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Clarification of new regulatory network for antibiotic production

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Research aims

Gram-positive bacteria of the genus *Streptomyces* are industrially important microorganisms, producing more than 70% of commercially important antibiotics. The production of these compounds is often regulated by diffusible signaling molecules called autoregulators. One well-studied family of autoregulators consists of the γ-butyrolactones, which are active at nanomolar concentrations and which elicit antibiotic production by modulating the DNA-binding activity of cognate receptor proteins, and hence have been referred to as bacterial hormones. Although 60% of *Streptomyces* strains may utilize γ-butyrolactone autoregulators, little is known about signaling molecules used by other members of this genus.

Streptomyces avermitilis produces a family of polyketide macrocyclic lactones known as avermectins, which have potent antiparasitic and broad-spectrum activities against nematodes and arthropod parasites, together with two other families of polyketide antibiotics, filipins and oligomycins. While the biosynthetic pathways of the three antibiotics have been predicted and partially characterized, the regulatory mechanism of antibiotics production in *S. avermitilis* has so far remained unclear. Moreover, nothing is known about the autoregulator itself that might control antibiotic production in *S. avermitilis*.

In this study, I purified a signaling molecule from *Streptomyces avermitilis*, and determined its structure, including stereochemistry. In addition, I investigated the functional role of *avaR3*, one of the clustered-receptor genes, to know its involvement in antibiotic production in *S. avermitilis*.

Methods

Two thousands liters of wild-type culture broth were prepared to isolate the ligand (avenolide) of AvaR1, which was expressed in *E. coli* and purified as a single protein. Two bioassays were used to detect avenolide activity: a DNA dissociation activity on the rAvaR1-DNA complex in the gel-shift experiment, and an avermectin inducing activity with *S. avermitilis aco* mutant, which lacks ability to produce both avenolide and avermectin. NMR (^{1}H: 500 MHz, ^{13}C: 125 MHz) spectra were obtained on JEOL JNM-ECP 500 FT NMR SYSTEM. The wild-type *avaR3* gene was replaced with the disrupted allele (Δ*avaR3*) by homologous recombination for construction of the Δ*avaR3* strain. The amounts of each antibiotic were quantified with HPLC system using authentic standards.

Results

1. Identification of avenolide, an *S. avermitilis* autoregulator belonging to a new class of *Streptomyces* hormone[1)]

Genes involved in autoregulator biosynthesis and those encoding autoregulator receptor proteins are frequently clustered at a same locus in various *Streptomyces* species. The genes *sav3702-3706* of *S. avermitilis* include three genes (*avaR1*, *avaR2*, and *avaR3*) encoding homologues of γ-butyrolactone-autoregulator receptor proteins and the *aco* gene that encode an acyl-CoA oxidase. To determine whether the *aco* gene is important in antibiotic production, an *aco* gene knockout mutant was evaluated for antibiotic production. The level of avermectin production in the *aco*

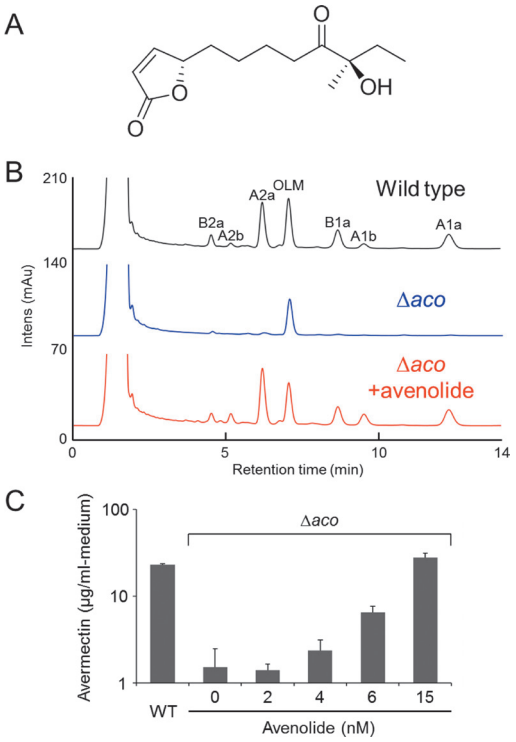

Fig. 1 (A) A chemical structure of avenolide. (B) Effect of avenolide on avermectin production. (C) Avermectin production with increasing concentrations of avenolide.

