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Production of doramectin by rational engineering of the avermectin biosynthetic pathway

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ABSTRACT

In an attempt to construct a strain that produces doramectin, the loading module of Ave polyketide synthase (PKS) from *Streptomyces avermitilis* M1 was replaced with a cyclohexanecarboxylic (CHC) unique loading module from phoslactomycin PKS. Additionally, the CHC-CoA biosynthetic gene cassette was introduced into the engineered strain, which provided the precursor for directed biosynthesis of doramectin. The doramectin production ability of the final mutant *S. avermitilis* TG2002 was increased about six times and the ratio of Dor to Ave was enhanced 300 times more than the original strain.

Avermectins (Ave) are widely used as insecticidal and antiparasitic agents and are composed of a 16-membered macrocyclic lactone with a disaccharide of methylated deoxysugar L-oleandrose. They are produced by *Streptomyces avermitilis*, and include eight major active compounds, while the B1a component (Fig. 1A, **2**) has the most antiparasitic activity.[1,2] Ave biosynthesis consists of (i) condensation of short-chain fatty acids to form the polyketide-derived initial 6,8a-deoxy-5-oxoavermectin aglycons, (ii) modifications of the initial aglycons including oxidative cyclization, ketoreduction, and/or O-methylation at C5 to generate avermectin aglycons, and (iii) O-glycosylation of avermectin aglycons at C13 and C4′ using deoxythymidine diphosphate-L-oleandrose to form Ave.[3] The biosynthetic gene cluster for Ave was cloned and characterized, and the biosynthetic pathway was thoroughly elucidated.[4–6] Four polyketide synthases (PKSs) encoded by *aveA1*, *aveA2*, *aveA3*, and *aveA4* catalyze the biosynthesis of the polyketide backbone.

Doramectin (Dor, Fig. 1A, **1**), an Ave analogue and one of the most successful veterinary antiparasitic drugs, is produced by fermenting a mutant *S. avermitilis*, which lacks a branched chain-2-oxo acid dehydrogenase (bkd), in the presence of cyclohexanecarboxylic acid (CHC).[7] The differences in the chemical structure of Ave and Dor are caused by the different starter units in their biosynthetic pathways. In the Δ*bkd* mutant, the biosynthesis of isobutyryl-CoA or (*2S*)-*sec*-butyryl-CoA is disrupted, which abolishes the supply of the original substrates of the acyltransferase domain (AT) in the loading module for the biosynthesis of Ave. However, it has been demonstrated that this AT domain could accept numerous C2 branched carboxylic acids as alternative starter units to generate novel Ave analogues including Dor, C_{25}-cyclohexyl-Ave Bl, by precursor-directed biosynthesis.[8] It has been made great progress to improve the production of Dor in industrial scale by metabolic engineering to improve the ratio of Dor to its CHC-B2 analogue (Fig. 1, **1a**).[9,10] However, all of the previous attempts to produce Dor were based on the compatible AT, which could accept numerous C2 branched carboxylic acids as starter units. In other words, CHC-CoA is one of the various substrates of the native AT, but not the best one. Therefore, there is a potential to produce Dor or improve the production of Dor by enhancing the substrate specificities of AT. We developed a new strategy to produce Dor by genetically engineering the loading module of the Ave PKS to use CHC-CoA as a substrate.

Combinatorial biosynthesis has been successfully developed to generate new polyketides according to the module PKS assembly line.[11–13] A loading module swap is one of the most effective strategies to create hybrid PKS. The native loading module of the erythromycin PKS or spinosyn PKS, was replaced with the Ave PKS loading module, resulting in the production of novel erythromycin or spinosyn analogues.[14,15] Phoslactomycin B (PN, Fig. 1A, **3**) is one of the few polyketide natural products that uses CHC-CoA as the starter unit,[16,17] which means the loading domain prefers CHC-CoA to isobutyryl-CoA. If the native loading module of the Ave PKS is replaced with the loading module of PN PKS, it would be possible to change the starter unit of the resultant PKS from isobutyryl-CoA to CHC-CoA and to produce Dor without knocking out the *bkd* gene (Fig. 1).

