (ramoplanin and calcium-dependent antibiotic), and are known, as in the case of p-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine, to be essential for rigidifying side-chain cross-links of the peptide scaffold. Although the biosynthetic pathways of p-hydroxyphenylglycine and 3,5-dihydroxyphenylglycine have been well studied (Hubbard et al., 2000; Tseng et al., 2004), there is little available information on genes or pathways for the biosynthesis of a similar nonproteinogenic amino acid, L-pheGly, that is only found in streptogramin antibiotics. In the present study, we verified that VisG is an L-pheGly provider that is essential for the biosynthesis of VS, a streptogramin type B antibiotic. VisG resembles the 3,5-dihydroxyphenylacetyl (DPA)-CoA 1,2-dioxygenase DpgC, which is reported to incorporate one oxygen atom of O2 at C-2 and another at C-1 of DPA-CoA with cleavage of the thioester bond to form dihydroxyphenylglyoxylate (Tseng et al., 2004). With respect to L-pheGly biosynthesis, we propose that phenylacetyl-CoA would be converted to benzoylformate by VisG, although in vitro experiments will be needed to clarify the detailed reaction mechanism. Following the VisG reaction, an aminotransferase would convert the benzoylformate to L-pheGly, which would eventually be activated and incorporated into VS by the action of VisF.

Four genes (visG, bkdC, bkdD and orf4) have start and stop codons which overlap each other sequentially and their transcription was induced by the external addition of VB, suggesting that visG might form a tetracistronic operon with bkdC, bkdD and orf4. Increased transcription of visF with the addition of VB was also observed, and the intergenic region between visF and visG is likely to be short, implying that visG would be expressed polycistronically with visF, bkdC, bkdD and orf4. In the course of
constructing the visG-disruption plasmid, one of the plasmids was unintentionally made to contain a 34 bp deletion in the coding region of bkdD, which presumably encodes the E1β subunit of the BCDH complex. Introduction of this plasmid resulted in double mutations (∆visG bkdD). The strain harbouring the double mutations showed no VS production, similar to that of the visG disruptant (results not shown). However, complementation by intact visG completely restored VS production to a level similar to that of the wild-type strain without any change of VM production, indicating that bkdD (presumably as well as bkdC encoding the cognate E1z subunit) at least is not involved in the biosynthesis of virginiamycins. Although phenotypic analysis of the orf4 mutant is under way to further clarify the borders of the virginiamycin cluster, at present, visG is the VS biosynthetic gene in the most left-hand extremity within the clustered virginiamycin biosynthetic genes.

Expanding the diversity of the virginiamycin scaffold could facilitate chemical modification, affecting the water solubility, size and receptor affinity of the molecules. With respect to alteration of non-ribosomal peptide scaffolds, p-hydroxyphenylglycine (HpG) or 1,3,5-dihydroxyphenylglycine (DPG) residues are attractive targets for chemical modification, because the hydroxyl groups on the aromatic ring are easily modified by sulfation (Lamb et al., 2006), and chlorine atoms can be introduced into the DPG residue by a halogenase (Puk et al., 2002). In the present study, we attempted to make VS derivatives by feeding the visG disruptant with L-pheGly analogues harbouring a mono- or dihydroxyl group on the aromatic ring. However, the incorporation of L-pheGly analogues seems to be hampered by strict substrate recognition of the A domain, VisF A4, of the corresponding NRPS. The HpG-incorporating A domains show many variants of the substrate recognition sequence, such as BspB-m1 and BspB-m2 for balhimycin biosynthesis (DIFHGLLCK and DAVHLGLLCK, respectively), and EndC-m2 for enduracidin biosynthesis (DAYHLGMLCK) (Recktenwald et al., 2002; Yin & Zabriskie, 2006). Mutagenesis and replacement analyses of the L-pheGly-incorporating A domain (VisF A4) are currently under way in our laboratory. Together with the biochemical analysis, these studies will provide useful information for forced incorporation of HpG and DPG into the VS core structure.

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