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Engineering of avermectin biosynthetic genes to improve production of ivermectin in *Streptomyces avermitilis*

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ABSTRACT

Two new recombinants of avermectin polyketide synthases were constructed by domain and module swapping in *Streptomyces avermitilis* 73-12. However, only the strain, *S. avermitilis* OI-31, formed by domain substitution could produce ivermectin. Analysis of the ivermectin synthesized gene cluster showed that decreased amount of *aveC* transcripts was one of the factors causing low yield of ivermectin. Overexpression of *aveC* could improve ivermectin yield.

Avermectins, a group of antiparasitic macrolides produced by *Streptomyces avermitilis*, are composed of eight structurally related polyketide compounds (Fig. 1).[1] Among those compounds, avermectin B1a has the strongest antiparasitic activity. Ivermectins (Fig. 1) (22, 23-dihydroavermectin B1), which are derived from avermectin B1, show lesser toxic side effects than avermectin B1, and are used worldwide in livestock production and in health care of animals. They have also been applied in human medicine, particularly treatment of onchocerciasis and strongyloidiasis.[2] The previously established industrial process for producing ivermectin involves extracting a mixture of avermectins from fermentation broth of *S. avermitilis*, isolating avermectin B1 from the mixture, and chemically reducing the double bond between C^{22} and C^{23} of avermectin B1 using rhodium chloride as a special catalyst for region-specific hydrogenation.[3] This process is expensive, and causes heavy metal pollution. A preferred alternative is to produce genetically engineered strains of *S. avermitilis* which produce ivermectins directly.

The complete nucleotide sequence of the linear chromosome of *S. avermitilis* was published, and sequence analysis revealed the organization of the avermectin biosynthetic genes and their deduced functions.[4] The responsible avermectin synthase (AVES) was a typical modular polyketide synthase (PKS). The avermectin PKS were consisted of four giant multifunctional polypeptides (AVES1, AVES2, AVES3, and AVES4), which were encoded by four large open reading frames (*aveA1-aveA2* and *aveA3-aveA4*).[4b] The unsaturated bond of C^{22}–C^{23} is determined by a putative partially active dehydratase (DH) and ketoreductase (KR) in module 2 without an enoylreductase (ER). In order to directly synthesize ivermectins and avoid any chemical steps, several attempts have been made to replace the DH2 domain of AVES1 with completely active DH and ER domain from other modular PKSs. In a previous study, we replaced *aveDH2* with the DNA fragment encoding DH4-ER4 from erythromycin PKS. However, neither ivermectins nor avermectins were detected from the resulting mutants. Next, *aveDH2-KR2* was replaced by the DNA fragment encoding DH4-ER4-KR4 from pikromycin PKS. The mutants had the ability to produce ivermectins, but the yield was only 1–4 μg/ml.[5] When the DNA fragment encoding DH13-ER13-KR13 from rapamycin PKS was used to replace the domains of AveDH2-KR2, the estimated total amount of avermectins and 22, 23-dihydroavermectins produced in the mutant was decreased approximately eightfold compared to the yield of avermectins of wild-type control.[6] Therefore, the yield of ivermectins produced by hybrid PKSs needs to be improved.

Oligomycins, strongly toxic compounds that specifically inhibit the oxidative-phosphorylation reaction in mammalian cells, are another family of macrocyclic lactones synthesized by *S. avermitilis*.[7] The oligomycin biosynthetic gene cluster (*olm*) contains 18 ORFs spanning a distance of 104 kb. The *olm* gene was deleted from the chromosome of *S. avermitilis* in our previous study,[8] and the *aveD* which encodes C5 *O*-methyltransferase in avermectin biosynthetic gene cluster was also deleted.[9] The resulting mutant strain *S.*

