Null mutation analysis of an *afsA*-family gene, *barX*, that is involved in biosynthesis of the γ -butyrolactone autoregulator in *Streptomyces virginiae*

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Virginiae butanolide (VB) is a γ -butyrolactone autoregulator that triggers production of the streptogramin antibiotic virginiamycin in *Streptomyces virginiae*. Our previous studies suggested that the *barX* gene, an *afsA*-family gene, is likely to participate in the regulatory pathway for the production of VB, rather than in the biosynthetic pathway of VB itself, in contrast to the function of other *afsA*-family genes. Mutation analysis now shows that BarX at least plays an enzymic role in the VB biosynthetic pathway. Heterologous expression of the *afsA* gene from *Streptomyces griseus* into the *barX* mutant partially restored the deficiency of virginiamycin production, suggesting that *afsA*-family genes have a common ability to synthesize the γ -butyrolactone autoregulators. Taken together with previous works relating to the function of an *afsA*-family gene, these results support the idea that streptomycetes have two biosynthetic pathways for the γ -butyrolactone autoregulators.

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INTRODUCTION

The Gram-positive filamentous bacterial genus *Streptomyces* is notable both for its complex morphological differentiation and for its ability to produce a plethora of secondary metabolites, including antibiotics, during its life cycle. One of the many factors affecting these characteristics of *Streptomyces* spp. is the family of γ -butyrolactone autoregulators, which are small diffusing signalling molecules that trigger the onset of secondary metabolism, aerial mycelium formation, or both (Takano, 2006). In particular, the A-factor from *Streptomyces griseus* is the best-characterized γ -butyrolactone autoregulator that activates production of streptomycin and other metabolites in concert with sporulation (Horinouchi, 2007). Another γ -butyrolactone autoregulator is SCB1, which controls the expression of the cryptic type I polyketide biosynthetic gene cluster in *Streptomyces coelicolor* A3(2) (Takano, 2006), which has been used for many years as a model organism in studies of the regulation of secondary metabolism and cytodifferentiation.

Virginiae butanolides (VBs) A–E are isolated as γ -butyrolactone autoregulators from the culture broth of

Streptomyces virginiae (Yamada *et al.*, 1987). They induce the coordinated production of two different antibiotics, virginiamycin M and virginiamycin S. So far, genetic information on the biosynthetic mechanism of VB itself has been limited by difficulties in identifying the specific pathways or enzymes involved. With respect to VB biosynthesis, we first reported that the skeleton of VB-A is formed from two molecules of acetic acid and one molecule each of isovaleric acid and glycerol, based on feeding experiments using ¹³C- and ²H-labelled precursors (Sakuda *et al.*, 1992). After coupling between a dihydroxyacetone (DHA)-type C3 unit and a β -keto acid derivative, intramolecular aldol condensation occurs, followed by two reduction steps, the NADH-dependent enoyl reductase-type reduction and the final NADPH-dependent keto-reduction by BarS1 (Shikura *et al.*, 2002), to form the VB molecules.

Recent progress at the molecular level in characterizing the A-factor biosynthesis pathway has indicated that AfsA catalyses β -keto acyl transfer, and that the resultant β -keto ester is reduced by BprA, followed by a dephosphorylation step to form the A-factor molecule (Kato *et al.*, 2007). Currently, the NCBI database indicates that there are 24 *afsA* homologue genes, although the function of most of these genes in γ -butyrolactone biosynthesis remains to be clarified. *In silico* analysis and mutagenesis analysis have

Abbreviations: DHA, dihydroxyacetone; VB, virginiae butanolide.

Three supplementary figures and two supplementary tables, with references, are available with the online version of this paper.

suggested that ScbA in *S. coelicolor* A3(2) has an enzymic activity to synthesize SCB1, but could not eliminate the possibility that ScbA also has a regulatory function for antibiotic production (Hsiao *et al.*, 2007). Previously, we suggested that *barX* might be involved in the regulation of VB biosynthesis rather than as an enzyme in VB biosynthesis itself (Kawachi *et al.*, 2000). Here, we recharacterized the function of *barX* in VB biosynthesis by null mutation analysis, and demonstrated that a *barX* product plays at least an enzymic role in the VB biosynthetic pathway. The present study also supports the idea that there are two distinctive pathways of γ -butyrolactone autoregulator biosynthesis in streptomycetes.

