RESEARCH LETTER

Gene targeting for *O*-methyltransferase genes, *mycE* and *mycF*, on the chromosome of *Micromonospora griseorubida* producing mycinamicin with a disruption cassette containing the bacteriophage ϕC31 *attB* attachment site

Shu-Ichi Tsukada[1], Yojiro Anzai[1], Shengying Li[2], Kenji Kinoshita[3], David H. Sherman[2] & Fumio Kato[1]

[1]Faculty of Pharmaceutical Sciences, Toho University, Funabashi, Chiba, Japan; [2]Life Sciences Institute, Department of Medicinal Chemistry, Chemistry, and Microbiology and Immunology, University of Michigan, Ann Arbor, MI, USA; and [3]School of Pharmaceutical Sciences, Mukogawa Women's University, Nishinomiya, Hyogo, Japan

Correspondence: Fumio Kato, Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan. Tel.: +81 47 472 2068. Fax: +81 47 472 2086; e-mail: fkato@phar.toho-u.ac.jp

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Abstract

Mycinamicin, a 16-membered macrolide antibiotic produced by *Micromonospora griseorubida*, comprises a macrolactone and two deoxysugars: desosamine and mycinose. Mycinose is synthesized through two modification steps: the methylation of 6-deoxyallose in mycinamicin VI and of javose in mycinamicin III. To confirm the role of *mycE* and *mycF* genes in mycinamicin biosynthesis in *M. griseorubida*, disruption mutants of *mycE* and *mycF* were constructed by disruption plasmids containing *attB* in the disruption cassette FRT-*neo-oriT*-FRT-*attB* for the integration of ϕC31-derivative vector plasmids; the disruption mutants were complemented through the integration of pSET152 derivatives containing intact *mycE* or *mycF* into the artificially inserted *attB* site. These disruption mutants did not produce mycinamicin II, but mainly accumulated mycinamicins VI and III, indicating that MycE and MycF methylated the C2″-OH group of 6-deoxyallose in mycinamicin VI and the C3″-OH group of C2″-methylated 6-deoxyallose in mycinamicin III, respectively. The complemented strains of *mycE* and *mycF* recovered the mycinamicin II productivity.

Introduction

In general, to confirm the function of a gene in a microorganism, the mutant with a disrupted gene should be isolated, and genetic complementation studies for the mutant should be performed. Recently, a simple and highly efficient PCR-targeting method was developed with the phage λ-Red recombinase to disrupt chromosomal genes in *Escherichia coli* in which PCR primers provide the homology to the targeted gene (Datsenko & Wanner, 2000), and a modified system was also developed for gene targeting of *Streptomyces* strains with a disruption cassette, which contained an *oriT* region with a selectable antibiotic resistance gene to efficiently transfer a targeted plasmid from *E. coli* to *Streptomyces* by intergeneric conjugation (Gust *et al.*, 2003). In *Streptomyces* strains, genetic complementation studies could be performed with transconjugation vectors, possessing a ϕC31 *int* gene and an *attP* site, that were site specifically inserted into the ϕC31 *attB* attachment site of a host chromosome. The *attB* site is distributed widely throughout *Streptomyces* strains, but there are few reports regarding the *attB* site of non-*Streptomyces* actinomycetes (Anzai *et al.*, 2009). *Saccharopolyspora erythraea*, which produces erythromycin, does not possess the ϕC31 *attB* site on its chromosome; the site was artificially introduced into the chromosome for antibiotic production using a combinatorial biosynthesis technique (Rodriguez *et al.*, 2003).

Mycinamicin, which is produced by *Micromonospora griseorubida* A11725, is a 16-membered macrolide antibiotic with strong antibacterial activity against gram-positive bacteria (Satoi *et al.*, 1980). Mycinamicin consists of a macrolactone substituted with two different sugars: desosamine and mycinose. The nucleotide sequence of the complete mycinamicin biosynthetic gene cluster has been reported (Anzai *et al.*, 2003), wherein two putative *O*-methyltransferase (OMT) genes *mycE* and *mycF* were identified. It was

