Direct Fermentative Production of Acyltylosins by Genetically-engineered Strains of *Streptomyces fradiae*

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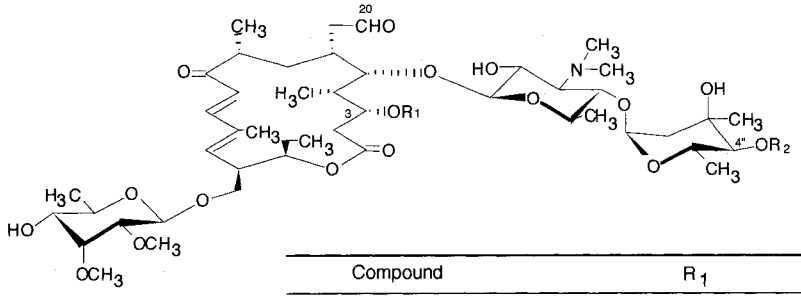
A tylosin-producer, *Streptomyces fradiae*, was transformed with plasmids carrying genes from *Streptomyces thermotolerans* that are involved in acyl modification of macrolide antibiotics. A transformant with pMAB3, in which macrolide 4″-*O*-acyltransferase gene (*acyB1*) and its regulatory gene (*acyB2*) are subcloned, produced several types of 4″-*O*-acyltylosins. A transformant with pAB11ΔEH containing macrolide 3-*O*-acyltransferase gene (*acyA*) in addition to the above two genes produced 3-*O*-acetyltylosin and 3-*O*-acetyl-4″-*O*-acyltylosins. Among the products of the latter transformant, 3-*O*-acetyl-4″-*O*-isovaleryltylosin (AIV) was detected as a minor component. When L-leucine, a precursor of isovaleryl-CoA, was added to the medium at the late stage of the fermentation, AIV content among the total macrolides increased ten-fold and AIV became a main product. This fact suggests that a high level of endogenous isovaleryl-CoA may be essential for the selective production of AIV by *S. fradiae* carrying pAB11ΔEH.

The acyl modified 16-membered macrolide antibiotic, 3-*O*-acetyl-4″-*O*-isovaleryltylosin (AIV) (Fig. 1), is a widely-used veterinary drug, since it inhibits growth of tylosin-resistant bacteria such as *Staphylococcus aureus* MS8710[1,2]. To date AIV has been manufactured by bioconversion of tylosin (Fig. 1), a product of *S. fradiae*[3,4], with a carbomycin producing *S. thermotolerans* as an acyl converter[5]. After a series of NTG (*N*-methyl *N'*-nitro *N*-nitrosoguanidine) mutageneses to *S. thermotolerans*, a strain that possesses high activities

Fig. 1. Tylosin and its acyl derivatives.



	Compound	R_1	R_2
I.	Tylosin	H	H
II.	4″-*O*-Acetyltylosin	H	$COCH_3$
III.	4″-*O*-Propionyltylosin	H	$COCH_2CH_3$
IV.	4″-*O*-Butyryltylosin	H	$COCH_2CH_2CH_3$
V.	4″-*O*-Isovaleryltylosin	H	$COCH_2CH(CH_3)_2$
VI.	3-*O*-Acetyltylosin	$COCH_3$	H
VII.	3,4″-Di-*O*-acetyltylosin	$COCH_3$	$COCH_3$
VIII.	3-*O*-Acetyl-4″-*O*-propionyltylosin	$COCH_3$	$COCH_2CH_3$
IX.	3-*O*-Acetyl-4″-*O*-butyryltylosin	$COCH_3$	$COCH_2CH_2CH_3$
X.	3-*O*-Acetyl-4″-*O*-isovaleryltylosin (AIV)	$COCH_3$	$COCH_2CH(CH_3)_2$

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of 3-*O*-acyltransferase and 4″-*O*-acyltransferase and lacks the ability of carbomycin production was obtained in Mercian and has been used for industrial manufacturing.