mutant was markedly reduced to about 6% of the wild-type level. The defect of the mutant in the avermectin production was restored completely by the addition of an ethyl acetate (AcOEt)-extract of the wild-type culture broth. Interestingly, an AcOEt-extract of the *aco* mutant was unable to induce avermectin production, which suggests that signaling molecule(s) eliciting avermectin production is present in the wild-type strain, but not in the *aco* mutant. Furthermore, the AcOEt-extract of the wild-type culture clearly prevented AvaR1 from forming the AvaR1-DNA complex, whereas the AcOEt-extract of the *aco* mutant showed almost no activity. These results implied that the factor inhibiting the DNA-binding activity of AvaR1 seems to be synthesized by enzymatic function of Aco and the factor is responsible for inducing avermectin production as a signaling molecule. Thus, I purified the factor from wild-type culture broth by several procedures including a silica gel column chromatography and HPLC with C_{18} reverse-phase column, and determined the structure of signaling molecule termed "avenolide", which triggered avermectin production at a minimum effective concentration of 4 nM (Fig. 1).

2. Functional analysis of AvaR3, an autoregulator-receptor homologue[2)]

avaR3, one of the clustered-receptor genes, encodes a protein containing an extra stretch of amino acid residues that has not been found in the family of γ-butyrolactone-autoregulator receptors. In order to investigate the *in vivo* role of *avaR3* in *S. avermitilis*, an *avaR3*-deletion mutant was constructed. In liquid cultivation, significantly decreased production of avermectins was observed in the *avaR3* mutant with delayed expression of avermectin biosynthetic genes, suggesting that AvaR3 positively controls the avermectin biosynthetic genes. Moreover, the disruption caused an increased production of filipin without any changes in the transcriptional profile of the filipin biosynthetic genes, suggesting that filipin production is indirectly controlled by AvaR3. AvaR3 is also involved in both antibiotic productions on solid medium. Furthermore, the *avaR3* mutant showed more fragmented mycelia with a remarkable decrease in the mean diameter of the pellet, indicating that AvaR3 plays an important role in the mycelial aggregation in liquid cultivation.

Conclusion

In this study, I identified avenolide, a novel signaling molecule controlling avermectin production in *S. avermitilis*, and showed functional roles of the *avaR3* gene encoding an autoregulator-receptor homologue in the secondary metabolism of this bacterium. The identification of avenolide as a new *Streptomyces* autoregulator should allow the construction of high level producers of antibiotics, including avermectin, as well as facilitating genetic understanding of regulatory mechanism for antibiotic production. On the other hand, the completed *Streptomyces* genome sequences have demonstrated that the putative autoregulator receptor genes are frequently located far from the antibiotic biosynthetic gene clusters on the genomes. Thus, it is expected that further understanding of the regulatory mechanism by AvaR3, whose gene is located far away from the avermectin biosynthetic gene cluster, will lead to higher yields in antibiotic production.

The results obtained in this study suggest that we could discover clinically-useful natural compounds by activating the numerous silent natural product gene clusters prevalent in actinomycete genomes.

References

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2) Miyamoto, K. T., Kitani, S., Komatsu, M., Ikeda, H., and Nihira, T. (2011) The autoregulator receptor homologue AvaR3 plays a regulatory role in antibiotic production, mycelial aggregation and colony development of *Streptomyces avermitilis*. *Microbiology* **157**: 2266–2275.