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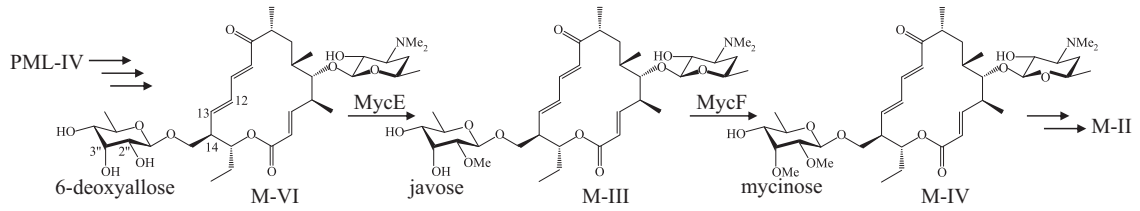


Fig. 1. Function of MycE and MycF in mycinamicin biosynthesis. PML-IV, protomycinolide IV; M-VI, mycinamicin VI; M-III, mycinamicin III; M-IV, mycinamicin IV; and M-II, mycinamicin II.

reported previously by Inouye *et al*. (1994) that mycinamicin III (M-III) was converted to mycinamicin IV (M-IV) by the crude *E. coli* cell extract overexpressed MycF protein. Moreover, the *in vitro* functions of MycE and MycF proteins were characterized using the purified MycE and MycF proteins overexpressed in *E. coli* cells (Li *et al*., 2009; Fig. 1). The purified MycE and MycF proteins methylated the C2″-OH group of 6-deoxyallose in mycinamicin VI (M-VI) and the C3″-OH group of javose (i.e. C2″-methylated 6-deoxyallose) in M-III, respectively. Here, we have demonstrated the isolation and characterization of *mycE* and *mycF* disruption mutants obtained from *M. griseorubida* A11725, which would not possess the ϕC31 *attB* site on the chromosome, by the disruption cassette FRT-*neo*-*oriT*-FRT-*attB* and the genetic complemented strains, in which plasmids including each OMT gene – *mycE* or *mycF* – were inserted into the artificially inserted *attB* site.

Materials and methods

Strains, media, and culture conditions

The strains used in this study are shown in Table 1. The culture conditions of *M. griseorubida* and *E. coli* were according to our previous report (Anzai *et al*., 2004a). FMM broth containing 7% dextrin, 0.5% glucose, 0.5% yeast extract, 0.5% soybean meal (Ajinomoto, Japan), 0.5% $CaCO_3$, 0.1% K_2HPO_4, 0.4% $MgSO_4 \cdot 7H_2O$, and 0.0002% $CoCl_2 \cdot 6H_2O$ was used for fermentation of *M. griseorubida*.

Vectors, DNA manipulation, PCR, and Southern blot

The vectors used in this study are shown in Table 1. TaKaRa ExTaq® (TaKaRa, Japan) and PfuTurbo® (Stratagene) DNA polymerase used for the DNA fragment were amplified by PCR. Plasmid and genomic DNA amplification, restriction enzyme digestion, fragment isolation, cloning, and DNA fragment amplification were performed according to standard procedures. Southern blot analysis was performed according to our previous procedure (Anzai *et al*., 2004a).

Construction of the gene disruption cassette FRT-*neo*-*oriT*-FRT-*attB*

Using pIJ776 containing FRT-*neo*-*oriT*-FRT as the template, the gene disruption cassette FRT-*neo*-*oriT*-FRT-*attB* was amplified by PfuTurbo® DNA polymerase with the primers FRTF+attB containing the sequence of the bacteriophage ϕC31 *attB* attachment site and FRTR (Table 2). The PCR fragment was cloned into the EcoRV site of pLITMUS38 to generate pMG501.

Construction of plasmids

The *mycE*-disrupted plasmid, pMG502, was constructed using three restriction fragments (3.2 kb BamHI–MluI, 0.7 kb MluI–EcoRI, and 3.8 kb StuI–BamHI) derived from pMR01, and the 1.5-kb EcoRV fragment containing the disruption cassette FRT-*neo*-*oriT*-FRT-*attB* derived from pMG501. The 9.5-kb DNA fragment linking these three restriction fragments and the disruption cassette together was inserted into the BglII and EcoRI sites on pSAN-lac to create pMG502. To generate pMG503 whose *neo* gene was in the opposite direction from the mycinose biosynthesis gene cluster, the 1.3-kb XbaI fragment including *neo* and *oriT* derived from pMG501 was ligated with the 15-kb XbaI fragment derived from pMG502.

