Microbiology (2011), **157**, 3213–3220 **DOI** 10.1099/mic.0.050203-0

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

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Received 5 April 2011
Revised 1 August 2011
Accepted 3 August 2011

A streptogramin type B antibiotic, virginiamycin S (VS), is produced by *Streptomyces virginiae*, together with a streptogramin type A antibiotic, virginiamycin M_1 (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 virginiae*, VS is coproduced with a polyunsaturated macrolactone antibiotic, virginiamycin M_1 (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 water-soluble 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-pipecolic 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

Abbreviations: A, adenylation; D-amBu, D-α-aminobutyric acid; BCDH, branched-chain α-keto acid dehydrogenase; C, condensation; Hpg, *p*-hydroxyphenylglycine; M, methylation; NRPS, nonribosomal peptide synthetase; PCP, peptidyl carrier protein; L-pheGly, L-phenylglycine; qRT-PCR, quantitative RT-PCR; VB, virginiae butanolide; TE, thioesterase; VM, virginiamycin M_1; VS, virginiamycin S.

The DDBJ/EMBL/GenBank accession number for the sequence reported in this paper is AB621357.

A supplementary table, listing oligonucleotide primers used in this study, is available with the online version of this paper.

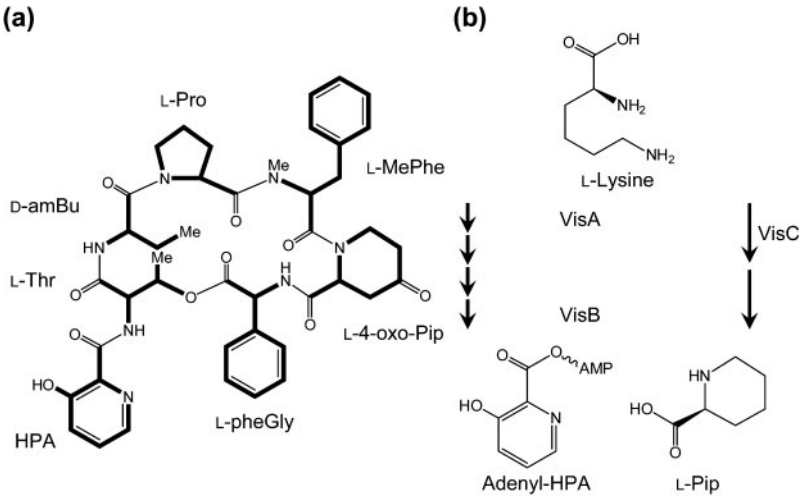

Fig. 1. Chemical structure of virginiamycin S from *S. virginiae* and schematic representation of adenyl-HPA (hydroxypicolinic acid) and L-Pip (pipecolic acid) formation by VisA/B/C proteins. (a) Virginiamycin S is composed of the following seven amino acids: HPA, L-Thr, D-α-aminobutyric acid (D-amBu), L-Pro, *N*-methyl-L-phenylalanine (L-MePhe), L-4-oxopipecolic 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.

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-pipecolic 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-pipecolic 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 future efforts in combinatorial biosynthetic manipulation. However, although the committed synthesis of hydroxyphenylglycines is usually achieved by specialized enzymes encoded by the clustered biosynthetic genes for hydroxyphenylglycine-containing natural products (Hubbard *et al.*, 2000), and this synthetic mechanism has been well studied, very little information is available for the biosynthetic gene(s) for L-pheGly.

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.

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 $MgCl_2$ 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* ET12567 (*dam-13*::Tn*9 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 *tfd* 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^- Reverse Transcriptase (Invitrogen) and Random Primers (Invitrogen) 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 consequently 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 mRNA level of the *hrdB*-like gene [*hrdB* encodes the major sigma factor in *Streptomyces coelicolor* A3(2) (Buttner *et al.*, 1990)] 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 *Xba*I and

*Spe*I. Similarly, a 2.3 kb *visG*-downstream fragment was amplified using the primer pair visG-BS/visG-BH, and digested with *Spe*I and *Hin*dIII. PCR was performed using high-fidelity PrimeSTAR HS DNA polymerase (Takara Bio) according to the manufacturer's recommendations. The fidelity of the amplified regions was confirmed by sequencing. The two resulting fragments were cloned together into the *Xba*I and *Hin*dIII sites of pBluescript II SK, and were recovered as a 4.5 kb *Xba*I/*Hin*dIII fragment, which was then inserted into the *Xba*I and *Hin*dIII sites of pKC1132 to generate pLT208 for *visG* disruption. *E. coli* ET12567(pUZ8002) harbouring pLT208 was conjugated with *S. virginiae*, and the wild-type gene was replaced with the disrupted allele (Δ*visG*) by homologous recombination. The genotype of the Δ*visG* disrupted candidates was confirmed by PCR analysis, and the *visG* disruptant was designated *S. virginiae* strain IC109.

