APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1994, p. 2657–2660
0099-2240/94/$04.00+0

Vol. 60, No. 7

Cloning of the Macrolide Antibiotic Biosynthesis Gene *acyA*, Which Encodes 3-*O*-Acyltransferase, from *Streptomyces thermotolerans* and Its Use for Direct Fermentative Production of a Hybrid Macrolide Antibiotic

AKIRA ARISAWA,* NAOTO KAWAMURA, KOUJI TAKEDA, HIROSHI TSUNEKAWA, KAZUHIKO OKAMURA, AND ROKUROU OKAMOTO

Mercian Corporation, Central Research Laboratories, 4-9-1 Johnan, Fujisawa, Kanagawa 251, Japan

Received 20 December 1993/Accepted 2 May 1994

A gene encoding the macrolide modification enzyme 3-*O*-acyltransferase (*acyA*) was cloned by chromosome walking onto the carbomycin biosynthetic region in *Streptomyces thermotolerans* TH475, with the 3′ region of the gene encoding the macrolide modification enzyme 4″-*O*-acyltransferase (*acyB1*) as a probe. A shortened fragment (1.8 kb) containing *acyA* was subcloned with pIJ350. A high-level tylosin producer, *Streptomyces fradiae* MBBF, transformed with the plasmid could produce a hybrid macrolide, 3-*O*-acetyltylosin, most efficiently.

Several reports have demonstrated production of specific components or new types of antibiotics by disruption of targeted genes or introduction of genes encoding antibiotic modification enzymes, respectively, in actinomycetes (2, 10–12). The use of such a genetically engineered strain would be more beneficial than the indirect and conventional bioconversion method for production of a hybrid macrolide antibiotic.

3-*O*-Acetyl 4″-*O*-isovaleryltylosin (AIV) (Fig. 1B) is an industrially important 16-membered macrolide antibiotic that is widely used by veterinarians and is known to be effective for preventing the growth of tylosin-resistant bacteria (9). The current process for producing AIV consists of two steps of fermentation: first, tylosin fermentation by *Streptomyces fradiae* and second, acyl conversion by a mutant strain of *Streptomyces thermotolerans* that does not produce carbomycin (Fig. 1A) (8). Two distinct acyltransferases (3-*O*-acyltransferase and 4-*O*-acyltransferase) of macrolide antibiotics have key roles in this acyl conversion. We have studied the possibility of innovating direct fermentation in place of the indirect method by use of a genetically engineered strain. During this study, we cloned from *S. thermotolerans* the 4″-*O*-acyltransferase gene (*acyB1*) together with its regulatory gene (*acyB2*) (1). *acyB1* was found to be identical to *carE*, whose sequence has been published elsewhere by other workers (2). A macrolide biosynthesis gene, *mdmC*, encoding 3-*O*-acetyltransferase, was recently cloned from midecamycin-producing *Streptomyces mycarofaciens* by Hara and Hutchinson (3), but it has not yet been cloned from carbomycin-producing *S. thermotolerans*.

In this communication, we report the production of 3-*O*-acetyltylosin (3-AT) by a transformant of *S. fradiae*, into which the macrolide antibiotic biosynthesis gene *acyA*, which encodes 3-*O*-acyltransferase, cloned from *S. thermotolerans* was introduced. This is the preliminary step for the direct production of AIV.