To construct pMG504 containing *myrB*, *mycG*, *mycF*, *mycCI*, and *mycCII*, the 2.4-kb BsiWI–StuI and 3.8-kb StuI–MluI fragments obtained from pMR01 were cloned into pLITMUS28 and pLITMUS38, respectively; then, the 2.4-kb BglII–StuI and 3.8-kb StuI–SpeI fragments derived from these resulting plasmids were inserted into the BglII and XbaI sites on pSAN-lac. The *mycF* region on pMG504 was replaced with the disruption cassette FRT-*neo*-*oriT*-FRT-*attB* using the λ-Red-mediated recombination system according to Gust *et al*. (2003). The cassette was amplified by PCR with the primers mycFendF and mycFendR using the 1.5-kb EcoRV fragment from pMG501 as a template DNA. *Escherichia coli* BW25113/pIJ790 cells containing pMG504 were transformed with the amplified cassette by electroporation. The resulting transformants were characterized by PCR with a set of oligonucleotides (mycFF and mycFCIendR) priming outside of the recombination region, and the plasmid pMG505 was used for disruption of the

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype/comments	Source or reference
M. griseorubida		
A11725	Wild type, mycinamicin producer	Satoi *et al*. (1980)
TPMA0003	Δ*mycE*: aa6-393 replaced with an *oriT-neo-attB* cassette	This study
TPMA0004	Δ*mycF*: aa2-253 replaced with an *oriT-neo-attB* cassette	This study
TPMA0006	pMG507 including a *mycE*-controlled *mycCI* promoter *mycCI*p induced in *mycE* deletion mutant TPMA0003	This study
TPMA0009	pMG508 including *mycF* induced in the *mycF* deletion mutant TPMA0004	This study
TPMA0014	Δ*mycE*: aa6-393 replaced with a *neo-oriT-attB* cassette	This study
TPMA0016	Δ*mycF*: aa2-253 replaced with a *neo-oriT- attB* cassette	This study
E. coli		
S17-1	Conjugation donor	American Type Culture Collection
BW25113	K12 derivative: Δ*araBAD*, Δ*rhaBAD*	Gust *et al*. (2003)
JM109	General cloning host	
Plasmids		
pIJ790	λ-Red (*gam*, *bet, exo*), *cat*, *araC*, rep101ts	Gust *et al*. (2003)
pIJ776	Source of *neo-oriT* for pMG501	K.F. Chater, (pers. commun.)
pLITMUS28	Cloning vector: *bla*	NewEngland BioLabs
pLITMUS38	Cloning vector: *bla*	NewEngland BioLabs
pDrive	TA-cloning vector: *bla*, *aph*	Qiagen
pSET152	*aac(3)IV*(Apra^r), *oriT*, *attP*, *int*	Kieser *et al*. (2000)
pSAN-lac	*bla*, *tsr*	Arisawa *et al*. (1995)
pMR01	Cosmid clone that contains some mycinose biosynthesis genes including *mycE* and *mycF*	Anzai *et al*. (2003)
pMG501	1.5-kb FRT-*neo-oriT*-FRT-*attB* cassette inserted into pLITMUS38	This study
pMG502	9.5-kb fragment containing a mycinose biosynthetic gene cluster region replacing *mycE* with a FRT-*neo-oriT*-FRT-*attB* cassette inserted into pSAN-lac	This study
pMG503	9.5-kb fragment containing a mycinose biosynthetic gene cluster region replacing *mycE* with an FRT-*neo-oriT*-FRT-*attB* cassette inserted into pSAN-lac	This study
pMG504	6.9-kb fragment containing *myrB*, *mycG*, *mycF*, *mycCI*, and *mycCII* inserted into pSAN-lac	This study
pMG505	*mycF* replaced with a FRT-*neo-oriT*-FRT-*attB* cassette on pMG504	This study
pMG506	*mycF* replaced with an FRT-*neo-oriT*-FRT-*attB* cassette on pMG504	This study
pMG507	*mycE*-controlled *mycCI* promoter *mycCI*p inserted into pSET152	This study
pMG508	1.4-kb BamHI–EcoRI fragment including *mycF* inserted into pSET152	This study