Genetic complementation of the *visG* disruptant. The *visG* ORFs starting from different initiation codons were amplified by the primer pairs visGS-N/visG-C and visGL-N/visG-C, respectively, and each fragment was inserted into the *Eco*RV site of pBluescript II SK. The resulting plasmids were digested with *Bam*HI, and were then cloned into the *Bam*HI site of pLT101 (Pulsawat *et al.*, 2009) to place the *visG* ORF under the control of *ermEp**, resulting in pLT209 and pLT210, respectively. By intergenic conjugation and integration, each plasmid was introduced into *S. virginiae* strain IC109, creating *S. virginiae* strains IC110 and IC111, respectively. The correct integration in the exconjugants was confirmed by PCR analysis.

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

Feeding of the *visG* mutant with L-pheGly and L-pheGly analogues. *S. virginiae* strain IC109 was grown in 70 ml f-medium as described previously. After incubation for 12 h, L-pheGly (Tokyo Chemical Industry), D-(−)-2-(*p*-hydroxyphenyl)glycine (Wako Pure Chemical Industries), L-phenylalanine (Wako Pure Chemical Industries) or L-3,4-dihydroxyphenylalanine (Wako Pure Chemical Industries) was added to the culture. After incubation for 24 h, the culture supernatants were analysed by a bioassay and reversed-phase HPLC.

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 the *S. virginiae* genome 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 *visE* 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 methylation (M) domain and one thioesterase (TE) domain. Bioinformatic analysis to predict the substrate

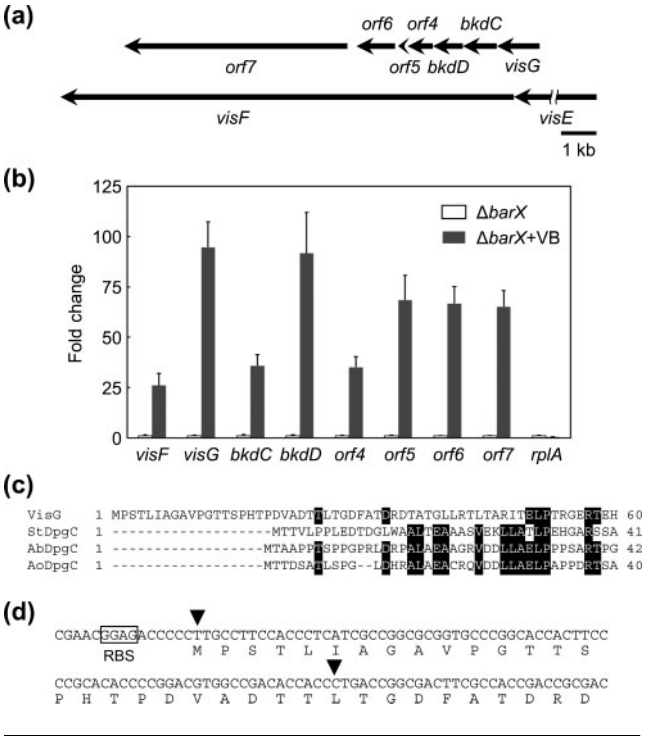

Fig. 2. (a) Gene organization of the sequenced 28 kb DNA region in the *visE*-flanking region, (b) transcriptional analysis of the identified genes by qRT-PCR, (c) amino acid sequence alignment of the N-terminal region of VisG with those of other actinomycetes' DpgC, and (d) the nucleotide sequence encoding the N-terminus of VisG and the *visG*-upstream region. (a) The direction of *visG* transcription is the same as those of the *visE* and *vmsT* genes. (b) Total RNA was extracted from mycelia harvested after incubation for 10 h. VB-C_6 (60 ng ml^{-1}) was added into the culture of the *barX* mutant after incubation for 8 h. Virginiamycin production was observed after incubation for 10 h with exogenous VB addition. Fold change is relative to the expression of each gene without VB addition after incubation for 10 h. Error bars represent standard deviations from triplicate experiments. (c) The numbers indicate the amino acid positions within each sequence. Black boxes in the alignment indicate positions at which the same amino acid is found in at least three of the four sequences: StDpgC (AAM80546) from *S. toyocaensis*, AbDpgC (CAC48380) from *A. balhimycina* and AoDpgC (CAA11787) from *Amycolatopsis orientalis*. (d) The putative ribosome-binding site (RBS) is shown in a box. The black triangles together with either TTG or CTG indicate the positions of probable initiation codons for the longer and shorter VisG protein, respectively.