METHODS

Bacterial strains and plasmids. The microbial strains and plasmids used in this study are listed in Supplementary Table S1, and all the primers are listed in Supplementary Table S2.

Construction of a *barX*-null mutant and complementation. The two fragments flanking *barX* were prepared by PCR with high-fidelity PrimeSTAR HS DNA polymerase (Takara Bio). A *barX*-upstream DNA fragment amplified by the primer pair barX-UF/barX-UR was cloned into pUC19. Similarly, a *barX*-downstream fragment amplified by the primer pair barX-DF/barX-DR was cloned into pUC19. Both fragments were inserted together into pUC19 and were recovered as a *Hind*III/*Eco*RI fragment, which was then transferred into pKC1132, thereby yielding pLT261. *Escherichia coli* ET12567 (pUZ8002) harbouring pLT261 was conjugated with *S. virginiae*. Candidates for the *barX*-deleted mutants were analysed by Southern blot analysis using a 0.6 kb *Sac*I-*Pvu*I fragment as a probe.

For complementation, three DNA fragments having *barX*-upstream regions of different lengths were amplified by the primer pairs barX-CF2/barX-CR, barX-CF3/barX-CR and barX-CF4/barX-CR, respectively. Each fragment was cloned into pUC19, and *Eco*RI-*Bam*HI fragments were inserted into pSET152 to generate pLT263, pLT264 and pLT265, respectively. The resulting plasmids were introduced into *S. virginiae* strain IC160, creating *S. virginiae* strains IC163, IC164 and IC165, respectively.

Overexpression of *barX* in a *barX*-null mutant using the *ermEp promoter.** The *barX* ORFs starting from different putative starting codons were amplified by the primer pairs barX-SF1/barX-SR, barX-SF2/barX-SR, barX-SF3/barX-SR and barX-SF4/barX-SR, respectively. Each fragment was cloned into pUC19, and an *Xba*I fragment was inserted into pLT101, resulting in pLT266, pLT267, pLT268 and pLT269, respectively. The resultant plasmids were introduced into *S. virginiae* strain IC160, creating *S. virginiae* strains IC166, IC167, IC168 and IC169, respectively.

Cross-complementation of a *barX*-null mutant by introduction of *afsA*. The *afsA* coding sequence was amplified by the primer pair afsA-E-F/afsA-R using genomic DNA of *S. griseus* IFO13350, and was cloned into pUC19. An *Xba*I fragment was inserted into pLT101, yielding pLT270. The resultant plasmid was introduced into *S. virginiae* strain IC160, creating *S. virginiae* strain IC170.

Transcriptional analysis by RT-PCR. Total RNAs were extracted from fresh mycelia by using an RNeasy Mini kit (Qiagen). RT-PCR was performed as described by Pulsawat *et al.* (2009). The primers (afsA-RT-F/afsA-RT-R, as listed in Supplementary Table S2) were designed to generate a PCR product of 379 bp from *afsA*.