However, manufacturing of AIV requires two steps of fermentation: *i.e.*, tylosin fermentation by *S. fradiae* and subsequent acyl conversion of tylosin by *S. thermotolerans*. In order to improve the existing production, especially in terms of cost performance, we started to construct genetically-engineered strains that enables production of AIV in a single step fermentation. Genes for 3-*O*-acyltransferase (*acyA*)[6,7], 4″-*O*-acyltransferase (*acyB1*[8]: identical to *carE*[9]), and a regulatory gene probably for the macrolide acylation by *acyB1* (*acyB2*)[8], had been cloned from the *S. thermotolerans* genome and a tylosin-producer of *S. fradiae* produced 3-*O*-acetyltylosin with a high productivity[6] when transformed with the *acyA* gene subcloned in a vector plasmid pIJ350.

Apart from our work, applications of recombinant DNA techniques to antibiotic-producing actionomycetes to obtain genetically-engineered strains beneficial to antibiotic production have been reported and some of them were useful to produce specific components or an intermediate of certain antibiotics[10,11] or hybrid antibiotics[9,12~14].

This paper describes (i) construction of the two plasmids carrying *acyB1-acyB2* with and without *acyA* and subsequent establishment of a suitable host strain of *S. fradiae* for their expression; (ii) direct production of 4″-*O*-acyltylosins; and finally, (iii) direct selective production of industrially-important AIV from the group of the hybrid tylosins.

Materials and Methods

Strains Used

Streptomyces lividans TK24[15]: a host strain for plasmid construction and large scale plasmid preparation was obtained from D. A. HOPWOOD (John Innes Institute, Norwich, UK).

Streptomyces fradiae ATCC19609: a tylosin producer was purchased from ATCC.

Streptomyces fradiae MBBF: a tylosin high-titer strain was obtained at Mercian Central Research Laboratories.

Streptomyces fradiae MBBF-c: a host strain for expression of the genes subcloned into plasmids pMAB3 and pAB11ΔEH was derived from *S. fradiae* MBBF.

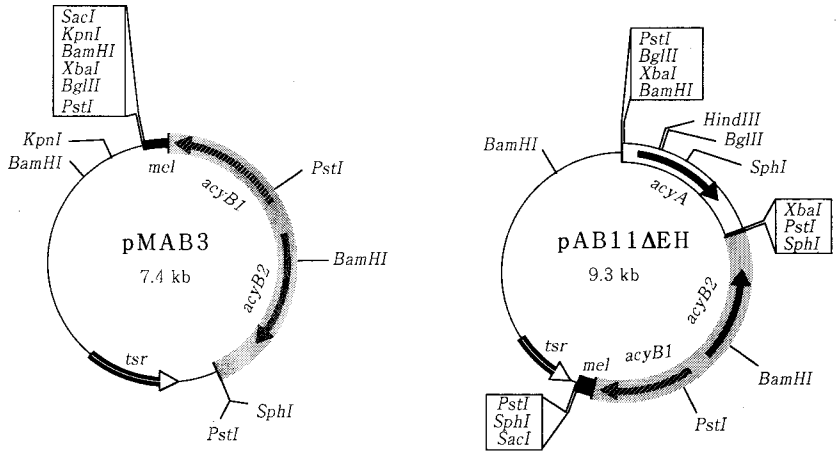
Construction of Plasmids

A 3.4-kb *Sac*I-*Sph*I fragment containing *acyB1-acyB2* from pUS81[8] was ligated to a 4.1-kb *Sac*I-*Sph*I-digested fragment corresponding to pIJ350 portion of pUJ350[8] to construct pMAB3 (Fig. 2). A 1.8-kb *Bam*HI-*Sal*I fragment containing *acyA* from p53A[6] was subcloned into the *Xba*I site of pMAB1[8] with a *Bam*HI-*Xba*I-*Sal*I synthetic adapter. A 2.7-kb pUC18 fragment was deleted from the resulting plasmid (pAB11) by self-circulization after *Eco*RI-*Hin*dIII digestion and end-filling reaction with T4 DNA polymerase (Toyobo), to obtain pAB11ΔEH (Fig. 2).

