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Alternative Production of Avermectin Components in *Streptomyces avermitilis* by Gene Replacement

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The avermectins are composed of eight compounds, which exhibit structural differences at three positions. A family of four closely-related major components, A1a, A2a, B1a and B2a, has been identified. Of these components, B1a exhibits the most potent antihelminthic activity. The coexistence of the "1" components and "2" components has been accounted for by the defective dehydratase of *ave*AI module 2, which appears to be responsible for C22-23 dehydration. Therefore, we have attempted to replace the dehydratase of *ave*AI module 2 with the functional dehydratase from the erythromycin *ery*AII module 4, via homologous recombination. Erythromycin polyketide synthetase should contain the sole dehydratase domain, thus generating a saturated chain at the C6-7 of erythromycin. We constructed replacement plasmids with PCR products, by using primers which had been derived from the sequences of avermectin *ave*AI and the erythromycin *ery*AII biosynthetic gene cluster. If the original dehydratase of *Streptomyces avermitilis* were exchanged with the corresponding erythromycin gene located on the replacement plasmid, it would be expected to result in the formation of precursors which contain alkene at C22-23, formed by the dehydratase of erythromycin module 4, and further processed by avermectin polyketide synthase. Consequently, the resulting recombinant strain JW3105, which harbors the dehydratase gene derived from erythromycin, was shown to produce only C22,23-unsaturated avermectin compounds. Our research indicates that the desired compound may be produced via polyketide gene replacement.

Key words: *Streptomyces avermitilis*, avermectin, polyketide, gene replacement, dehydratase

The avermectins (AVMs) are complex polyketides produced by *Streptomyces avermitilis*. The avermectins have been shown to manifest potent antiparasitic effects, and exhibit a broad spectrum of activity against the Nemathelminthes (nematodes) and arthropod parasites, although they appear to lack significant antibacterial and antifungal properties (Burg *et al.*, 1979; Campbell, 1982; Hotson, 1982; Needleman, 1986; Arena *et al.*, 1991).

The AVMs are pentacyclic, 16-membered macrocyclic lactones, which harbor a disaccharide of the methylated sugar, oleandrose. AVMs consist of eight compounds: four closely related primary components, A1a, A2a, B1a and B2a, and four minor components, A1b, A2b, B1b and B2b, which are homologous to their corresponding primary components, as is shown in Fig. 1. Among these compounds, the B1a compound exhibits the most potent anthelmintic activity (Egerton *et al.*, 1979; Ikeda and Omura, 1997).

The "A" components are believed to originate from the "B" components, via methylation at C-5, and group "1" is believed to originate from the precursor group "2", via dehydration at C-22 and C-23. The "a" components possess a 2-methylbutyryl group, derived from isoleucine, located at C-25 , whereas the "b" components possess an isobutyl group, derived from valine, at C-25 (Cane *et al.*, 1983; Omura *et al.*, 1991; Ikeda and Omura, 1997).

The dehydratase (DH) of *ave*AI module 2 is responsible for C22-23 dehydration (Ikeda *et al.*, 1999). This DH lacks the ordinary active site motif sequence (HXXXGXXXXP) (Bevitt *et al.*, 1992), rather containing a partially conserved DH consensus sequence with one mismatched amino acid in the active motif (HXXXGXXXXS). However, when the sequence of the ordinary active site motif was introduced by gene replacement into the corresponding region of the DH domain in module 2 on the chromosome, the resulting recombinants generated both the "1" and "2" components (Ikeda *et al.*, 1999). The fact that the DH of *ave*AI module 2, which is responsible for C22-23 dehydration, appears to exhibit partial activity might be attributable to other regions of the DH or downstream domain, which may act prematurely on the substrate in the subsequent acyl con-

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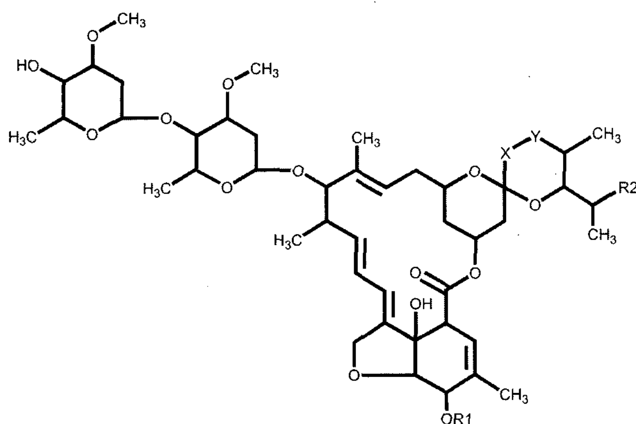

	R1	R2	X-Y
Avermectin A1a	CH_3	C_2H_5	CH=CH
A1b	CH_3	CH_3	CH=CH
A2a	CH_3	C_2H_5	CH_2-CH(OH)
A2b	CH_3	CH_3	CH_2-CH(OH)
B1a	H	C_2H_5	CH=CH
B1b	H	CH_3	CH=CH
B2a	H	C_2H_5	CH_2-CH(OH)
B2b	H	CH_3	CH_2-CH(OH)

Fig. 1. Structure of Avermectins.

densation reaction (Ikeda *et al.*, 1999).