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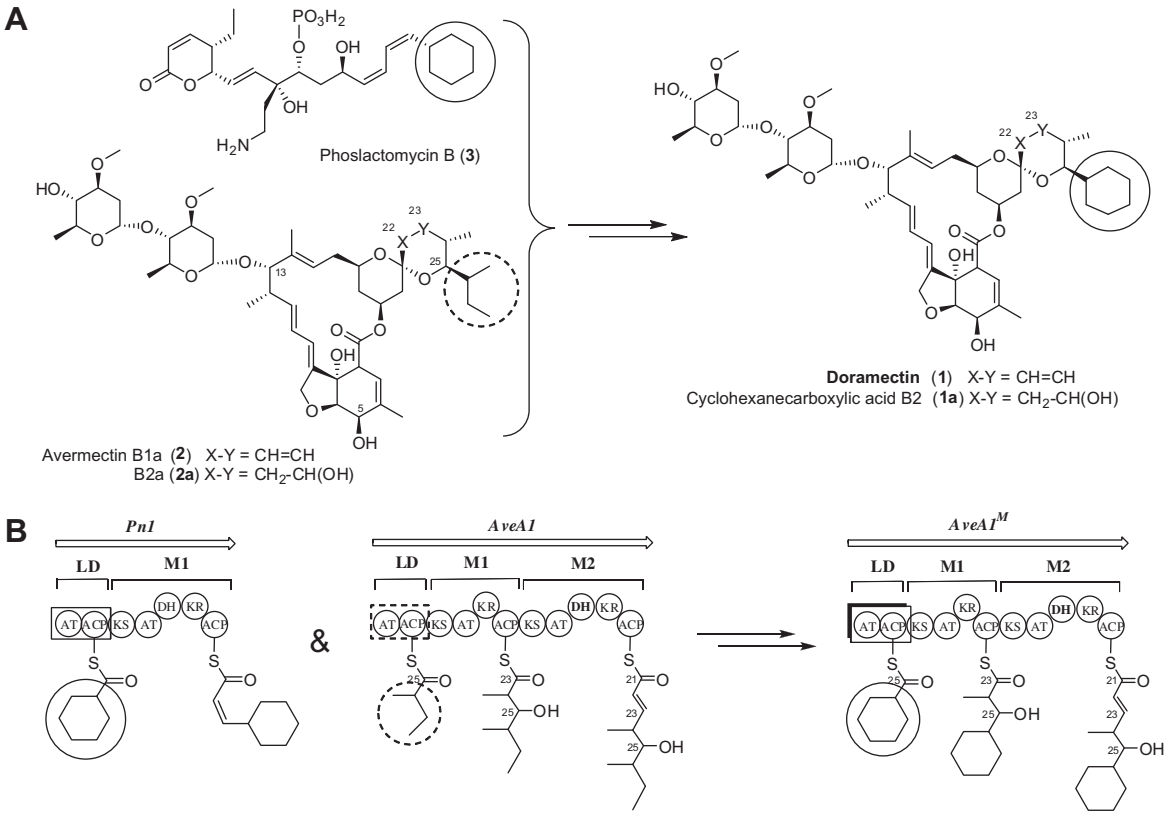

Figure 1. Engineering of the avermectin biosynthetic pathway to produce doramectins by polyketide synthase loading module swap.