RESULTS

Functional analysis of the *barX*-null mutant

A Pfam search to find a conserved domain in BarX revealed that both the N terminus and the C terminus have an A-factor biosynthesis repeat (Pfam03756), which is considered to be essential for forming an intramolecular dimer-like structure (Kato *et al.*, 2007), although no experimental evidence has yet been reported. Previously, Kawachi *et al.* (2000) demonstrated that multiple phenotypic changes are associated with a *barX* mutation that results from a 554 bp deletion in the middle of *barX* through homologous recombination by an unknown mechanism. Because the *barX* mutation would generate an aberrant BarX protein still containing a part of the A-factor biosynthesis repeat at the N terminus, which could have been the reason for the observed phenotypic changes, we here attempted to gain further insight into the function of BarX by constructing a *barX*-null mutant (Supplementary Fig. S1). The wild-type strain started producing virginiamycin after 14 h of cultivation, whereas the *barX*-null mutant IC160 was unable to produce any biologically active substance throughout cultivation (Fig. 1a), demonstrating that BarX is necessary to trigger virginiamycin biosynthesis. Complementation of the *barX*-null mutant IC160 was carried out using *barX* fragments with upstream regions of different lengths (Fig. 1b). Introduction of *barX* with the 572 bp upstream region efficiently restored virginiamycin production in strain IC165, although the shorter upstream regions did not give full complementation in strains IC163 and IC164. The wild-type strain produced 496 nM VB in the culture broth, but no VB activity was detected in the culture broth of mutant IC160 (Fig. 1c). These observations suggested that BarX plays a positive role in the biosynthesis of VB that is necessary for eliciting virginiamycin production.

Because transcription proceeds in a growth-dependent manner (Pulsawat *et al.*, 2009), the *barX* gene was expressed by inserting the *barX* gene under the control of the constitutive *ermEp** promoter (Fig. 2a). Strain IC166 began to produce both virginiamycin and VB 2 h earlier than either strain IC165 or the wild-type (Fig. 2b, c). In strain IC168, transcription of *barX* and *barS1* is constant during cultivation, while that of *barS2* (Lee *et al.*, 2008) increases in a growth-dependent manner (Supplementary Fig. S2), suggesting that an enzymic reaction by BarS2 might be the plausible rate-limiting step in VB biosynthesis.

Addition of VB restores the deficiency of virginiamycin production

To determine whether the deficiency of virginiamycin production in the *barX*-null mutant was due to a lack of VB production, chemically synthesized VB-C₆ was added to the culture medium after 8 h of cultivation of mutant IC160. Virginiamycin production after 10 h of cultivation

Fig. 1. Virginiamycin and VB production in different strains. (a) Virginiamycin production in the wild-type and the *barX* mutant (IC160) strains by bioassay with *Bacillus subtilis* PCI219. (b) Virginiamycin production in derivative strains. Virginiamycin in the culture broth was detected as described by Lee *et al.* (2008). $\blacklozenge$, Wild-type strain; $\blacktriangle$, mutant IC160; $\circ$, mutant IC163; $\diamond$, mutant IC164; $\triangle$, mutant IC165. (c) VB production after 24 h of cultivation in derivative strains. The amount of VB in culture broth was estimated by measuring the VB-dependent production of virginiamycin essentially as described by Nihira *et al.* (1988).

was observed in the culture filtrate at a level equivalent to that of the wild-type strain (Fig. 3a), suggesting that the VB-dependent signalling mechanism for virginiamycin biosynthesis was not impaired in the *barX*-null mutant. These results indicated that the lack of VB production in cells with the *barX*-null mutation was the sole reason for the lack of virginiamycin production, implying that BarX plays the main role in the catalytic pathway of VB biosynthesis.

Fig. 2. Effect of the *ermEp**-driven *barX* on virginiamycin production. (a) Map of the fragments used in this experiment. The numbers indicate the distance in nucleotides from the plausible start codon of *barX*. (b) Virginiamycin production determined by bioassay in strains derived from IC160. (c) VB production in the IC165 and IC168 strains. Mid-grey and dark-grey bars represent the concentration of VB in cultures of the IC165 and IC168 strains, respectively.

afsA is able to substitute for the function of *barX*

To test whether *afsA* acts as a functional substitute for *barX* in the biosynthesis of VB by *S. virginiae*, the *afsA* gene was expressed under the control of *ermEp** in the *barX*-null mutant. Strain IC170, in which heterologous expression of *afsA* was successfully achieved (Fig. 3b), produced 248 nM VB at 24 h of cultivation (Fig. 1c), and 75 % less virginiamycin (Fig. 3c) than was produced by strain IC168, in which the intact *barX* gene was overexpressed in the *barX*-null mutant. These results demonstrated that

Fig. 3. Effect of external VB addition (a) and heterologous *afsA* expression (b, c) on virginiamycin production. (a) Virginiamycin in each culture supernatant was detected by bioassay. (+VB), VB-C₆ dissolved in methanol was added to a final concentration of 280 nM after 8 h of cultivation, and further cultivation was carried out; (–VB), the same volume of methanol was added at 8 h of cultivation. (b) Transcriptional analysis of the introduced *afsA* gene by semiquantitative RT-PCR. *afsA* expression was examined in the *barX* mutant harbouring pLT270 (strain IC170). (c) Strain IC170 was cultivated for detection of virginiamycin production by bioassay.