Chromosome walking and cloning of *acyA*. For preparation of the cosmid library, chromosomal DNA was isolated from *S. thermotolerans* TH475, a mutant of carbomycin-producing strain ATCC 11416 in which carbomycin-lactone formation is blocked but acyltransferase activities are intact. *Escherichia coli* cosmid pHC79 (4) (Boehringer Mannheim, Penzberg, Germany) and *E. coli* HB101 were used as a vector and a host strain, respectively. Packaging to λ phage was done by using the Giga Pack Gold in vitro packaging kit (Stratagene, La Jolla, Calif.). Transduction to *E. coli*, labeling of DNA, colony hybridization, and other basic methods for genetic manipulation were done by following the procedures of Maniatis et al. (7) and Hopwood et al. (5). On primary screening of the cosmid library, with a 1.0-kb *Sac*I-*Eco*RI fragment (3′ region of *acyB1*) of pAOY-17 (1) as a probe, some positive clones were isolated. One of the clones, carrying pSE26, was selected for an additional walking step. Secondary screening of the library was done to extend the cloned region. A probe for the cloned region was prepared from a terminal region (1.0-kb *Bam*HI fragment) of the insert of pSE26. One of the positive secondary clones, carrying pBM73, which shared only a small region with pSE26 (<0.5 kb), was selected. The total length covered by the two cosmids (pSE26 and pBM73) was estimated to be about 80 kb. They were partially digested with *Sal*I, ligated into the *Xho*I site of pIJ922 (5), and then transformed into *Streptomyces lividans* TK24 (5). The transformants were grown on GYM plates (1.0% glucose, 0.5% yeast extract, 1.0% malt extract, 1.5% agar; pH 7.2) supplemented with thiopeptin to 50 μg/ml. Next, leucomycin A_1 was overlaid on the plates together with soft nutrient agar (5) to a final concentration of 200 μg/ml. After incubation for 20 h at 28°C, agar pieces were cut from the center of the colonies with a cork borer (inside diameter, 6 mm) and each was transferred to a separate lane on thin-layer chromatography (TLC) plates (no. 13143; Merck). After being left for 30 min at room temperature, the dry agar pieces were removed gently and TLC was done with *n*-hexane-toluene-ethylacetate-acetone-methanol (30:25:20:10:8). For coloration of TLC spots, the TLC plates were immersed in 10% H_2SO_4 and baked at 120°C for 10 min. In the assay of acyltransferase activity, a transformant with 3-*O*-acyltransferase activity that could convert leucomycin A_1 (a 3-hydroxy 4″-*O*-isovaleryl type of macrolide antibiotic with an R_f value of 0.35) to leucomycin A_3 (3-*O*-acetylated leucomycin A_1 with an R_f value of 0.46) was obtained easily from 250 recombinants of *S. lividans* (Fig. 2). A plasmid harboring a 3.2-kb *Sal*I insert was isolated from the clone and designated

* Corresponding author. Mailing address: Mercian Corporation Central Research Laboratories, 4-9-1 Johnan, Fujisawa, Kanagawa, Japan 251. Phone: 0466-35-1511. Fax: 0466-35-1530.