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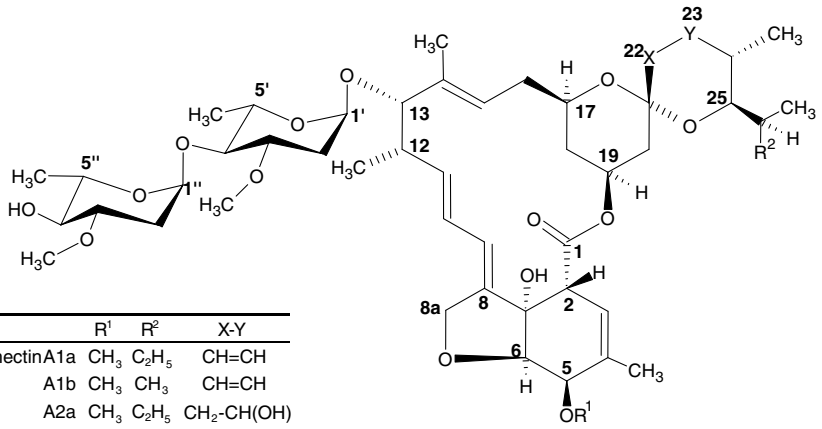


		R^1	R^2	X-Y
Avermectin	A1a	CH_3	C_2H_5	CH=CH
	A1b	CH_3	CH_3	CH=CH
	A2a	CH_3	C_2H_5	CH_2-CH(OH)
	A2b	CH_3	CH_3	CH_2-CH(OH)
	B1a	H	C_2H_5	CH=CH
	B1b	H	CH_3	CH=CH
	B2a	H	C_2H_5	CH_2-CH(OH)
	B2b	H	CH_3	CH_2-CH(OH)
Ivermectin	B1a	H	C_2H_5	CH_2-CH_2
	B1b	H	CH_3	CH_2-CH_2

Figure 1. Structures of avermectins and ivermectins.

avermitilis Olm73-12 produces only avermectins B, and no oligomycin. And it was reported that by employing naturally occurring subunits from different PKSs, the yield of a new compound can be improved over 100-fold.[10] The goal of the present study was to substitute AVES1 module 2 or DH2-KR2 domain (Scheme 1A) by module or domains in oligomycin synthase (OLMS), to increase the yield of ivermectins.