On the other hand, the avermectin polyketide synthase (PKS) polypeptides bear a strong similarity to those of the erythromycin PKS (Ikeda and Omura, 1997). Module 4 of the erythromycin (Ery) PKS of *Saccharopolyspora erythraea* appears to contain the sole DH domain, and is fully activated to generate an unsaturated chain at C6-7 of Ery (Cortes *et al.*, 1990; Donadio *et al.*, 1990; Donadio *et al.*, 1993). If the gene which encodes for the C22,23-unsaturated polyketide in *S. avermitilis* could be exchanged with the corresponding gene in *S. erythraea*, the AVMs containing alkene at C22-23 would then be formed by the DH domain of *ery*AII module 4.

In this study, we have attempted to replace the DH domain of *ave*AI module 2 in *S. avermitilis* with the DH of Ery module 4 in *S. erythraea,* and then determined whether or not this engineered system could produce only C22, 23-dehydroavermectin compounds.

Materials and Methods

Bacterial strains

Streptomyces lividans 1326 was used as a standard cloned gene host. The AVM production strain, *S. avermitilis* ATCC 31272, was obtained from the ATCC (American Type Culture Collection, USA), and was used as the host strain for the only C22,23-unsaturated AVM producer. *Escherichia coli* DH5 and *E. coli* 12567 (*dam-13*::Tn9, *dcm-6, hsdM*) (MacNeil *et al.,* 1992) were used for DNA manipulation and demethylation, respectively. The transformation of *E. coli* was conducted via the $CaCl_2$ method, as described by Sambrook. We also developed the temperature-sensitive replicative *E. coli-Streptomyces* shuttle vector, pKC1139, in order to induce gene disruption. This vector can be replicated in both *E. coli* and *Streptomyces spp.*, but cannot be replicated in *Streptomyces spp.* at temperatures higher than 34°C (Bierman *et al.*, 1992). The temperature-sensitive replicative plasmid was used to mediate homologous recombination for the gene replacement.

Media for regeneration and cultivation

The morphological and physiological properties of the recombinant *Streptomyces* strain were examined on YM medium (3 g yeast extract, 3 g malt extract, 5 g peptone from soybeans, 10 g glucose, 15 g agar per liter). The isolation of recombinant spores was conducted as described by Hopwood (1985).

In order to transform the protoplasts, R2YE agar medium (103 g sucrose, 0.25 g K_2SO_4, 10.12 g $MgCl_2 \cdot 6H_2O$, 0.1 g Casamino acid (Difco, USA) 5 g yeast extract, 10 ml K_2HPO_4 (0.5%), 80 ml $CaCl_2 \cdot 2H_2O$ (3.68%), 15 ml L-proline (20%), 100 ml TES (5.73%, pH 7.2), 2 ml trace elements per liter) was used for the regeneration. *E. coli* DH5 and *E. coli* 12567 were grown in Luria-Bertani medium.

General genetic manipulation of DNA

Techniques for DNA manipulation, including plasmid DNA preparations, DNA restriction endonuclease digestions, ligation, transformation into *E. coli* spp., PCR, and gel electrophoresis, were all conducted according to the standard protocols (Hopwood *et al.*, 1985; Bierman *et al.*, 1992). The DNA fragments were purified from agarose gel using a Jetsorb kit (Genomed, USA). The plasmid DNA was isolated from *E. coli* using a QIAZEN kit (Qiagen, Germany). The transformation of *Streptomyces* spp. and the genomic DNA from *Streptomyces* spp. were isolated according to the method described by Hopwood (Hopwood *et al.*, 1985).

PCR

In order to construct the DH domain of the Ery-conferring replacement plasmid, we used the DH domain of *S. erythraea ery*AII module 4, as well as the acyltransferase (AT) and ketoreductase (KR) domains of *S. avermitilis ave*AII module 2. The AT and KR domains are located upstream and downstream of the DH, respectively. The region containing the open reading frame (ORF) of the AVMs AT domain was additionally connected with the partial DH domain, which encompasses 18 amino-acid residues which were amplified by PCR, using the following primers: primer 1, 5'-AAGCTTCTCGCCATCGCCGCCATCAAC-3' (underlined indicates a *Hind* III site) and primer 2, 5'-GTCGACGGCGCCGAGTAGGGGTGTTC-3' (under-