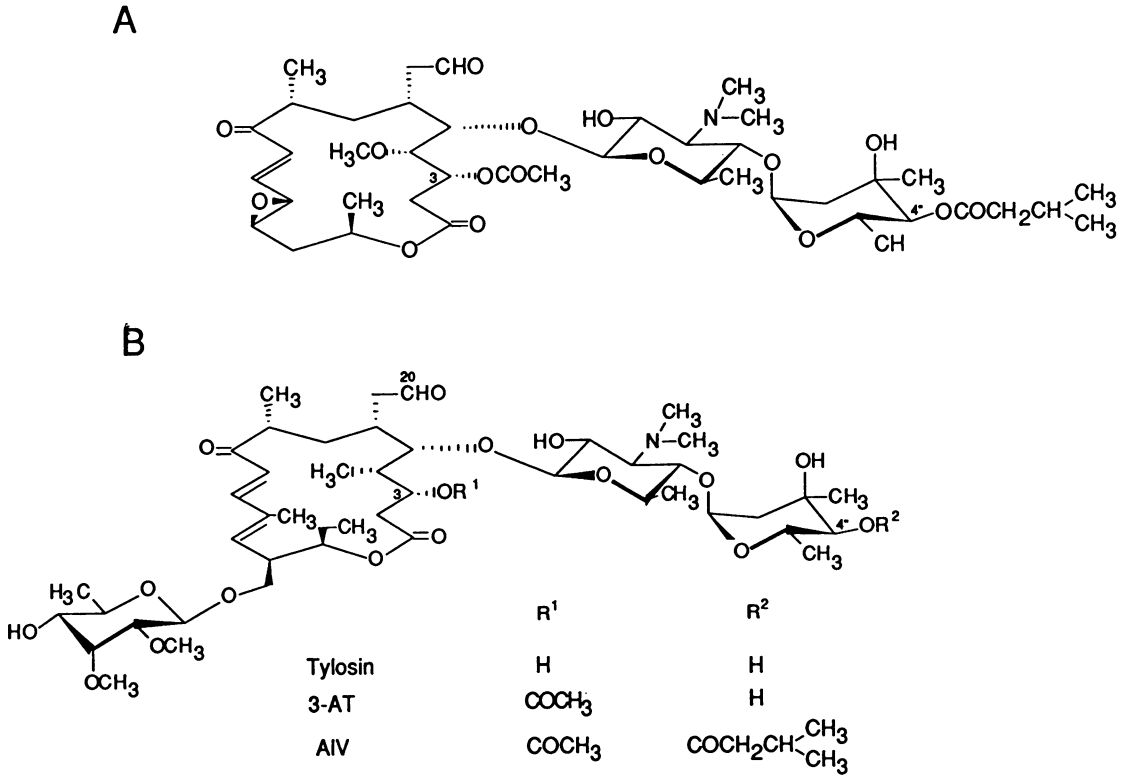

FIG. 1. Chemical structures of carbomycin (A) and acyltylosins (B).

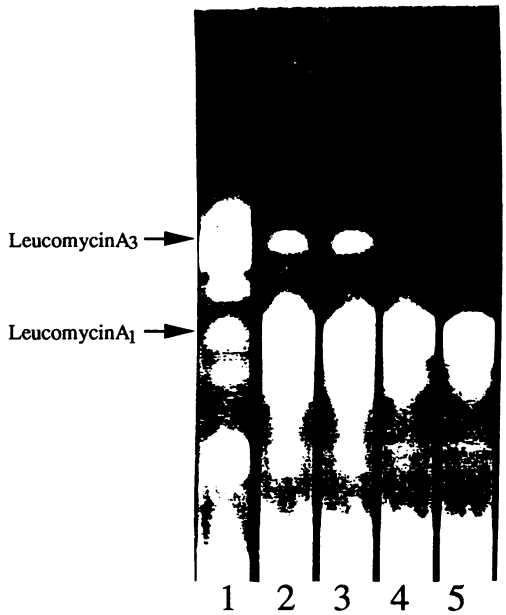

FIG. 2. TLC analysis of 3-*O*-acyltransferase activity for macrolide antibiotics. Lanes: 1, *S. thermotolerans* TH475; 2, *S. lividans*(p53A); 3, *S. lividans*(pMAA25); 4, *S. lividans*(pIJ922); 5, *S. lividans*(pIJ350). The TLC spots were copied from the original TLC plate to a sheet of paper with a QUICK COPY apparatus (Fuji Film, Tokyo, Japan).

p53A (Fig. 3). *S. lividans* transformed with p53A converted leucomycin A_1 or tylosin added exogenously to the growth medium into leucomycin A_3 or 3-AT, respectively. The insert of p53A was hybridized with pBM73 but not hybridized with pSE26 on Southern blot analysis (data not shown).

Expression of *acyA* in *S. fradiae* and production of hybrid macrolide antibiotic. A 1.8-kb *Bam*HI-*Sal*I fragment from the 3.2-kb region containing *acyA* was isolated from a 0.8% agarose gel and purified with GENE CLEAN II (Bio 101, Inc., La Jolla, Calif.). The DNA fragment was subcloned into a modified pUC18, the *Sma*I site of which was replaced by a *Pst*I