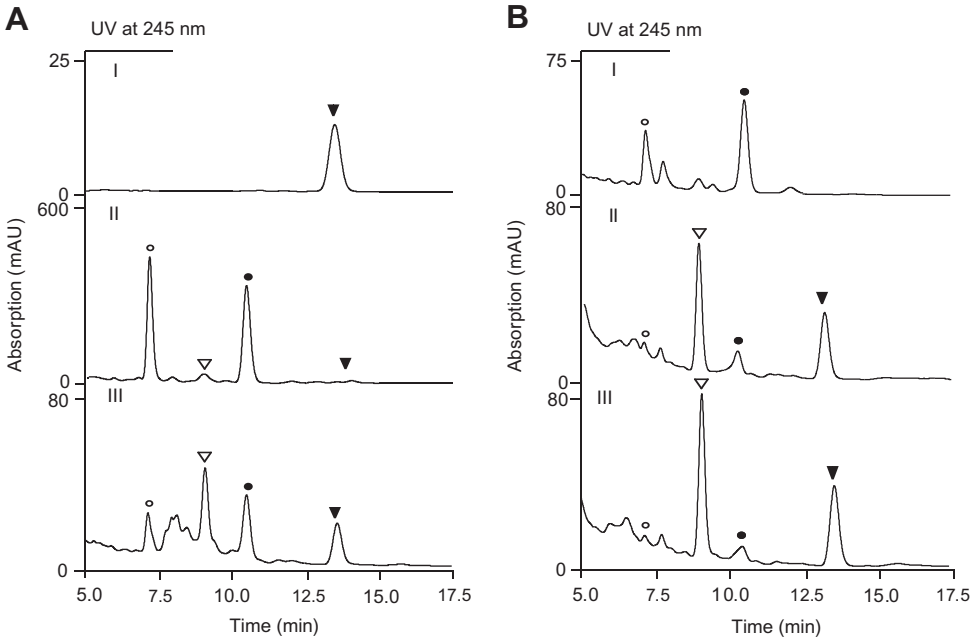

Figure 2. HPLC analysis of avermectins and doramectins production by *S. avermitilis* recombinant strains. Loading module swap strain (A): (I) Doramectin standard; (II) *S. avermitilis* M1 in the presence of cyclohexanecarboxylic acid (CHC); (III) mutant *S. avermitilis* TG2001 in the presence of CHC; and mutant strain with heterologous expression of CHC-CoA gene cassette (B): (I) mutant *S. avermitilis* TG2001 in the absence of CHC; (II) mutant *S. avermitilis* TG2002 (TG2001 harboring the expression plasmid pTG2002) in the absence of CHC; (III) mutant *S. avermitilis* TG2002 in the presence of CHC. (▼), doramectin (**1**); (▽), CHC-B2 (**1a**); (○), avermectin B2a (**2a**); (●), avermectin B1a (**2**).

According to loading module swapping stratagies,[14,15,18] we selected the AT-acyl carrier protein (ACP) didomain of the loading module as the exchange unit (Fig. 1B). *Xho*I and a *Bgl*II restriction enzyme sequences were introduced at the accessible linker region and the junction between ACPL and KS1, according to the homologous comparison among the AveA1 loading module, Pn1 loading module (Fig. S1, ESI) and the crystal structure of PKS.[19] To replace the aveATL-ACPL with pnATL-ACPL, a gene replacement plasmid

Table 1
Production of doramectin (**1**) and avermectin B1a (**2**) from recombinant *S. avermitilis* strains

Strain	Genotype	Media[a]	**1** (μg/mL)	**2** (μg/mL)	**1**:**2**
S. avermitilis M1	aveA1	FM + CHC	9 ± 1	500 ± 5	1:55
S. avermitilis TG2001	aveA1^{M}	FM + CHC	29 ± 1	40 ± 1	1:1.3
S. avermitilis TG2002	aveA1^{M} + pTG2002	FM	53 ± 1	20 ± 2	3:1
S. avermitilis TG2002	aveA1^{M} + pTG2002	FM + CHC	58 ± 2	9 ± 1	6:1

[a] Standard fermentation medium (FM) described in the ESI, no antibiotics were added.

pTG2001 was constructed, which contained a 2-kb external fragment of aveA1 upstream, an *Xho*I–*Bgl*II fragment of pnATL-ACPL, and a 2-kb DNA fragment of the aveATL-ACPL downstream region. This plasmid was introduced into *S. avermitilis* M1 (production of avermectin B1a: 500 μg/mL) by conjugation to select for the resulting double-crossover mutants *S. avermitilis* TG2001, and the genotype was confirmed by PCR analysis (Figs. S2 and S3, ESI).

Cultures of *S. avermitilis* TG2001 were grown in fermentation medium supplemented with exogenous CHC (200 mg/L), and the resulting productions were analyzed by HPLC and LC–MS. Just as expected, the production of Ave B1a and B2a decreased dramatically in the mutant strain, and about 30 mg/L Dor (**1**) and 50 mg/L CHC-B2 (**1a**) could be detected (Fig. 2A, Table 1). All compounds were further confirmed by LC–MS analysis and compared with a standard sample (Fig. S4). However, the production of Dor was still not as high as expected. One of the possible reasons might be the limited supplementation of CHC-CoA, however, higher concentration of CHC (>200 mg/L) would inhibit the growth of *S. avermitilis* strains. So, the production of Dor by mutant *S. avermitilis* TG2001 will possibly be further increased if we could enhance the supplementation of the precursor CHC-CoA in vivo.