Table 2. PCR primers used in this study

Primer	Sequence (5′–3′)	Restriction site
FRTF+attB	ATCC**GGGTGCCAGGGCGTGCCCTTGGGCTCCCCGGGCGCGTA**attccggggatccgtcgacc	–
FRTR	ATCtgtaggctggagctgcttc	–
mycFendF	CTGAGTCGCCCACCAGGCAAGGAGGTATTTCTGCCCATGgggtgccagggcgtgccctt	–
mycFendR	CGGATCGACCCGACCGCCGGCAGTGGACCGGGAACGTCAtgtaggctggagctgcttc	–
152attPF	AATCGCTCTTCGTTCGTCTG	–
152intR	AATGCCCGACGAACCTGAAC	–
MGneo860F	TCTCCTGTCATCTCACCTTG	–
mycCIPFNh	CTCATGCTAGCAAATACCTCCTTGCCTGG	NheI
mycCIPRNd	GCGGTCCATATGCCAGACCACCATCTTCTG	NdeI
mycEF	ATGACCGCACAGACCGA	–
mycEFNd	CAGCATATGACCGCACAGACCGAATTC	NdeI
mycERBam	GCGGATCCGGGAGGAGCTCATGTCGCGCCT	BamHI
mycFCIendR	AGGAATTCTGACGGGAAGTATT	–
mycFF	TGCCGGTCGTGTGGTGACAG	–
NeoFEV	GATATCGGTTCATGTGCAGCTCCATC	–

Bacteriophage ϕC31 *attB* site was bold. The unique priming site P1 and P2 of disruption cassette FRT-*neo-oriT*-FRT on pIJ776 and the unique priming site 5′-end of *attB* and P2 of disruption cassette FRT-*neo-oriT*-FRT-*attB* on pMG501 are lowercase. The relevant restriction sites for genetic manipulation are underlined.

mycF gene. To generate pMG506 whose *neo* gene was in the same direction as the disrupted *mycF* gene, the 1.3-kb XbaI fragment including *neo* and *oriT* derived from pMG501 was ligated with the 12-kb XbaI fragment derived from pMG505.

pMG507 and pMG508 were constructed to perform the genetic complementation studies for *mycE* and *mycF* disruption mutants, respectively. The *mycCI* gene promoter region, *mycCI*p, was amplified by PCR with the primers mycCIPFNh and mycCIPRNd; the protein-coding region of *mycE* was also amplified with the primers mycEFNd and mycERBam. After determining the sequences of these amplified fragments, the 0.5-kb NheI–NdeI fragment that included the *mycCI*p region along with the 1.2-kb NdeI–BamHI fragment coding *mycE* were inserted into the XbaI and BamHI sites on pSET152 to generate pMG507. To construct pMG508, the 1.4-kb BamHI–EcoRI fragment derived from pMR01 was cloned into pSET152.

Conjugation procedure

The intergeneric conjugation from *E. coli* S17-1 to *M. griseorubida* was performed using a modified protocol of our previous procedure (Anzai *et al*., 2004a). A mixture of the *E. coli* donor cells and the *M. griseorubida* recipient cells was spread on MR0.1S plates or AS-1 agar plates (Alexander *et al*., 2003). The plates were incubated at 32 °C for 20–24 h and then overlaid with 1 mL water containing 500 μg of nalidixic acid to inhibit further growth of *E. coli* and 1 mg of neomycin or apramycin for selecting the *M. griseorubida* exconjugants. The plates were then reincubated at 32 °C for 7–10 days for the growth of the exconjugants. The genetic conditions of the exconjugants were confirmed by PCR and Southern hybridization. The result of Southern blot analysis is shown in Supporting Information.