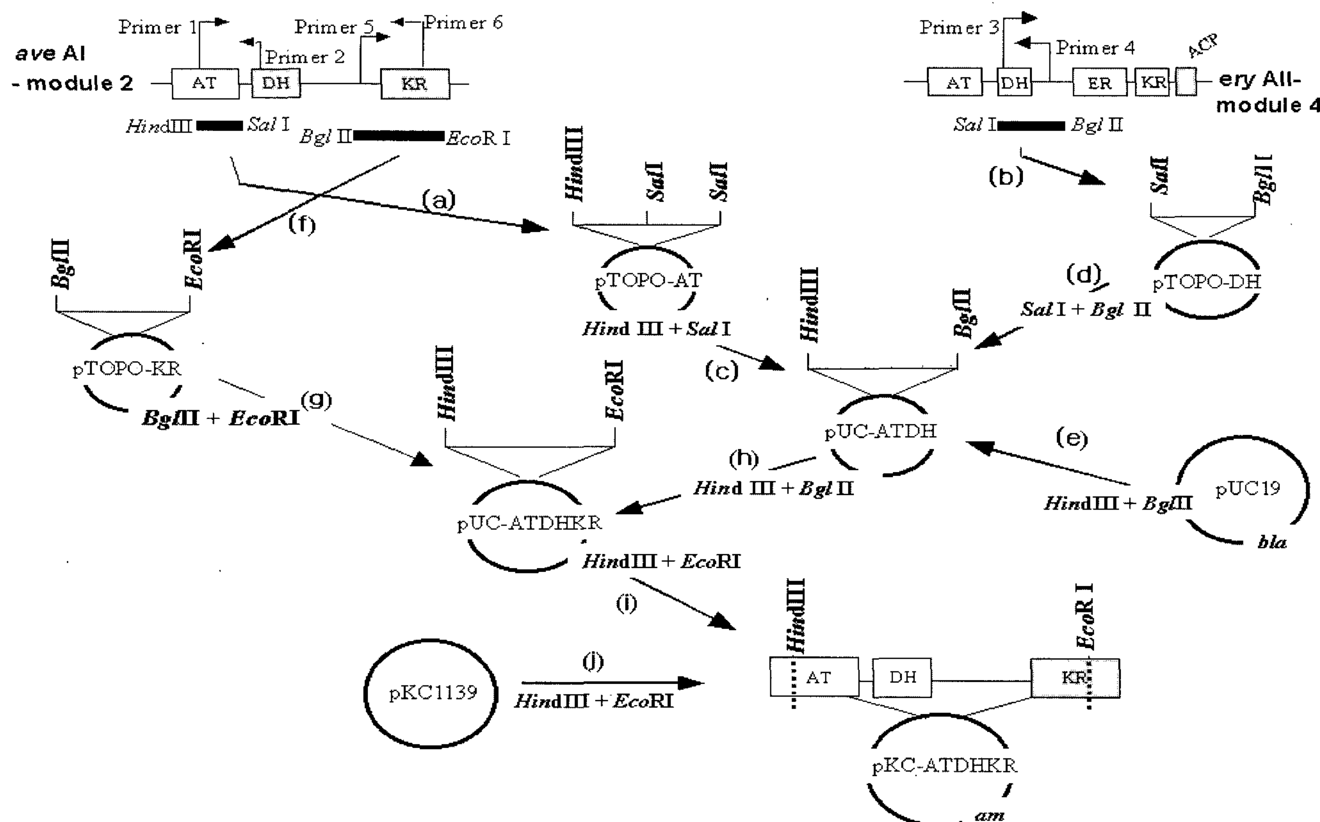

Fig. 2. Construction of DH replacement plasmid. The PCR products were cloned in *bla* (β-lactamase) conferring pCR-TopoII (Invitrogene, USA). (a), (b) and (f) demonstrate the TA cloning procedure using Tag polymerase and pCR-TopoII. (c), (d) and (e) demonstrate three-fragment ligation, and (g) and (h) demonstrate two-fragment ligation. (i) and (j) demonstrate the recombination of pUC-ATDHKR with the apramycin resistance (*am*^{r}) *E. coli-Streptomyces* shuttle vector, pKC1139. The resultant replacement plasmid was designated pKC-ATDHKR. AT, acyltransferase; DH, dehydratase; KR, ketoreductase.

lined indicates a *Sal* I site). In order to replace the DH domain, the erythromycin DH domain was amplified via PCR, using primer 3, 5'-GTCGACCTGCTGCTCGCCGC-GGTCGACGTG-3' (underlined indicates a *Sal* I site) and primer 4, 5'-AGATCTGACCGTGGCGACCAGGTGGCC-TGC-3' (underlined indicates a *Bgl*II site). The AVMs KR domain was amplified via PCR using primer 5, 5'-AGATCTCTGCGTGCGGTGTGGCGTGAT-3' (underlined indicates a *Bgl*II site) and primer 6, 5'-GAATTCTCCG-GATCGAGGTAGTCGGTG-3' (underlined indicates an *Eco*RI site). Each of the PCR products was cloned with pCR-TopoII (Invitrogen, USA), then amplified. Three PKS domains were subsequently connected to pKC1139, in the order AT-DH-KR (Fig. 2).