Establishment of Host-Vector System in a High Tylosin Producer

First of all, *S. fradiae* ATCC19609 was transformed with pIJ702 by the method of HOPWOOD *et al.*[15]. The plasmid DNA was isolated from the transformant resistant to thiopeptin (over 25 μg/ml) and then transformed *S. fradiae* MBBF. Among several thiopeptin-resistant colonies, one colony was selected for further curing experiment carried out as described previously[15]: *i.e.*, by cultivation, protoplast-preparation

Fig. 2. Structures of plasmids for direct production of acyltylosins.



Construction of the plasmids are described in the Materials and Methods. *mel*; partial (0.24-kb *Sac*I-*Bgl*II) fragment of melanoid pigment producing (tyrosinase) gene[22]. *tsr*; thiostrepton resistance gene[15].

and regeneration in the absence of thiopeptin. One of the strains obtained through this procedure was named as *S. fradiae* MBBF-c.

Culture Conditions

In general manipulations, *S. lividans* and *S. fradiae* were grown in Tryptic Soy Broth (Difco) at 28°C and their transformants were cultivated in the medium with thiopeptin (5 μg/ml). For the fermentation experiments of acyltylosins, transformants of *S. fradiae* were grown in the conditions described previously[6].

HPLC Analyses of Acyltylosins in Culture Broth

At the end of the fermentation, 0.2 ml of the culture broth was sampled and diluted 20-fold with 0.1 M phosphate buffer (pH 9.0). A 2-ml portion of the dilution was extracted with an equal volume of ethyl acetate. A dry pellet obtained after evaporation of the extract was dissolved in 1 ml of 80% acetonitrile. The sample solution (10 μl) was applied to HPLC (Shimadzu C-R5A) equipped with an ODS column (YMC-Pack ODS-A, 150 mm × 6 mm i.d. (internal diameter), YMC Co. Ltd.,). Solvent-system, its flow rate and temperature applied to the analyses were 0.2 M NaH_2PO_4 - 0.6 M $NaClO_4$ (pH 2.5) - methanol (1 : 1 : 6) at 0.5 ml/minute at 30°C and 0.85 M $NaClO_4$ (pH 2.5) - acetonitrile (17 : 15) 1.5 ml/minute at 40°C for 4″-*O*-acyltylosins and 3-*O*-acetyl-4″-*O*-acyltylosins, respectively.

Results and Discussion

Introduction of Plasmids Carrying the Acyltransferase Genes into *S. fradiae*

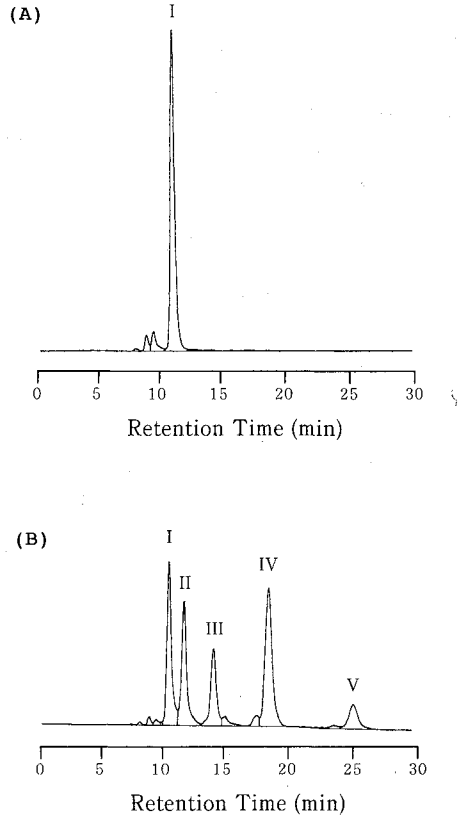
The plasmids pMAB3 and pAB11ΔEH (Fig. 2) were constructed as described in Materials and Methods. Initially, however, no transformants of *S. fradiae* MBBF with pMAB3 were obtained, probably because of a difference of restriction-modification systems between *S. lividans* and *S. fradiae*. Therefore, a host-vector system of *S. fradiae* MBBF was established as described Materials and Methods, using *S. fradiae* ATCC 19609 as an intermediate host. Consequently, two types of transformants of strain MBBF-c carrying either pMAB3 or pAB11ΔEH were obtained. Both of these plasmids were stably-maintained and their copy numbers seemed not to be reduced under a thiopeptin-selective condition since amounts of the plasmid DNAs recovered from the *S. fradiae* transformants were comparable to those of the same plasmids from the *S. lividans* transformants (unpublished data).