Table 1. Summary of ORFs in the cloned DNA

Protein	Location	Amino acids	Proposed function	Sequence similarity (protein, origin)	Identity/ Similarity (%)	Accession no.
VisF	1–14763	4920	NRPS for VS	SnbDE, *S. pristinaespiralis*	67/92	CBH31051
VisG	14796–16190	464	Phenylacetyl-CoA dioxygenase	DpgC, *S. toyocaensis*	48/83	AAM80546
BkdC	16187–17290	367	BCDH E1 α subunit	BkdA2, *S. coelicolor* A3(2)	54/83	CAD55333
BkdD	17290–18276	328	BCDH E1 β subunit	BkdB2, *S. coelicolor* A3(2)	74/93	CAB46953
ORF4	18273–19100	275	Type II thioesterase	Ken1, *Streptomyces violaceoruber*	41/77	CAQ52621
ORF5	19184–19402	72	MbtH-like protein	Orf46, *Streptomyces fungicidicus*	66/87	ABD65966
ORF6	19489–20754	421	Phenylglycine aminotransferase	PgaT, *A. balhimycina*	56/88	CAC48367
ORF7	21052–28320	2422	NRPS	SnaD, *S. pristinaespiralis*	69/92	CBW45640

specificity of the four A domains using NRPSpredictor (http://ab.inf.uni-tuebingen.de/software/NRPSpredictor/welcome.html) revealed that the first A domain [VisFA$_1$, 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 (VisFA$_2$, DAWTVAAVCK) recognizes phenylalanine as a substrate, and the third and fourth A domains (VisFA$_3$, DFQFFGVAVK; VisFA$_4$, DIYLWGLLGK) specifically incorporate pipecolic acid and L-pheGly, respectively. The M domain followed the VisFA$_2$ domain, presumably giving methylphenylalanine as the fourth amino acid of the VS structure. In addition, VisF showed high similarity with SnbDE, 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, methyl-L-phenylalanine, L-pipecolic acid and L-pheGly) into the VS core structure. The *visF*-downstream gene *visG* encodes a protein with homology to a hydroxyphenylacetyl-CoA dioxygenase, such as DpgC of *Streptomyces toyocaensis* and *Amycolatopsis balhimycina*, which are necessary for dihydroxyphenylglycine biosynthesis (Pfeifer *et al.*, 2001; Pootoolal *et al.*, 2002). The next two genes, *bkdC* and *bkdD*, encode E1α and E1β subunits of the branched-chain α-keto acid dehydrogenase (BCDH) complex that is responsible for oxidative decarboxylation (Skinner *et al.*, 1995). The following gene, *orf4*, encodes a type II thioesterase. Type II thioesterases are present in many biosynthetic gene clusters for nonribosomal peptides, where they have corrective functions by hydrolysing aberrant substrates from the respective PCP domains (Heathcote *et al.*, 2001). The gene product of *orf5* exhibits similarity to the MbtH-like protein. *mbtH*-like genes are frequently found in the biosynthetic gene clusters of nonribosomal peptides and siderophores, and have recently been reported to be necessary for modulation of the enzymic activity of NRPS (Zhang *et al.*, 2010). The protein encoded by the next gene, *orf6*, has homology to aminotransferases involved in di- or mono-hydroxyphenylglycine biosynthesis, such as PgaT of *A. balhimycina* (Pfeifer *et al.*, 2001). The last gene, *orf7*, encodes an NRPS with homology to SnaD, a pristinamycin II synthetase, which 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 (ORF7A$_1$, DVHHVTAYSK) 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.

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).