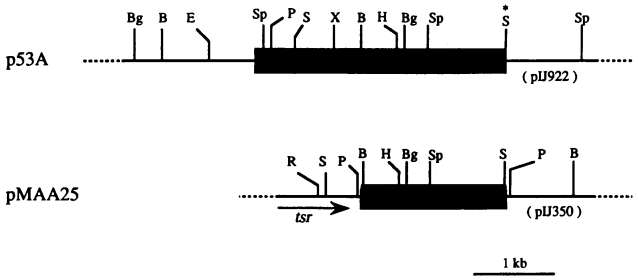

FIG. 3. Restriction map of DNA fragments carrying the *acyA* gene from *S. thermotolerans*. The solid boxes represent *S. thermotolerans* DNA cloned or subcloned into the *Xho*I site of pIJ922 or the *Pst*I site of pIJ350, respectively. A *Sal*I site at one end of the insert, indicated by an asterisk, was not, for an unknown reason, inactivated after being ligated to an *Xho*I cohesive end of pIJ922. The arrow indicates the location and orientation of the thiostrepton resistance gene (*tsr*) in pIJ350. Restriction site abbreviations: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RV; S, *Sal*I; Sp, *Sph*I; X, *Xho*I.

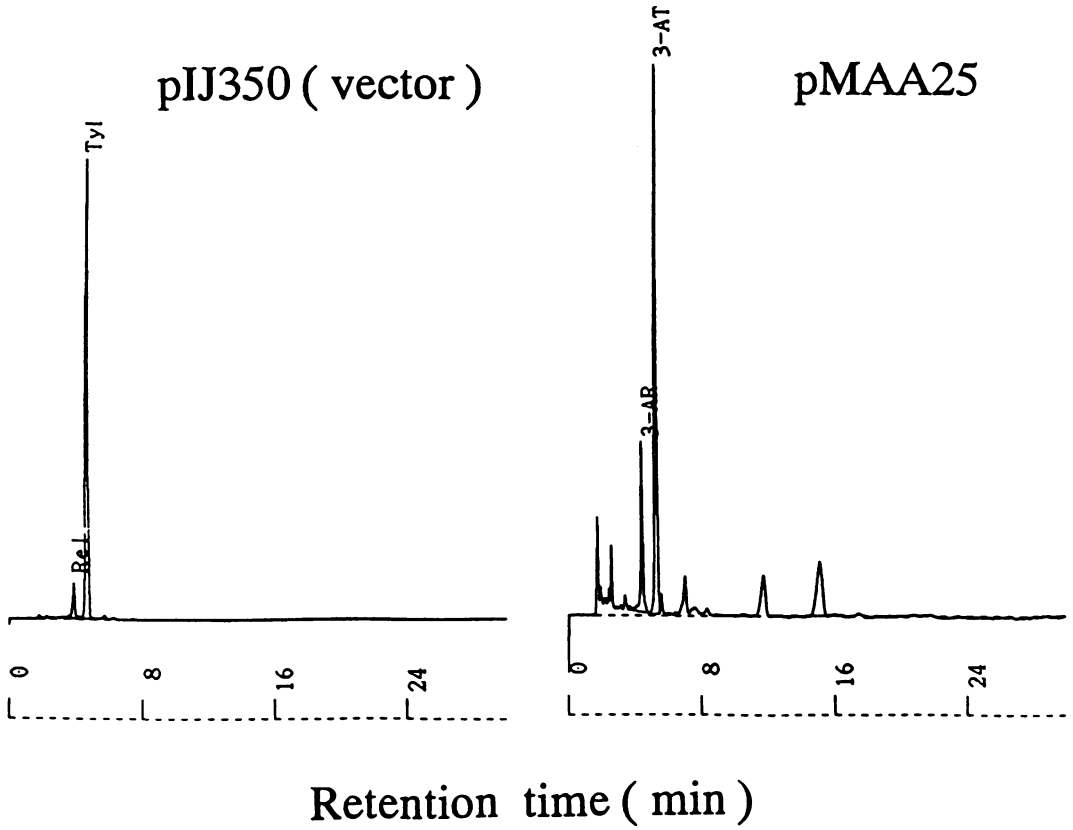

FIG. 4. HPLC analysis of culture broths from transformants with the indicated plasmids. The main products detected in the analysis were tylosin (Tyl; retention time of 4.6 min), relomycin (Rel; 3.8 min), 3-AT (5.3 min), and 3-*O*-acetylrelomycin (3-AR; 4.4 min).