Direct Production of 4″-*O*-Acyltylosins by *S. fradiae* (pMAB3)

The strain MBBF-c and the transformant with pMAB3 were cultivated in the production medium for 7 days and macrolides produced were compared with HPLC (Fig. 3). Although the host strain MBBF-c produced tylosin as a main product, with a retention time (RT) of 10.4 minutes (Fig. 3A), the MBBF-c transformant with pMAB3 produced several compounds in addition to tylosin, which were thought to be 4″-*O*-aclytylosin derivatives in the RT range of 11.6 to 25.1 minutes (Fig. 3B). Total amounts of the macrolide produced by MBBF-c and MBBF-c (pMAB3) were 3320 μg/ml and 2480 μg/ml as a tylosin equivalent basis, respectively.

Four major components (II, III, IV and V with RTs of 11.6, 14.0, 18.3 and 25.1 minutes, respectively, in Fig. 3B) were isolated and purified by the silica gel column chromatography as described previously[5] and analyzed for their chemical structures using ^1H NMR and MS. As a result, compounds II, III, IV and V turned out to be 4″-*O*-acetyltylosin, 4″-*O*-propionyltylosin, 4″-*O*-butyryltylosin and 4″-*O*-isovaleryltylosin (Fig. 1), respectively[5], which demonstrates that 4″-*O*-acyltylosins

Fig. 3. HPLC profiles of ethyl acetate extracts from culture broths of *S. fradiae* MBBF-c (A) and *S. fradiae* MBBF-c (pMAB3) (B).



Tylosin and 4″-*O*-acyltylosins identified were shown with I (tylosin), II (4″-*O*-acetyltylosin), III (4″-*O*-propionyltylosin), IV (4″-*O*-butyryltylosin) and V (4″-*O*-isovaleryltylosin.

were directly produced by the tylosin-producer due to the expression of genes introduced into the strain.