PCR was performed using Taq polymerase (TaKaRa, Japan) and MinicyclerTM (MJ Research, USA). Amplification was conducted with 25 cycles of denaturation (95°C for 60 sec), annealing (68°C for 60 sec), and elongation (72°C for 120 sec), and the amplified products were separated via agarose gel electrophoresis.

Gene replacement

We conducted a gene replacement experiment in order to develop a method for the generation of specific mutations in the chromosomes of *Streptomyces* spp. (Anzai *et al.*, 1988; Bierman *et al.*, 1992; Lomovskaya *et al.*, 1997).

Homologous recombination was conducted by exchanging the wild-type gene on the chromosome with the corresponding gene on a plasmid, which also elicited the disruption of the RNA polymerase sigma factor and the ppGpp synthetase genes (Butter *et al.*, 1990; Chakraburtty *et al.*, 1996). The results of this process were expected to include several genetic changes, including changes in beta-carbonyl processing, polyketide side chains, the starter side chain, and acyl chain length, due to either losses or gains of specific functions (Kartz, 1997).

The replacement plasmid, which contained the DH domain from Ery, was then transformed into *S. avermitilis* ATCC 31272.

The transformants which are resistant on apramycin medium (50 μg/ml) were selected and transferred to non-selective medium. Then, transferred transformants were incubated at 39°C to eliminate any autonomously replicating plasmids from the transformants (Bierman *et al.*, 1992). The apramycin-sensitive strain was screened as the candidate of the replacement strains and was cultured in

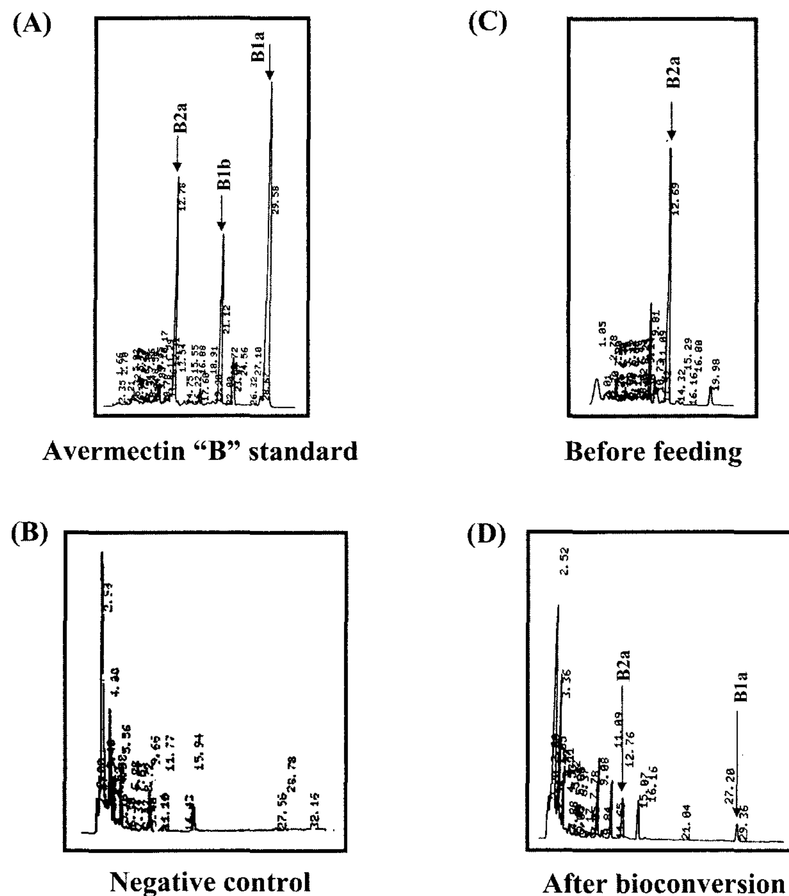

Fig. 3. HPLC profiles of bioconversion experiments. (A) Avermectin B compounds. (B) Only avermectin B2a component. (C) Metabolite of *Streptomyces lividans* 1326. (D) Metabolite of transformed *Streptomyces lividans*.

AVM production media. Genetic replacement was confirmed by Southern blot hybridization.

Biotransformation experiment

Streptomyces lividans 1326 was used as a standard host for the cloned gene. Biotransformation was conducted in 250 ml baffled flasks, with 20 ml of seed culture. Each flask was incubated at 28°C and 250 rpm on an orbital shaker. Biotransformation efficiency was determined by comparing the amounts of precursor that remained with the product transformed in the mycelia.