site with a *Pst*I linker. Next, a 1.8-kb *Pst*I fragment was recloned from the resulting plasmid into the single *Pst*I site of pIJ350 (6) to construct pMAA25 (Fig. 3). *S. lividans* transformed with pMAA25 expressed the macrolide 3-*O*-acyltransferase without any loss of activity compared with that of *S. lividans* transformed with p53A (Fig. 2). To acquire a strain that produces 3-AT directly, the tylosin producer *S. fradiae* MBBF, a high-level tylosin producer derived from ATCC 19609 by *N*-methyl *N'*-nitro *N*-nitrosoguanidine (NTG) mutagenesis, was transformed with pMAA25. One of the stable pMAA25 transformants was cultivated in seed medium (2% potato starch, 2% soybean meal, 0.1% yeast extract, 0.05% K_2HPO_4, 0.05% $MgSO_4$, 0.3% $CaCO_3$) at 28°C for 3 days and then in oil medium (6% rape oil, 2.5% dry yeast, 0.1% $CaCO_3$, 0.1% choline chloride) at 28°C for 10 days. The culture broth was diluted 20-fold with 0.1 M phosphate buffer (pH 9.0), and 2 ml of the solution was extracted with an equal volume of ethylacetate. After evaporation of the extract, the dry pellet was dissolved with 1 ml of 80% acetonitrile. Next, 10 μl of the solution was injected for high-performance liquid chromatography (HPLC). The column (YMC-Pack ODS-A; inner diameter, 6 mm; length, 150 mm; YMC Co., Ltd., Kyoto, Japan) was developed with 0.85 M $NaClO_4$ (pH 2.5)–acetonitrile (17:15), and the flow rate was 1.5 ml/min. Tylosin and its derivatives were detected by UV absorbance at 280 nm. The analysis of the culture broth showed a successful production of the hybrid macrolides, while control strains (the host strain with no plasmid and the transformant with pIJ350) produced no acyl macrolides (Fig. 4). The strain into which *acyA* was introduced produced 3-AT principally and 3-*O*-acetylrelomycin (3-*O*-acetyl 20-dihydrotylosin) as a minor product. Production of macrolides in batch culture (10th day) of transformants is summarized in Table 1. Epp et al. succeeded in producing 4″-*O*-isovalerylspiramycin directly from spiramycin-producing *Streptomyces ambofaciens*, into which the *carE* gene from *S. thermotolerans* was introduced (2). Their data showed that the unconverted spiramycin was also observed in the total products. No description of how much of the hybrid macrolide the recombinant strain could produce was found. We speculate that a lower level of endogenous isovaleryl coenzyme A (CoA) than acetyl-CoA might have been responsible for the imperfect 4″-*O*-isovalerylation of spiramycin.

Our strain satisfied both qualitative and quantitative demands: no unacylated antibiotic was produced, and the efficiency with which the hybrid antibiotic was produced was noticeable (Table 1).

Our final aim is to generate an AIV-producing strain by expression of both *acyA* and *acyB1-acyB2* genes simultaneously

TABLE 1. Production of Macrolides by *S. fradiae* transformants

Strain/plasmid	Macrolide production (μg/ml)			
	Tyl[a]	Rel[a]	3-AT[a]	3-AR[a]
MBBF	3,540	260	ND[b]	ND[b]
MBBF/pIJ350 (vector)	3,250	240	ND[b]	ND[b]
MBBF/pMAA25	ND[b]	ND[b]	3,690	650

[a] Abbreviations of the macrolides are given in the text or in the legend to Fig. 4.
[b] Not detected.

in *S. fradiae*. In this regard, the problem of how to enhance the level of the endogenous pool of isovaleryl-CoA merits further study.

Nucleotide sequence accession number. The nucleotide sequence data of the insert region of p53A (3.2-kb *SalI* fragment) containing the *acyA* gene will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under the accession number D30759.

We thank H. Asou-Takahashi for excellent technical assistance.

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