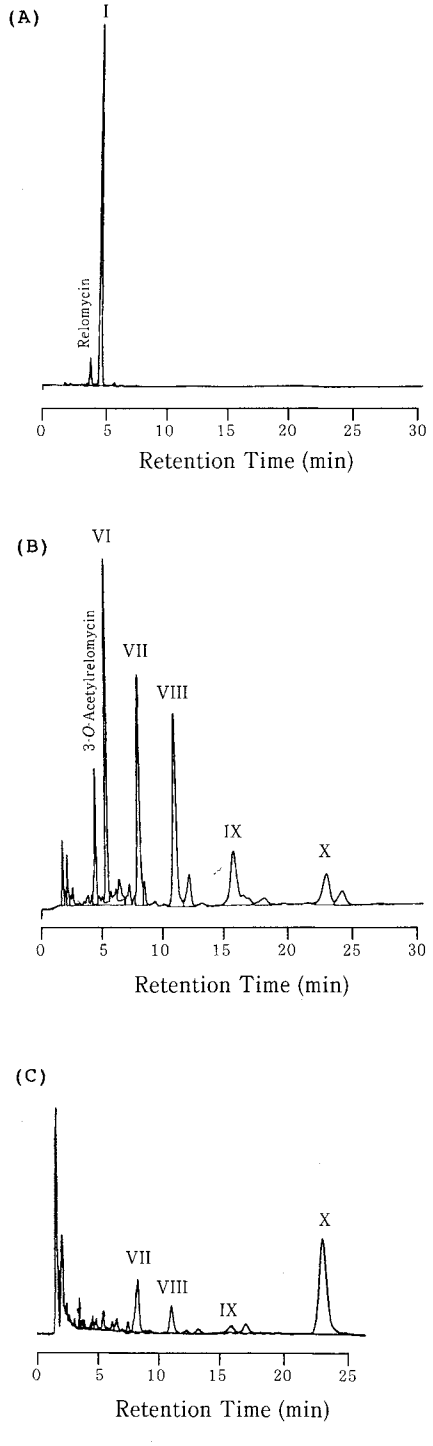
In this fermentation experiment, 74.4% of the total macrolide produced by MBBF-c (pMAB3) was 4″-*O*-acylated tylosins. Specifically, order of productivity of the acyl derivatives were 4″-*O*-butyryltylosin (IV, 772 μg/ml), 4″-*O*-acetyltylosin (II, 524 μg/ml), 4″-*O*-propionyltylosin (III, 359 μg/ml) and 4″-*O*-isovaleryltylosin (V, 190 μg/ml), which indicates that the 4″-*O*-acyltransferase can utilize several types of acyl-CoAs as substrates. Productivities of the 4″-*O*-acyltylosins differ as observed due to the amounts of acyl-CoAs accumulated intracellularly.

Although EPP *et al.* reported that when a spiramycin-producer of *S. ambofaciens* was transformed with a plasmid carrying the 4″-*O*-acyltransferase gene (*carE*) with a truncated *acyB2*, it produced 4″-*O*-isovalerylspiramycin[9], our similar experiment using an *acyB2*-deleted plasmid revealed that intact *acyB2* is essential for the expression of 4″-*O*-acyltransferase activity in *S. fradiae* (unpublished data) as well as *S. lividans*[8]. We performed a hybridization experiment using the *acyB2* probe which indicates that *S. ambofaciens* has an *acyB2*-homologous region in the genomic DNA while *S. fradiae* and *S. lividans* do not (unpublished data); thus, *S. ambofaciens* is thought to possess a gene of its own that can express and work as a 4″-*O*-acyltransferase.

Direct Production of AIV by *S. fradiae* (pAB11ΔEH)

Since it was confirmed that *S. fradiae* with *acyA* produced 3-*O*-acetyltylosin[6], *S. fradiae* with pAB11ΔEH containing both *acyA* and *acyB1-acyB2* was examined for production of 3-*O*-acetyl-4″-*O*-acyltylosins including AIV. The macrolides were extracted from culture broth of the strain MBBF-c (pAB11ΔEH) and analyzed with HPLC system suitable for detection of 3-*O*-acetyl-4″-*O*-acyltylosin derivatives in which tylosin is eluted at RT of 4.6 minutes (component I in Fig. 4A). As a result of both activities of 3-*O*-acetylation and 4″-*O*-acylation of tylosin, several new components were observed in the RT range of 5.4 to 23.4 minutes, most of which were thought to be 3-*O*-acetyl-4″-*O*-acyltylosins (Fig. 4B). Further chemical analyses identified compounds VI, VII, VIII, IX and X at RTs of 5.4, 8.0, 11.0, 15.8 and 23.4 minutes as 3-*O*-acetyltylosin, 3,4″-di-*O*-acetyltylosin, 3-*O*-acetyl-4″-*O*-propionyltylosin, 3-*O*-acetyl-4″-*O*-butyryltylosin and AIV (Fig.1), respectively, with reference to data obtained previously[5]. Productivity of each component after the 7-day fermentation is summarized

Fig. 4. HPLC profiles of culture broths of *S. fradiae* MBBF-c (A) and *S. fradiae* MBBF-c (pAB11ΔEH) (B, C).