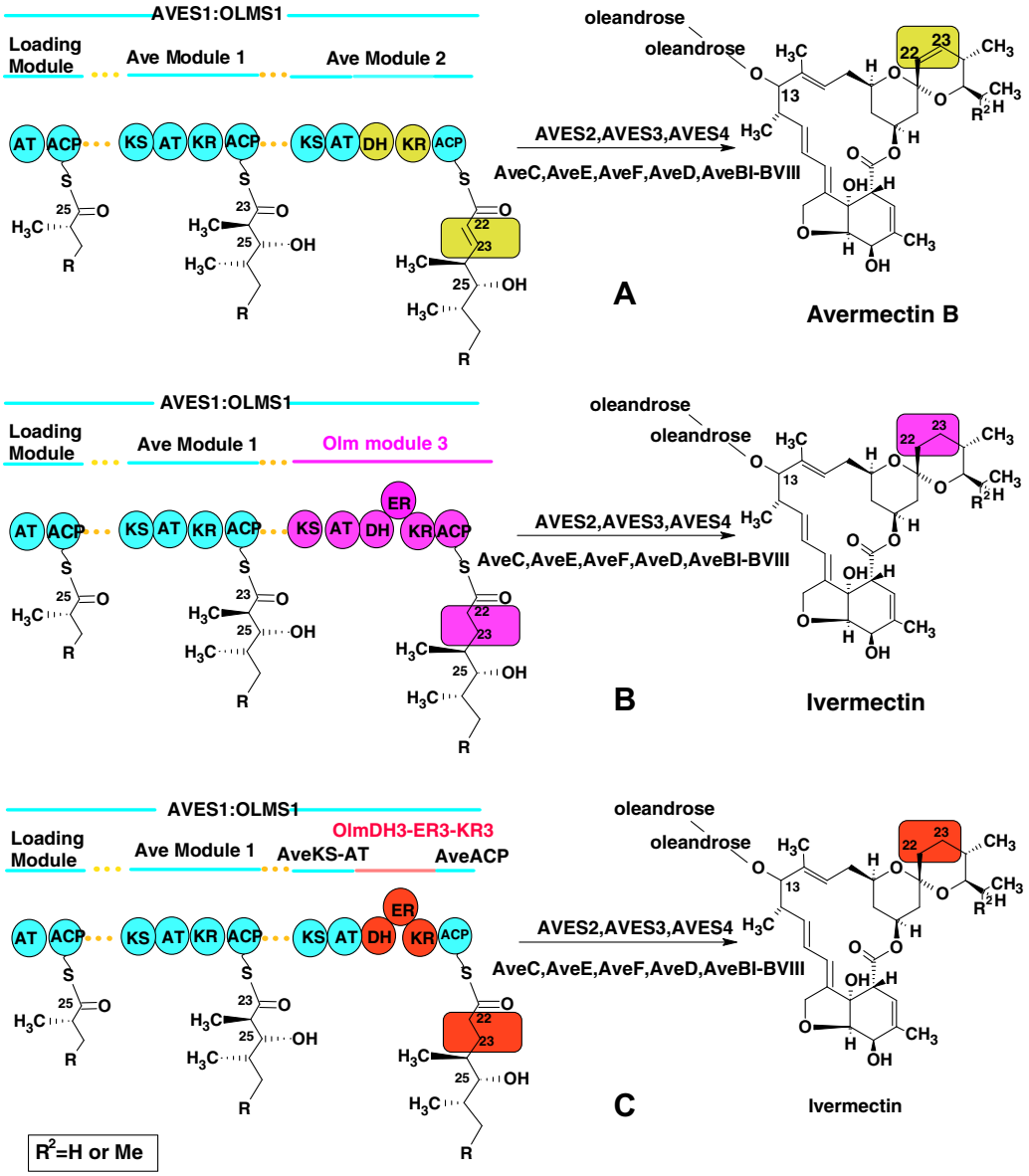
The OLMS was analyzed by the database SEARCHPKS (http://www.nii.res.in/pksdb.html),[11] and found to consist of 17 modules carrying 79 catalytic domains. Among these, modules 3, 7, and 9 contain DH-ER-KR domains. It was reported that relative substrate tolerance of diverse modules is essential for the rational design of hybrid multimodular PKS. Especially the substrate specificity of the KS domains is the critical determinant for generating functional hybrid PKS systems.[12] The native substrates of AVES module 2 contain sp^3 hybridized carbons (α-methyl-β-hydroxy) would only process sp^3 hybridized substrates. In these three OLMS modules, only module 3 contains sp^3 hybridized carbons. Native substrates of modules 7 and 9 contain sp^2 hybridized carbons (β-keto or α,β-unsaturated) may not process the sp^3 hybridized substrates of avermectin module 2. To improve the yield of ivermectins, we chose OLMS module 3 to substitute the AVES1 module 2, to generate hybrid PKSs that produce ivermectins (Scheme 1B). The putative substituted mutants were named OM-9. The double-crossover recombination event in the mutant was confirmed by PCR analysis. Yields of avermectins and ivermectins in Olm73-12 and OM-9 were determined by fermentation experiments and HPLC analysis. HPLC results showed that the module swapping strain produced no avermectins and ivermectins, indicating that substituting the AVES1 module 2 inactivated the avermectin PKS.

Next, OlmDH3-ER3-KR3 was used to substitute AveDH2-KR2, to construct a functional hybrid PKS with reduction of the double bond between C^{22} and C^{23} of avermectins B1 (Scheme 1C). The putative substitution mutant was named OI-31. The double-crossover recombination event in these mutants was confirmed by PCR analysis. Cultures of the substituted mutant OI-31 and the parental strain *S. avermitilis* Olm73-12 were extracted with methanol and the extracts were analyzed by RP-HPLC/UV. A new compound has the same retention time of the authentic sample 22, 23-dihydroavermectin B1a yield in OI-31, which was not detected in Olm73-12, as monitored by RP-HPLC with UV detection at 246 nm (Fig. 2).