The mycelia of each of the cultures were harvested and extracted, using 10 ml of mixed solvent of chloroform: methanol (9:1). The extract was then dried by evaporation, and dissolved in 100 μl of methanol. The biotransformed product was separated via silica gel column chromatography, and confirmed by HPLC. We also conducted a control experiment with a culture of *S. lividans* 1326.

Secondary metabolite production and detection

The seed culturing of *S. avermitilis* was conducted in a primary culture medium (20 g glucose, 5 g yeast extract, 1.5 g soybean meal, 3 ml soybean oil, pH 7.0 per liter) at 28°C, with agitation at 200 rpm. After 48 h, the cell culture was diluted 10-fold with the secondary production medium (45 g glucose, 2.5 g yeast extract, 1.4 g soybean meal, 1 g NaCl, 5 ml soybean oil, pH 7.0 per liter) in order to elicit the production of metabolites. Culturing continued for an additional 5 days. The mycelia from the cultured suspension were then extracted with equal volumes of methanol, and the mycelia were extracted via filtration. The extract was then evaporated under reduced pressure, and the viscous residue was dissolved in 1 ml of chloroform. After centrifugation, the lower layer of chloroform was withdrawn, and dried by evaporation. The crude extract was then dissolved in 100 μl methanol, and spotted onto a thin layer chromatography (TLC) plate (60F-254, Merck, USA). A hexane-isopropanol (85:15) mixture was then used to develop the sample. The compounds were then detected under 254 nm UV light provided by a portable illuminator (UVGL58, UVP, USA). For the HPLC analysis, the dried residue was then eluted with a mobile phase consisting of acetonitrile-methanol-water (60:16:24), and developed in a YMC ODS-A column (250 × 4.6 mm, S-5 μm, YMC, Japan) at a flow rate

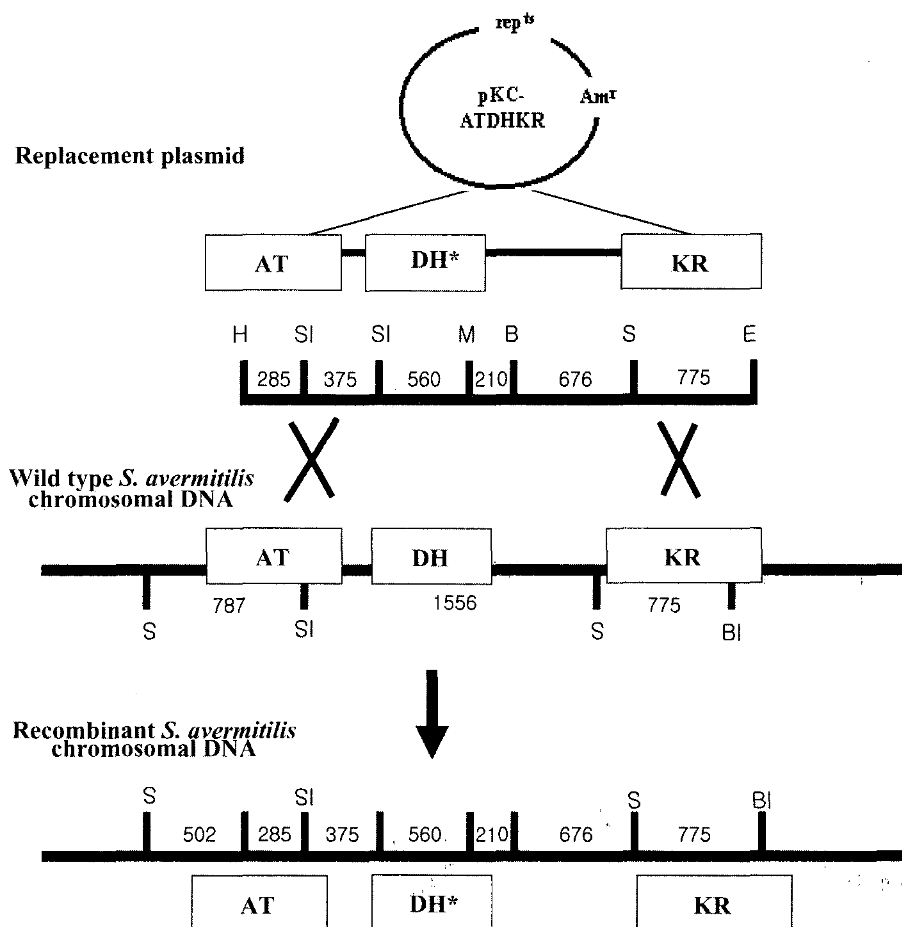

Fig. 4. Scheme for DH gene replacement by homologous recombination. DH, dehydratase gene of *Streptomyces avermitilis*; DH*, dehydratase gene of *Saccharopolyspora erythraea*; Am^r, apramycin resistance gene; Rep^{ts}, temperature sensitive replication origin; H, *Hin*dIII; SI, *Sal*I; M, *Mlu*I; B, *Bgl*II; S, *Sma*I; E, *Eco*RI.

of 1.25 ml/min, monitored at 245 nm, and compared with the standard compound, avermectin B1a.