AfsA can functionally complement BarX, and strongly suggest that BarX is a catalytic enzyme for VB biosynthesis in *S. virginiae*.

DISCUSSION

We have previously shown that a *barX* deletion mutant that was generated by an unknown mechanism loses the ability to produce both virginiamycin and VB (Kawachi *et al.*, 2000). Because the lack of virginiamycin production was not restored by exogenous addition of VB, we could rule out the possibility that *barX* is enzymically involved in VB biosynthesis. However, in the present experiment, we verified that *barX* is involved in VB biosynthesis by reconstructing and analysing the *barX*-null mutant and the VB addition experiment. Our results, when considered together with recent reports of other AfsA-family proteins (Kato *et al.*, 2007; Hsiao *et al.*, 2007), suggest that BarX probably functions as an enzyme in VB biosynthesis, and may play other roles as well. In response to the presence of VB, virginiamycin production usually initiates 2 h later than VB production. Overexpression by *ermEp**-driven *barX* caused earlier production of virginiamycin, suggesting that VB production is successfully induced at an early stage by *barX* overexpression, which supports the idea that BarX has an enzymic activity to synthesize VB.

Regarding the VB biosynthetic pathway proposed by Sakuda *et al.* (1992), a β -keto ester precursor is formed by coupling between a DHA-type C₃ unit and β -ketoacyl-CoA. Recently, Kato *et al.* (2007) showed that AfsA catalyses condensation of a β -keto acid precursor and

dihydroxyacetone phosphate (DHAP), but DHA was not recognized as a substrate of AfsA. In the present experiment, AfsA was able to functionally substitute for *barX* in VB production. In the A-factor biosynthetic pathway, 8-methyl-3-oxononanoyl-DHAP ester is formed by AfsA. Therefore, it is probable that the β -keto ester phosphate is biosynthesized as a VB intermediate in the IC170 strain. However, we have previously shown that transformation of the β -keto ester phosphate into VB-A is less effective than that of the β -keto ester (Sakuda *et al.*, 1993). The lower production of VB in the *afsA*-introduced *barX* mutant might be explained by the low efficiency of the former conversion.

With respect to the VB biosynthetic pathway in *S. virginiae*, a possible route is via the β -keto ester derived from DHA and β -keto acid derivative(s), as proposed by Sakuda *et al.* (1992) and Kato *et al.* (2007) (Supplementary Fig. S3). The β -keto ester produced is converted into the butenolide skeleton by intramolecular aldol condensation, followed by successive dehydration and NAD-dependent reduction, leading to a C-1'-keto skeleton. BarS2 is presumably responsible for the reaction step(s) succeeding the aldol condensation to produce 1'-keto VB A–E. Finally, the keto group is reduced to a hydroxyl group with BarS1 to give the VB A–E molecules. Currently, there are two proposed pathways for γ -butyrolactone biosynthesis; one pathway utilizes a butenolide phosphate, and the other utilizes a butenolide. The former is the main pathway for the biosynthesis of A-factor, and also probably for SCB1, because a *barS2* homologue is not present on the genome of either *S. griseus* or *S. coelicolor*, although a *bprA* homologue (SCO6267) is found in the region next to

scbA and *scbR*. The latter pathway seems to be the major one for VB biosynthesis in *S. virginiae* and *Streptomyces antibioticus*. Further knowledge of the VB biosynthetic mechanism in *S. virginiae* will clarify why streptomycetes have two distinctive routes for γ -butyrolactone autoregulator biosynthesis.

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