Structure of the new compound was determined by LC/MS and NMR analyses. The mass of the compound was observed at *m*/*z* 897 ($[M+Na]^+$), which corresponds to the mass of 22, 23-dihydroavermectin B1a, and the mass fragmentation pattern was fully consistent with 22, 23-dihydroavermectin B1a. Results of ^1H and ^{13}C NMR analysis were also consistent with 22, 23-dihydroavermectin B1a. These findings confirmed that the new compound produced by OI-31 is ivermectin B1a. The ivermectin yield of OI-31 was 15.6 μg/ml, which was about fivefold higher than that of Ive12-4, an ivermectin-producing strain constructed by replacement of AveDH2-KR2 with DH4-ER4-KR4 domains from pikromycin PKS in our previous study.[5] To evaluate transcription of exogenous domain ER in the substitution mutant OI-31, total RNA was isolated from exponentially growing cultures. A 542-bp fragment inside *olmER3* was detected by RT-PCR. When primer pairs ER1/ER2 were used for PCR analysis of the mutant OI-31, a 0.5-kb band appeared, whereas no such band was detected when total RNA of Olm73-12 was used as template. Thus, *olmER3* could be transcribed in the substituted mutant.

Oligomycin is another secondary metabolite synthesized by type-I PKS of *S. avermitilis* besides avermectins. And the spatial relationships among domains within its modules may approximate those of avermectin. It may improve the ability of mutual recognition of modules in hybrid PKS and recognized to substrate. Our results indicate that domains from oligomycin PKS may be more suitable for swapping AveDH2-KR2 than domains from pikromycin PKS or erythromycin PKS. However, the yield of ivermectins is still too low for large-scale production and it still produces avermectin B, indicating that the β-processing steps at C^{22}–C^{23} are incomplete. The module-substituted mutants produce no avermectins or ivermectins. It is likely that module swapping disrupts interactions between protein modules in the assembly line.

Although genetic engineering of modular PKSs has been reported frequently, and many kinds of new polyketides have been produced, how to investigate the hybrid modules inter identified



Scheme 1. Predicted products of engineered avermectin PKS. (A) AVES module2. (B) AveDH2-KR2 was replaced with OLMS module3. (C) AveDH2-KR2 was replaced with OlmDH3-ER3-KR3.

and to be functional is still unknown. There are many possible reasons for low yield of the new metabolite, including decrease in transcription level or mRNA stability. For example, reduced yield of ivermectins and avermectins in substituted mutant strain OI-31 may result from low transcription level of hybrid PKS. To test this hypothesis, expression levels of several ORFs in the avermectin gene cluster were analyzed by semi-quantitative RT-PCR applied to total RNA obtained from *S. avermitilis* OI-31 after growth on SYFT for 48 and 72 h, and concurrently from parental *S. avermitilis* Olm73-12. Three genes were chosen for this analysis: *aveA1*, *aveA2*, and *aveC*. The PCR primers are specific to the sequence within each gene, and designed to produce PCR products ranging from 200 to 450 bp. After agarose gel electrophoresis, band intensities of PCR products were compared between Olm73-12 and OI-31. In OI-31, the amount of *aveC* transcripts decreased drastically, whereas those of *aveA1* and *aveA2* were almost unchanged (Fig. 3). Decreased expression of *aveC* affects the combined efficiency of avermectin and ivermectin biosynthesis. The *aveC* overexpression vector pMC2 was constructed and introduced into OI-31 in order to test whether overexpression of *aveC* could increase ivermectin production. The resulting transformants, OI-31C2, displayed a two-fold increase in ivermectin production, although the biomass of OI-31C2 was similar to that of OI-31 (Fig. 4). AveC is the product of an additional open reading frame of the avermectin biosynthetic cluster. Sequence analysis suggested that *aveC* may be transcriptionally coupled to *aveA2*. Random mutation of *aveC* leads to enhanced production of the hydrated avermectin series 2.[4b] And several mutations of *aveC* by site-specific mutagenesis, error-prone PCR[13a] and semi-synthetic DNA shuffling[13b] conduce to significantly improved ratios of B1:B2. However, the function of AveC is not obvious, because there is no homology between its deduced amino acid sequence and the active-site motif of the putative dehydratase. Our results suggest that *aveC* plays an important role in ivermectin biosynthesis. We are currently investigating the function of AveC in avermectin biosynthesis, and the relationship between AveC and AveDH2.