Ethyl acetate extracts from the culture broths of the host strain (A) and the recombinant strain (B) were applied to the HPLC analysis while, in the culture supplemented with L-leucine, supernatant from the recombinant strain diluted 20-fold with acetonitrile was applied (C). Tylosin was indicated as component I while acyltylosins identified were shown with VI (3-*O*-acetyltylosin), VII (3,4″-di-*O*-acetyltylosin), VIII (3-*O*-acetyl-4″-*O*-propionyltylosin), IX (3-*O*-acetyl-4″-*O*-butyryltylosin) and X (AIV).

Table 1. Productivity of acylmacrolides by *Streptomyces fradiae* (pAB11ΔEH).

Strain	Leucine addition*	Macrolide production (μg/ml)					
		TY	RM	3AT	3AR	3,4″ AAT	AIV
MBBF-c	–	3320	255	ND	ND	ND	ND
MBBF-c (pAB11ΔEH)	–	ND	7	325	71	329	56
MBBF-c (pAB11ΔEH)	+	ND	ND	29	15	194	602

* L-Leucine was added to 500 μg/ml on the 5th day of 7 days culture (+) or not (−).
Symbols; TY: tylosin, RM: relomycin (20-dihydrotylosin), 3AT: 3-*O*-acetyltylosin, 3AR: 3-*O*-acetylrelomycin, 3,4″ AAT: total amount of 3-*O*-acetyl-4″-*O*-acyltylosins except AIV (VII to IX), ND: not detected (< 1 μg/ml).

in Table 1.

The macrolide yield for the recombinant strain was only 22~23% of the macrolides produced by MBBF-c. The reduced titers for the recombinant strain were not due to the toxicity on the producing host of the tylosin derivatives because the host strain was resistant to acyl derivatives of tylosin including AIV as well as tylosin (data not shown). The introduction of plasmid pAB11ΔEH into *S. fradiae* rather seems to have perturbed its secondary metabolism and reduced the macrolide productivity[16].

The acyl modification at 3-OH of tylosin was complete because components un-acylated at 3-OH were not detected. This observation is consistent with the previous result obtained from the fermentation in which 3-*O*-acetyltylosin was produced by the MBBF strain with only the *acyA* gene introduced. By contrast, the acyl modification at 4″-OH was incomplete because 3-*O*-acetyltylosin (VI) was also co-produced. Among the macrolides produced, AIV was detected as a minor component (56 μg/ml, 7.1% of the total macrolide). Since this low productivity was thought to be caused by a low level of endogenous isovaleryl-CoA, L-leucine, a starting material of isovaleryl-CoA, was added to the production medium (500 μg/ml) at fifth day when the culture just begins tylosin production. HPLC analysis of the culture broth showed that considerable amounts of AIV was produced with lower amounts of the other components (Fig. 4C, Table 1). Content of AIV reached 71.6% in the total macrolides, which was 10-fold higher than that in the initial condition without L-leucine. This result strongly suggested that a high level of endogenous isovaleryl-CoA may be important for the selective production of AIV.

In order to enhance the AIV productivity in *S. fradiae*, one of our next targets is to control a pathway for leucine catabolism. Although dehydrogenase (Vdh) and α-keto dehydrogenase (BCDH), the first and second enzymes of the leucine catabolism pathway in streptomycetes, respectively, were biochemically and genetically characterized[17~21], little has been known about the third catabolic enzyme, isovaleryl-CoA dehydrogenase (IVD), in streptomycetes. In *S. fradiae*, for instance, inactivation of the IVD together with enhancement of the catabolic flow towards isovaleryl-CoA by overexpression of the Vdh gene and the BCDH gene seems to be an effective approach to breed a strain capable of accumulating isovaleryl-CoA in a larger amount intracellularly.

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