Southern blot hybridization

The genomic DNAs from *S. avermitilis* ATCC 31272 and from the replacement strain, *S. avermitilis* JW3105, were isolated and digested with *Sma*I. The digested fragments were then electrophoretically separated on 1% agarose gel, and transferred to a nitrocellulose membrane with 10 × SSC (1.5 M NaCl, 0.15 M trisodium citrate). The biotin-labeled DH domain of Ery *ery*AII module 4 was employed as a probe. The hybridization and detection experiments were carried out according to the protocols described by the manufacturer (Boehringer Mannheim, Germany).

Results and Discussion

Construction of replacement plasmid for avermectin-producing dehydratase

As the two intermediates containing the β-hydroxyl or enoyl carbons at C22-23 were processed in subsequent acyl condensation reactions, the DH domain corresponding to C22-23 dehydration appeared to exhibit partial DH activity (Ikeda *et al.*, 1999). The Ery is comprised of pentacyclic macrocyclic lactones, which are related structurally to the avermectins. In addition, the AVMs PKS proteins are quite similar to those of Ery (MacNeil *et al.*, 1992; Ikeda and Omura, 1997).

In order to ensure only the generation of C22, 23-unsaturated AVMs, we constructed a replacement plasmid for the defective DH, which corresponded to C22-23 dehydration, as is shown in Fig. 2. The construction of the replacement plasmid took place in three steps, as follows.

Initially, we amplified the DH domain of *ery*AII module 4 via PCR, then inserted it into pCR-TopoII (Invitrogene, USA), which harbors a *bla* (β-lactamase) gene (Fig. 2b). The acyltransferase (AT) of pTopo-AT was then derived from the avermectin *ave*AI module 2 (Fig. 2a). The 3' end of the AT domain in *ave*AI module 2 was subsequently connected to the DH domain of *ery*AII module 4. For the in-frame connection of ORF, we artificially inserted the *Sal*I restriction enzyme recognition site into the 5' end of oligonucleotide primer 2, even though a pre-existing *Sal*I restriction enzyme recognition site was located in the cen-

ter of the AT domain. The partial digestion of the PCR product, which contained the AT domain, resulted in the formation of two *Sal*I fragments, the sizes of which were 285 bp and 375 bp, via *Sal*I restriction enzyme digestion.

We then obtained a 660 bp fragment from the *Hin*dIII-*Sal*I partial digestion of pTopo-AT. This 660 bp fragment was connected to the 770 bp *Sal*I-*Bgl*II fragment, which was in turn derived from pTopo-DH (Fig. 2c and d). Then, the 1430 bp *Hin*dIII-*Bgl*II fragment was inserted into the pUC19 via *Hin*dIII-*Bgl*II double digestion, and designated pUC-ATDH.

pTopo-KR harbors the *Bgl*II-*Eco*RI fragment of the KR of avermectin *ave*AI module 2 (Fig. 2f). The *Bgl*II site of the amplified KR was then artificially inserted into oligonucleotide primer 5, for convenient cloning. The 1451 bp small fragment generated by the *Bgl*II and *Eco*RI double digestion of pTopo-KR was isolated and joined with pUC-ATDH, and designated pUC-ATDHKR. Then, the *Hin*dIII-*Eco*RI fragment of this plasmid was recombined with the *E. coli-Streptomyces* shuttle vector, pKC1139, which harbored the apramycin resistance gene, and the resulting recombinant-plasmid was designated pKC-ATDHKR.

Many *Streptomyces* spp. harbor a potent methyl-specific restriction, which can present an effective barrier to the introduction of heterologous DNA. In order, then, to prepare the demethylated replacement plasmid, we used *E. coli* ET12567 (MacNeil, 1988) as the modification host.