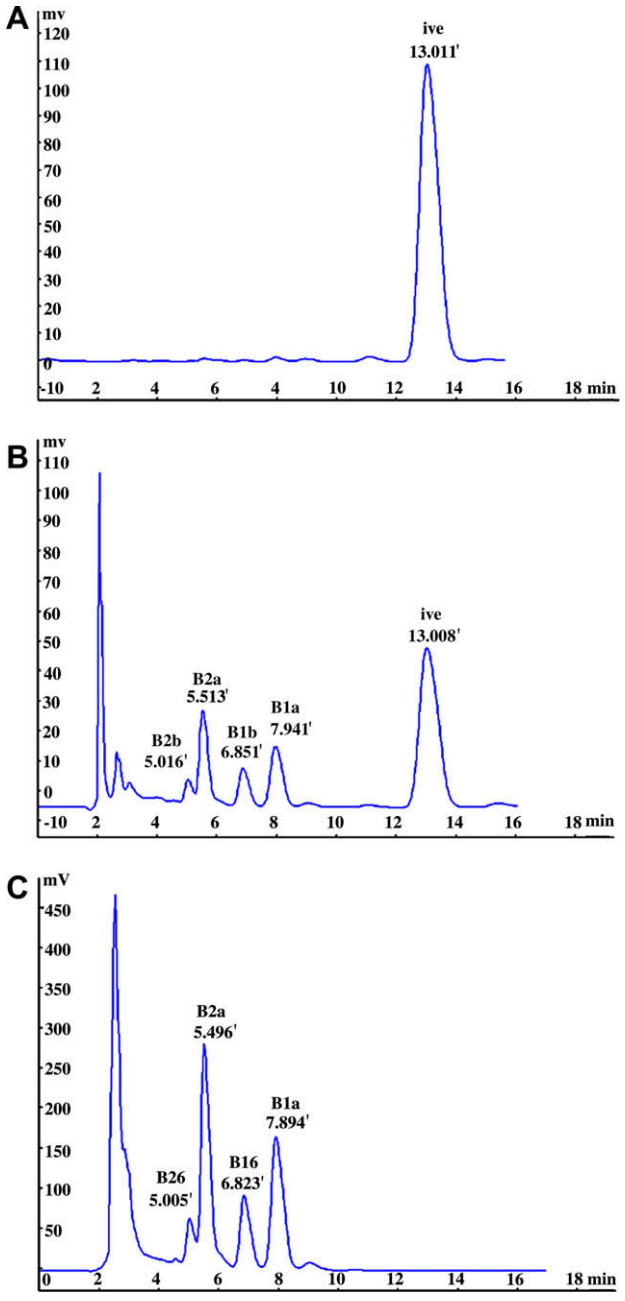


Figure 2. HPLC analysis of (A) pure 22, 23-dihydroavermectin B1a used as standard, (B) the mycelial extracts from the mutant OI-31, and (C) the parental strain *S. avermitilis* Olm73-12.