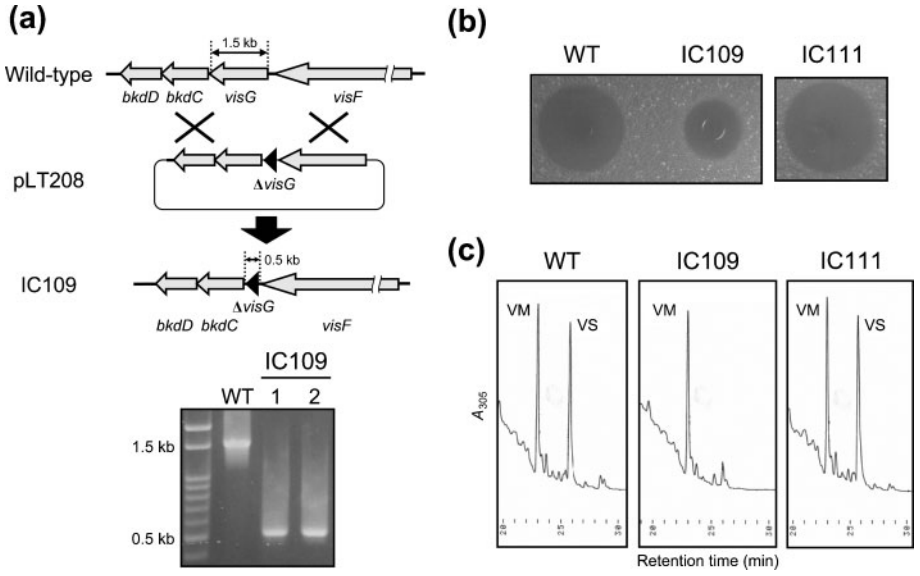

Fig. 3. Inactivation of *visG* by gene replacement (a), and bioassay (b) and HPLC analysis (c) of virginiamycin production. (a) Schematic representation of *visG* disruption. The black triangles indicate the deleted *visG* gene (Δ*visG*). PCR analysis of chromosomal DNA from the wild-type strain (WT) and Δ*visG* disruptants (IC109-1 and IC109-2) using the primer pair visG-DF/visG-DR for detection of the Δ*visG* 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 M_1 (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.

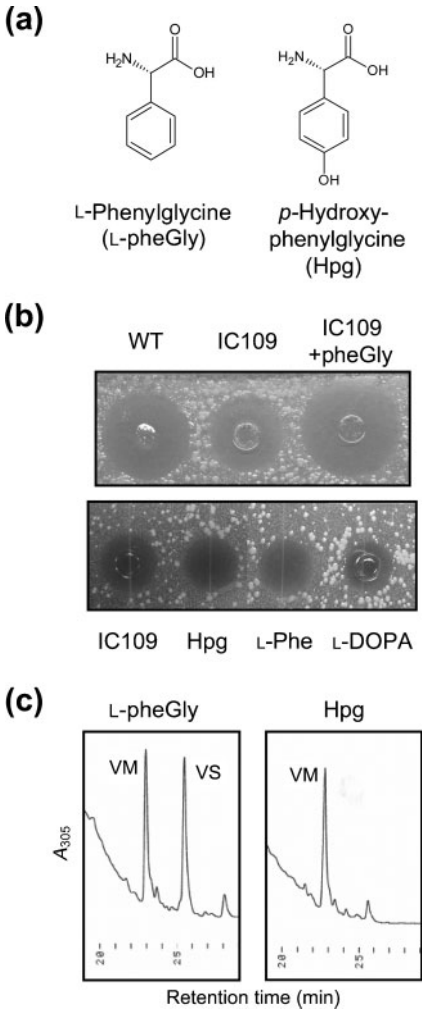

Fig. 4. External addition of L-pheGly for chemical complementation, and L-pheGly analogues for biosynthesis of the VS derivative. (a) Chemical structure of L-pheGly and Hpg. (b) L-pheGly was added to a final concentration of 100 μM after incubation for 12 h (upper panel), and the L-pheGly analogues Hpg, L-phenylalanine (L-Phe) and L-3,4-dihydroxyphenylalanine (L-DOPA) were added individually to a final concentration of 500 μM after incubation for 12 h (lower panel). (c) Production profiles corresponding to the retention time (19–29 min) with detection at 305 nm are shown. The peaks of VM and VS are indicated.

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 O_2 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 (Δ*visG* Δ*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 E1α 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 L-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, $VisFA_4$, of the corresponding NRPS. The Hpg-incorporating A domains show many variants of the substrate recognition sequence, such as BpsB-m1 and BpsB-m2 for balhimycin biosynthesis (DIFHLGLLCK 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 ($VisFA_4$) 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.

ACKNOWLEDGEMENTS

This work is a part of the PhD dissertation of F. N. We thank Satoshi Harashima, Minetaka Sugiyama and Kiyoko Miyamoto for assistance with qRT-PCR analysis. This work was supported in part by a grant for the Joint Program in the Field of Biotechnology under the Japan Society for Promotion of Science (JSPS), the National Research Council of Thailand and the National Science and Technology Development Agency of Thailand to T. N., by a Grant-in-Aid for Scientific Research (B) (no. 21360404) from JSPS to T. N. and S. K., and by a scholarship from the Ministry of Education, Culture, Sports, Science and Technology of Japan to F. N.

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Edited by: J.-H. Roe