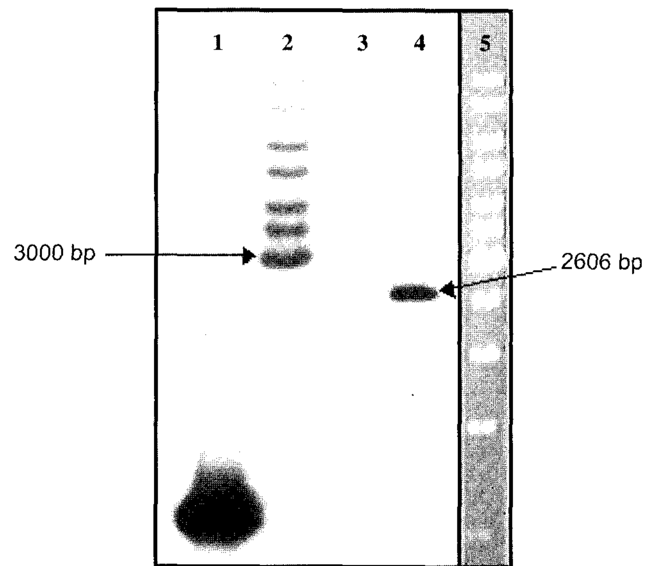

Fig. 5. Southern blot hybridization analysis of the recombinant JW 3105 genomic DNA and replacement plasmid pKC-ATDHKR. Lane 1: probe (biotin-labeled PCR product of DH (dehydratase) in *eryA*II module 4, which was amplified using primers 3 and 4). Lane 2: The 1 kb DNA ladder produced by MBI Fermentas Co. (The thickest band is 3,000 bp and the lower one is 2,500 bp.) Lane 3: The *Sma*I digested genomic DNA of the parent strain *S. avermitilis* ATCC 31272. Lane 4: *Sma*I digested genomic DNA of the recombinant strain JW3105. Lane 5: Et-Br stained 1 kb DNA ladder (MBI Fermentas Co.).

Avermectin biotransformation by replacement plasmid

In order to confirm the function of the pKC-ATDHKR replacement plasmid, we tested the biotransformation of AVMs B2a to B1a. This biotransformation was conducted using the transformant strain of this plasmid in *S. lividans* 1326. The transformant strain was initially cultivated at 30°C, with agitation at 220 rpm, for 3 days in R2YE medium which had been supplemented with apramycin (50 μg/ml). At this time, avermectin B2a was added to a final concentration of 10 μg/ml, and the sample was incubated for an additional 3 days.

After 3 days, the culture was extracted with a chloroform-methanol (9:1) mixture, and the mycelia were extracted via filtration. After the extract was dried under reduced pressure, the residue was dissolved in methanol. We then conducted an HPLC analysis, as was described in the Materials and Methods section.

Fig. 3 shows an HPLC analysis pattern of avermectin B (A) and avermectin B2a (B) prior to biotransformation. Fig. 3C and D are HPLC analysis patterns of the avermectin- related polyketides derived from *S. lividans* 1326, and the polyketides generated by biotransformation in the *S. lividans* 1326 transformant, respectively.

In the *S. lividans* which harbored the *ery*AII DH, the avermectin B2a concentration was decreased and B1a was newly produced instead, albeit in very small quantities. This result indicated that the replacement plasmids possessed dehydration activities, and suggested that the biosynthetic machinery exhibited by both avermectin and Ery would be complementary to one another (Fig. 3).

Gene replacement of the Erythromycin polyketide-derived dehydratase domain

As shown in Fig. 4, due to the temperature-sensitive replicative plasmid-mediated homologous recombination, a genetic exchange occurred between identical copies of the sequence (Hopwood, 1997; Xue *et al.*, 1998). After treatment of the replacement plasmid-harboring transformants at a non-permissive temperature (39°C) and under non-selective antibiotic pressure, we detected a strain which generated only C22,23-unsaturated avermectins.

As shown in the Southern blot hybridization profile in Fig. 5, the DH domain of the JW3105 replacement strain harbors the DH domain which was derived from *S. erythraea*. The replacement plasmid contained 659 bp of AVMs AT, 770 bp of Ery DH, and 1451 bp of AVMs KR. The AVM KR contained only a single *Sma*I site, and was divided into 676 bp and 775 bp fragments as the result of *Sma*I digestion. The smaller fragment was then connected to the DH domain derived from Ery in the replacement plasmid. Another *Sma*I site was determined to be located upstream of the AVM AT domain (Fig. 4). However, the genomic DNA from the replacement strain JW3105 was expected to exhibit a 2,606 bp *Sma*I fragment, and we