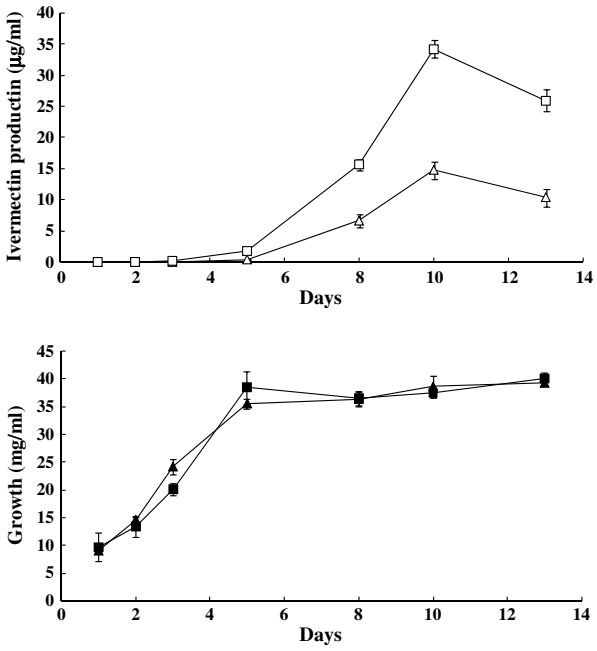


Figure 4. Growth and ivermectin production of *S. avermitilis* containing *aveC* on a multicopy plasmid. Growth curves: (■) transformants with pMC2; (▲) OI-31 with pKC1139. Ivermectin production: (□) transformants with pMC2; (Δ) OI-31 with pKC1139.

In this study we constructed the mutations which could produce ivermectin directly by domain substitution, although the parental strain could not produce ivermectin. However, the yields of ivermectins are much lower in OI-31 and OI-31C2 than the amount of avermectin B produced in the parental strain 73-12. There are presumably other reasons besides decreased *aveC* expression leading to low yield of ivermectins. The transcription level of mRNA of avermectin polyketide was not changed in the present study, implying that transcription level is not the factor causing low activity of ivermectin PKS. Poor folding of the hybrid PKSs, and unfavorable protein–protein interactions, may be responsible for this low activity. Function of individual domains with a preferred set of partner activities, and the fact that all domains have some degree of substrate specificity, are still problems in combinatorial biosynthesis. Spatial relationships among domains within the modules, that allow them to cooperate in a sequential fashion, remain to be studied in detail in order to improve the activity of hybrid PKSs.

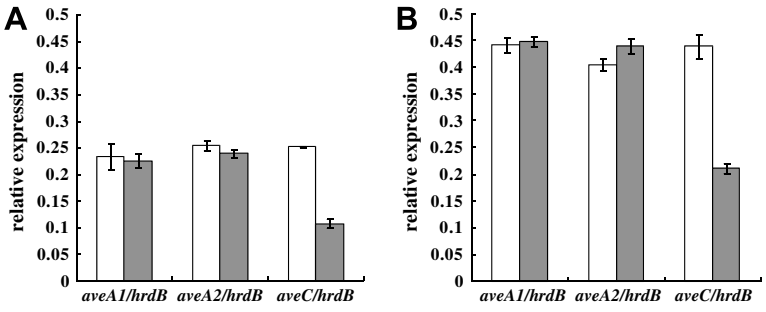


Figure 3. Semi-quantitative RT-PCR analysis for *aveA1*, *aveA2*, and *aveC* in *S. avermitilis* 73-12 (white) and OI-31 (gray), performed on total RNA isolated following two incubation periods: (A) 48 h (during exponential phase); (B) 72 h (early-stationary phase). Results are reported (mean ± SD) as relative expression of *aveA1*, *aveA2*, and *aveC* transcripts with respect to *hrdB* mRNA, at 48 and 72 h. In the two experiments with different time-points, the difference in relative *aveA1*, *aveA2*, and *aveC* mRNA levels may be attributed to difference in growth rates.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2008.09.061.

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