Production and analysis of mycinamicins

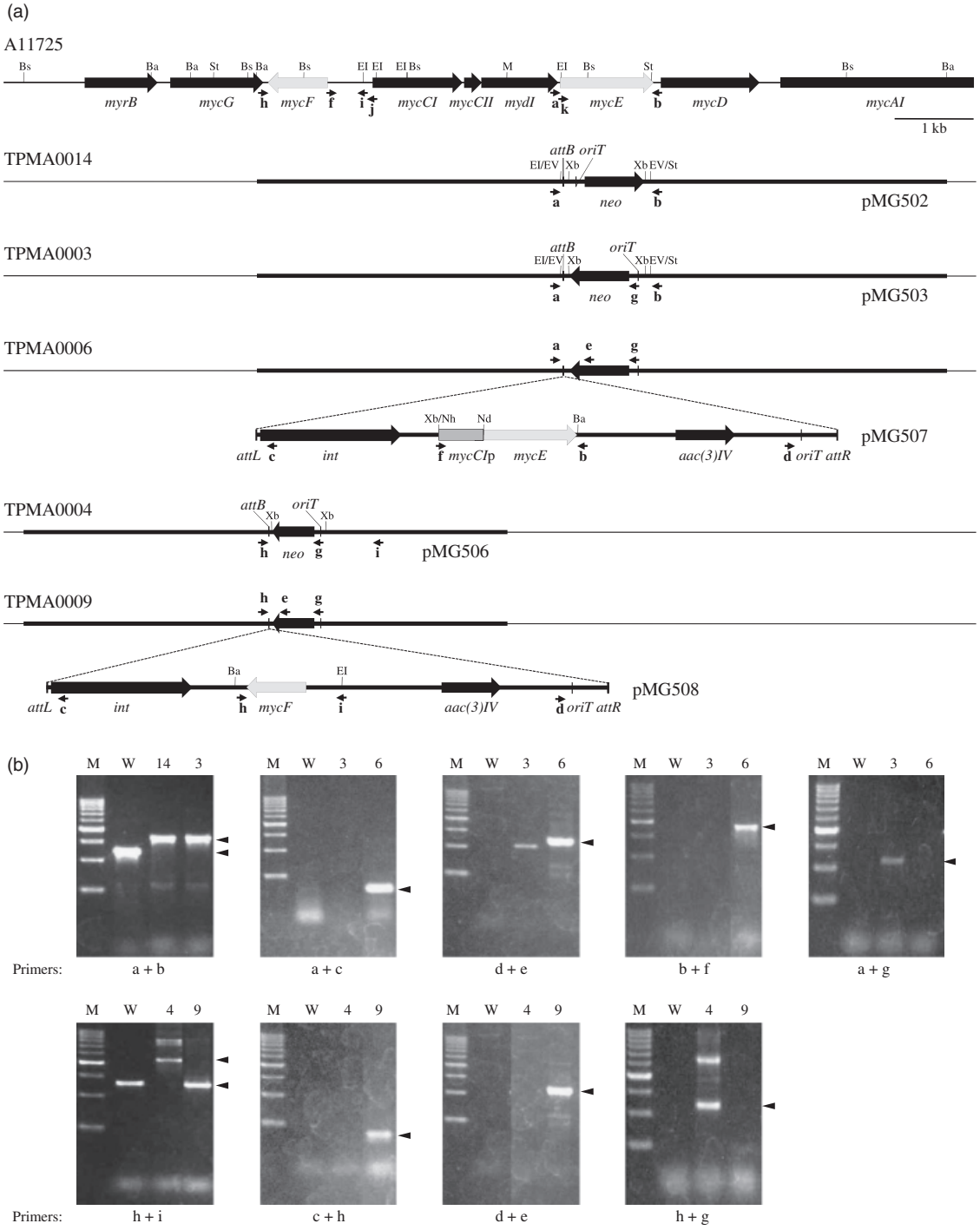
Micromonospora griseorubida was cultured in 5 mL MR0.1S broth or FMM broth with the appropriate antibiotics on a rotary shaker (150 r.p.m.) at 27 °C for 10 days. The broth was adjusted to pH 9–11 with 28% ammonia solution and extracted twice with an equal volume of ethyl acetate (EtOAc); the extract was then concentrated *in vacuo*. The crude extracts were dissolved with EtOAc, and then an equal volume of 0.1% trifluoroacetic acid (TFA) was added. The water layer containing mycinamicins was adjusted to pH 9–11 with 28% ammonia solution and extracted with an equal volume of EtOAc. The organic layer was concentrated *in vacuo*, and each residue was dissolved in MeOH for HPLC and liquid chromatography (LC-MS) analyses. HPLC analysis scanning was performed using a diode array detector model L-2450 (Hitachi, Japan) under the following conditions: ODS-80TM, i.d. = 150 × 4.6 mm (Toso Co., Japan); MeCN, 0.06% TFA (30 : 70); flow rate, 0.8 mL min^{-1}; and UV wavelength, 200–300 nm. LC-MS analysis was performed on LCMS2010 (Shimadzu) using reverse-phase HPLC [STR ODS-II, i.d. = 150 × 2.0 mm; MeCN, 0.06% TFA (35 : 65); flow rate, 0.2 mL min^{-1}; and UV wavelength, 220 nm]. Standard compounds of M-II, M-III, and M-VI were obtained from the fermentation broth of *M. griseorubida* A11725.