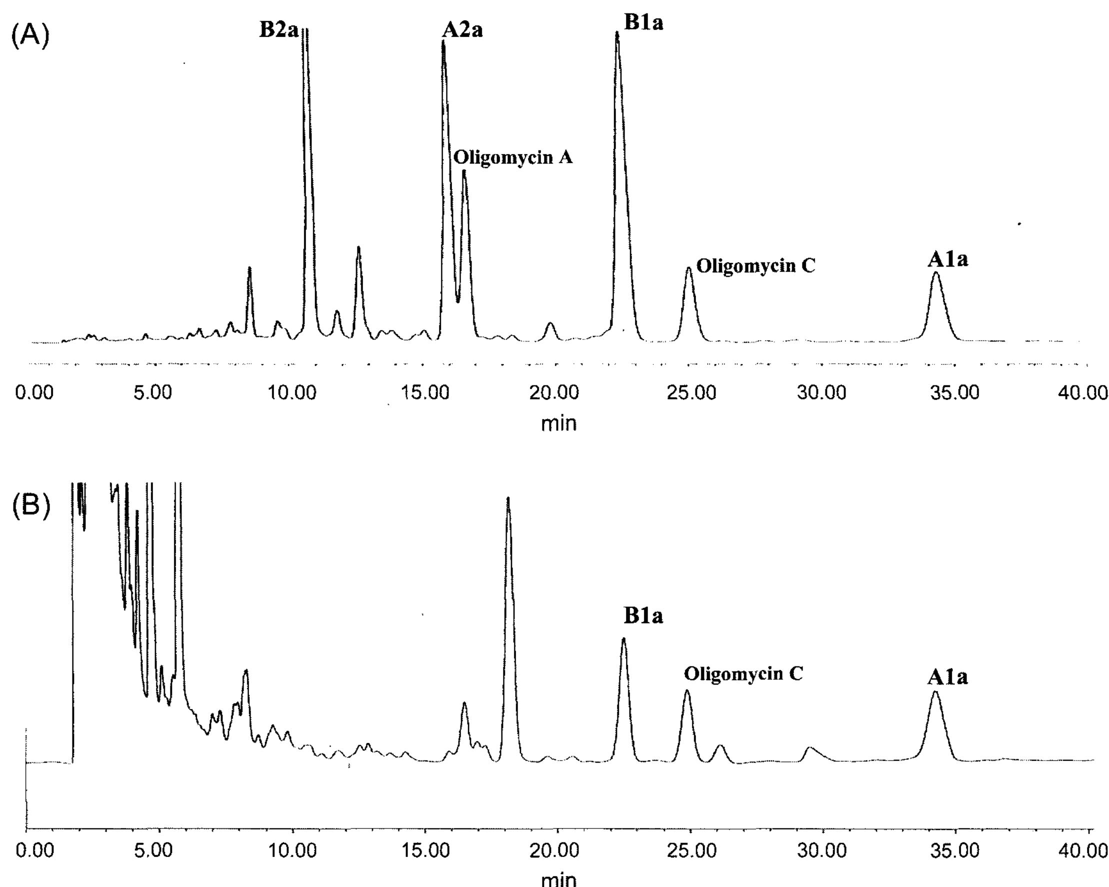

Fig. 6. Analytical HPLC of mycelium extract from the ATCC 31272 parent strain (A) and the JW 3105 replacement strain (B). Culture condition and preparation of the mycelia extract were accomplished as previously stated (see Materials and Methods). The dried extracts were eluted with a mobile phase of acetonitrile-methanol-water (60:16:24), and developed in a YMC ODS-A column (250 × 4.6 mm, S-5 μm) with a flow rate of 1.25 ml/min, which was monitored at 245 nm.

confirmed that this was the case, using the biotin-labeled PCR product of Ery DH, which was amplified using primers 3 and 4. The thickest band observed was 3,000 bp, and the lower band was 2,500 bp, in the DNA ladder of the Southern blot hybridization profile. We used the Southern hybridization to demonstrate that the desired gene replacement had, in fact, occurred (Fig. 5).

Production of only C22, 23-unsaturated avermectin compounds

The AVM-producing pattern of the wild-type strain was verified via HPLC, as is shown in Fig. 6A. The wild-type strain of *S. avermitilis* generates not only avermectins, but also polyketide-derived oligomycin compounds. Oligomycins consist of five compounds, exhibiting structural differences at four positions. Among the five oligomycin components (A, B, C, D, and E), the AVM-producing *S. avermitilis* strains tend to primarily generate oligomycin A. In addition, the avermectin-producing *S. avermitilis* generates polyketide-derived polyene compounds (Ikeda and Omura, 1997).

Although production levels were reduced as compared with the parent strain, the JW3105 replacement strain produced two major avermectin compounds, A1a and B1a. However, these C22,23-saturated avermectin compounds were not experimentally confirmed (Fig. 6B). As the result of our HPLC analysis of secondary metabolites, replacement strain JW3105 was confirmed to be a producer of C22,23-unsaturated avermectin compounds. This result implies that the equal production of "1" and "2" components, as observed in the wild-type strain of *S. avermitilis,* is not attributable to premature KR activity on the substrate, but rather, to the defective activity of the original DH on the substrate.

With the results presented in this paper, we have demonstrated that engineered polyketides are capable of generating polyketides with different structures and properties. Although AVMs lack antimicrobial activity, we believe that our research will boost interest in the development of PKS derivatives, which exhibit a broad spectrum of antimicrobial potency, for the treatment of a host of pathogens.

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