The CHC-CoA biosynthetic pathway is not present in *S. avermitilis*, so no detectable amounts of Dor could be produced by *S. avermitilis* grown in the absence of exogenous CHC (Fig. 2B I). Recent studies revealed that five putative genes are responsible for the biosynthesis of CHC-CoA in the ansatrienin biosynthetic gene cluster from *S. collinus*, and this gene cassette could be used to produce Dor in the Δ*bkd* mutant strain without CHC supplementation.[20,21] Similar genes also exist in the biosynthetic cluster of PN.[16,17] We then cloned the biosynthetic gene cluster of CHC-CoA from *Streptomyces platensis* SAM-0654 (Fig. 3A), another PN producer,[22,23] and put it downstream of the constitutive *ermE** promoter in the *Escherichia coli–Streptomyces* shuttle vector pSET152 to obtain the heterologous expression plasmid pTG2002 (Fig. 3B). This resulting plasmid was transformed into the mutant strain *S. avermitilis* TG2001 by conjugation and apramycin-resistant exconjugants were selected as the final mutants *S. avermitilis* TG2002.

Fermentation of *S. avermilitis* TG2002 was carried out with or without the addition of CHC respectively. Productions analysis revealed that the yield of Dor was further increased to 50∼60 mg/L even in the absence of CHC (Fig. 2B II and Table 1). These results showed that the CHC-CoA gene cassette was functionally expressed and supplied sufficient CHC-CoA precursors for the biosynthesis of Dor. Additional supplementation of exogenous CHC (200 mg/L) did not increase the production of Dor, but inhibited the recruitment of isobutyryl-CoA or butyryl-CoA and enhanced the ratio of Dor to Ave (Fig. 2B III and Table 1). Compared with the original strain, the production ability of Dor was increased about six times, and the ratio of Dor to Ave was enhanced more than 300-folds.

In conclusion, we have successfully constructed a Dor producing strain by loading module replacement and heterologous expression of the CHC-CoA biosynthetic cassette. The loading AT domain of the engineered PKS selected the CHC-CoA as its substrate more specifically than the original Ave PKS, so the final mutant's ability to produce doramectin is enhanced. Combining this with the heterologous expression of the CHC-CoA gene cluster, the production of Dor could be further increased in the absence of exogenous CHC. Additional supplementation of CHC into the fermentation medium could further inhibit the production of Aves and enhance the ratio of Dors to Aves. However, compared to the amount of Ave B produced in the original strain M1, the yield of Dor was still not high enough for industrial applications. There are several possible reasons for this result. First, the introduction of the exogenous gene could affect the fold of PKS, broke the interaction of the domains leading to the decreased activity of the hybrid PKS and the low production of doramectin. Secondly, as we can see, the replacement of the loading domain did not disappear the production of Ave completely, so the replacement position we selected could be further optimized. Thirdly, the activity of the loading module, which we selected, may be not the highest, because the production of the phoslactomycin is very low. In order to improve the production of Dor, it is necessary to study in more details the relationships between domains within the modules, and after successful domain substitution, use directed evolution technology to improve the activity of the hybrid PKSs.

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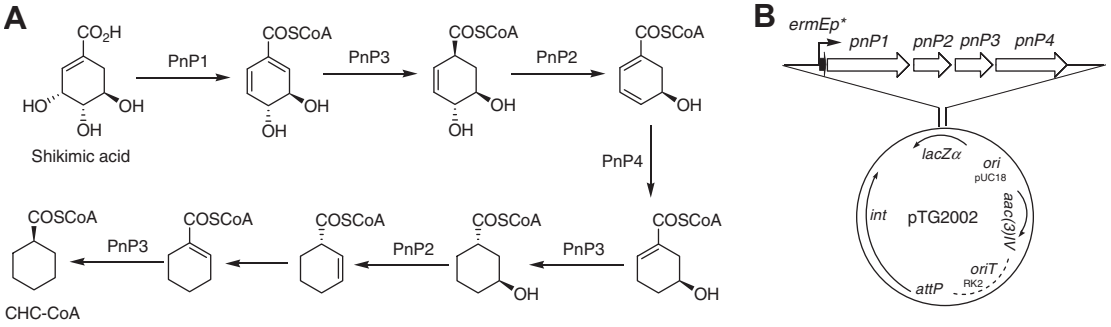

Figure 3. The biosynthetic pathway of CHC-CoA in *S. platensis* SAM-0654 (A) and the heterologous expression plasmid pTG2002 containing the CHC-CoA gene cassette (B).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2011.04.008.

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