Results

Disruption and complementation of the *mycE* gene

The disruption cassette FRT-*neo-oriT*-FRT-*attB* was used to obtain the *mycE* disruption mutant of *M. griseorubida*. In previous studies, the transconjugant of *M. griseorubida* has never been isolated with pSET152 as an intergeneric conjugation vector. Therefore, we estimated that *M. griseorubida* would not possess the bacteriophage ϕC31 *attB* site on the chromosome. The *mycE*-deleted plasmid pMG502, which had the mycinose biosynthetic gene cluster region in which *mycE* was replaced with the disruption cassette, was generated with pSAN-lac as the suicide vector. pSAN-lac was constructed with pUC18 and pIJ350 as an *E. coli–Streptomyces* shuttle vector, but the plasmid has never been amplified in *M. griseorubida* cells (data not shown). Plasmid pMG502 was transferred from *E. coli* to *M. griseorubida* A11725 by intergeneric conjugation, and some neomycin-resistant (neo^r) and thiostrepton-sensitive ($thio^s$) transconjugants were isolated. PCR was used to verify that the chromosomal copy of A11725 *mycE* was deleted by double cross-over. Using the primers mycEF and mycERBam annealing outside the disruption cassette, the 1.4- and 1.2-kb amplified fragments were observed in TPMA0014 and the wild strain A11725, respectively (Fig. 2b). The size difference indicated that TPMA0014 was the *mycE* disruption mutant. M-VI was detected in the EtOAc extract from the FMM culture broth of TPMA0014 at 7.63 min (Fig. 3). However, the productivity of M-VI by TPMA0014 was very low (0.08 μg mL^{-1}), and it was estimated that the direction of *neo* gene transcription had a negative effect on the productivity. We also isolated another *mycE* disruption mutant in which the direction of the *neo* gene was opposite to the mycinose biosynthesis gene cluster. The neo^r and $thio^s$ transconjugant TPMA0003, which was isolated by the introduction of pMG503 into A11725, was confirmed to be a *mycE* disruption mutant by PCR (Fig. 2b); the M-VI productivity (13.8 μg mL^{-1}) of TPMA0003 was higher than that of TPMA0014 (Fig. 3). Furthermore, three unknown peaks E-1, E-2, and E-3 were observed in the chromatogram of the extract of TPMA0003 at 5.62, 6.95, and 6.28 min, respectively. LC-MS was performed for the extract to measure the molecular weight of these metabolites (E-1; *m*/*z* 684, E-2; *m*/*z* 684, and E-3; *m*/*z* 698).

The expression of the *mycE* gene would be controlled with the *mycCI* intergenic *mycCI*p, which is located between *mycF* and *mycCI*, because there is no promoter region immediately upstream of *mycE*. The amplified *mycCI*p and *mycE* fragments were inserted into pSET152, and the resulting plasmid pMG507 possessing the *mycE* gene under the control of *mycCI*p was introduced into TPMA0003. The resulting apramycin-resistant (apr^r) transconjugant TPMA0006 produced M-II (2.4 $\mu g\,mL^{-1}$), and the amount of M-II produced by TPMA0006 was 14% of that produced by the wild strain A11725. It was confirmed by PCR that pMG507 was inserted into the artificial *attB* site on the TPMA0003 chromosome by a site-specific recombination between the *attB* site and the *attP* site derived from



pSET152. Using the primers mycEF and 152intR annealing outside *attL* and the primers 152attPF and MGneo860R annealing outside *attR*, 0.4- and 1.2-kb fragments were amplified from TPMA0006, respectively (Fig. 2b). These results proved that site-specific recombination between the artificial *attB* site and the *attP* derived from pSET152 occurred on the TPMA0003 chromosome. The existence of *mycE* combined with *mycCI*p was also confirmed by PCR with the primers mycCIPFNh and mycERBam annealing the 5′-end region of *mycCI*p and the 3′-end region of *mycE*, respectively (Fig. 2b). Moreover, using the primers mycEF and NeoFEV (annealing the 3′-end region of *neo*), the 1.1-kb amplified fragment – derived from TPMA0003 – was not observed in the TPMA0006 lane (Fig. 2b). These results indicated that the transconjugant TPMA0006 producing M-II was the homogenous *mycE* complementation strain on which the *mycE* gene under the control of *mycCI*p was located at the artificial *attB* site on the chromosome.

Disruption and complementation of the *mycF* gene

PCR targeting with the phage λ-Red recombinase was used to isolate the *mycF* disruption mutant. The *mycF* disruption cassette was amplified with long PCR primers, mycFendF and mycFendR, which included 39-nt targeting sequences and 20- or 19-nt priming sequences. The priming sequences of mycFendF and mycFendR were annealed at a part of the *attB* site and a flanked region of the FRT site, respectively. Replacement of *mycF* in pMG504 was achieved by the PCR-amplified gene disruption cassette FRT-*neo*-*oriT*-FRT-*attB* by electroporation into *E. coli* BW25113/pIJ790 containing pMG504, and the resulting plasmid pMG505 was introduced into A11725 by intergeneric conjugation. The resulting neo^r and thio^s transconjugant TPMA0016 produced M-III, whose productivity was the same as that of the following transconjugant TPMA0004 (data not shown). Plasmid pMG506, whose *neo* gene was in the same direction as the disrupted *mycF* gene, was also introduced into A11725. The resulting neo^r and thio^s transconjugant TPMA0004 was cultured in FMM broth, and M-III was detected in the EtOAc of the culture broth (7.9 μg mL^{-1}, Fig. 3). Furthermore, two unknown peaks F-1 and F-2 (5.33 and 10.7 min, respectively) were detected in the extract of TPMA0004; the molecular weight of these compounds was the same (*m*/*z* 698). When PCR was performed for TPMA0004 and A11725 using the primers mycFF and mycFCIendR annealing outside the FRT-*neo*-*oriT*-FRT-*attB* cassette, different-sized fragments (2.2 and 1.3 kb, respectively) were observed in the agarose gel (Fig. 2b). The difference in size indicated that TPMA0004 was the *mycF* disruption mutant.

For genetic complementation for the *mycF* disruption mutant TPMA0004, pMG508 including *mycF* was transferred to TPMA0004 by intergeneric conjugation. The transconjugant TPMA0009 isolated from the conjugation plate containing apramycin and nalidixic acid produced M-II (8.29 μg mL^{-1}) (Fig. 3). The amount of M-II produced by TPMA0009 was approximately 55% of that produced by the wild strain A11725. PCR was performed with several primer pairs to confirm the genetic condition of TPMA0009. As shown in Fig. 2b, the transconjugant TPMA0009 producing M-II was the homogenous *mycF* complementation strain in which the *mycF* gene was inserted into the chromosome by a site-specific recombination between the artificial *attB* site on the chromosome and the *attP* site on pMG508.

Discussion

The disruption cassette FRT-*neo*-*oriT*-FRT-*attB* was used to obtain the *mycE* disruption mutant TPMA0003 and the *mycF* disruption mutant TPMA0004 of *M. griseorubida*. In particular, PCR targeting with the phage λ-Red recombinase was performed to isolate the *mycF* disruption mutant. Furthermore, from these mutants, the homogenous complementation strains TPMA0003 and TPMA0004 were isolated by a site-specific recombination between the artificial *attB* site on the mutant chromosomes and the *attP* on pSET152 used as a vector. Recently, a simple and highly efficient PCR-targeting system was developed for the gene targeting of *Streptomyces* strains (Gust *et al.*, 2003). However, genetic engineering cannot be performed for actinomycete strains lacking the bacteriophage ϕC31 *attB* attachment site using vectors possessing a ϕC31 *int* gene and an *attP* site. In this study, gene disruption and

Fig. 2. (a) Physical maps of the region including *mycE*, *mycF*, and those flanking the genes on the chromosomes of wild strain *Micromonospora griseorubida*, *mycE* and *myc*F disruption mutants, and the complementation strains. Oligonucleotide priming nucleotide sequences on the mycinamicin biosynthetic gene cluster are shown as small arrows with lowercase alphabets: a, mycEF; b, mycERBam; c, 152intR; d, 152attPF; e, MGneo860F; f, mycCIPFNh; g, NeoFEV; h, mycFF; i, mycFCIendR; j, mycCIPRNd; and k, mycEFNd. The relevant restriction sites (Ba, BamHI; Bs, BsiWI; EI, EcoRI; EV, EcoRV; M, MluI; Nd, NdeI; Nh, NheI; St, StuI; and Xb, XbaI) are indicated. (b) Agarose gel electrophoresis of PCR products from wild strain *M. griseorubida*, *mycE* and *myc*F disruption mutants, and corresponding complementation strains. The PCR product was separated by electrophoresis using a 1.6% agarose gel. Expected PCR fragments on each agarose gel are indicated with arrows. Nonspecific fragments additionally appeared on the following lanes: a 0.5-kb fragment on lane 14 using primers a+b; a 0.5-kb fragment on lane 3 using primers a+b; 0.5- and 0.6-kb fragments on lane 6 using primers d+e; 3.5- and 4.0-kb fragments on lane 4 using primers h+i; 0.5- and 0.6-kb fragments on lane 9 using primers d+e; and a 2.5-kb fragment on lane 4 using primers h+g. Using primers d+e, an unexpected 1.0-kb fragment was observed on lane 3. W, A11725 (wild); 3, TPMA0003 (Δ*mycE*), TPMA0014 (Δ*mycE*); 6, TPMA0006 (*mycE* complemented); 4, TPMA0004 (Δ*mycF*), TPMA0009 (*mycF* complemented); and M, 500-bp ladder marker (MR500, Takara).

complementation studies could be performed for *M. griseorubida*, which lacked the bacteriophage ϕC31 *attB* site on the chromosome, using the disruption cassette FRT-*neo*-*oriT*-FRT-*attB*. A multiple gene disruption and complementation scheme using the disruption cassette is shown in Fig. 4. In this study, the complementation plasmid pMG508 possessing the *int* gene encoding integrase, the *attP* site, and the resistant marker *aac(3)IV* was inserted into the ϕC31 *attB* attachment site, which was flanked by the resistant marker *neo* and *oriT* on the *mycF* disruption mutant. For additional gene disruption and complementation studies of the complementation strain TPMA0009, resistant markers other than *neo* and *aac(3)IV* should be used. However, if a gene disruption mutant with the resistant marker eliminated was obtained by in-frame disruption, additional gene disruption studies can be performed with the same resistant marker. This multiple system would be a useful tool for genetic engineering studies of not only actinomycete strains lacking the *attB* site but also other strains possessing an *attB* site, such as *Streptomyces* strains.

The *mycE* disruption mutant TPMA0003 and the *mycF* disruption mutant TPMA0004 mainly produced the M-II intermediates M-VI and M-III, respectively. Based on the nucleotide sequence data, we have already proposed that the genes *mycE* and *mycF* encode OMTs and that these OMT proteins convert M-VI to M-III and M-III to M-IV, respectively (Anzai *et al.*, 2003). Moreover, based on enzymatic studies, it was proved that MycE and MycF proteins catalyze methylation at the C2″-OH group of 6-deoxyallose in M-VI and methylation at the C3″-OH group of javose (i.e. C2″-methylated 6-deoxyallose) in M-III, respectively (Inouye *et al.*, 1994; Li *et al.*, 2009). Therefore, the results from these disruption mutants supported these previous studies. In the EtOAc extract from the culture broth of TPMA0003, three new minor peaks E-1, E-2, and E-3 were detected. TPMA0003 had intact *mycG* genes, which encoded the cytochrome P450 enzyme catalyzing both hydroxylation and epoxidation at C14 and C12/13 on the macrolactone ring of mycinamicin. The overexpressed MycG protein recognized M-VI as its substrate (Anzai *et al.*, 2008). Therefore, the compounds of E-1 and E-2 were hypothesized to be C14-hydroxy-M-VI and C12/13-epoxy-M-VI, respectively, from their molecular weights, UV absorption spectra, and retention times. C14-hydroxy-M-VI has already been published as mycinamicin XV by Kinoshita *et al.* (1992), but

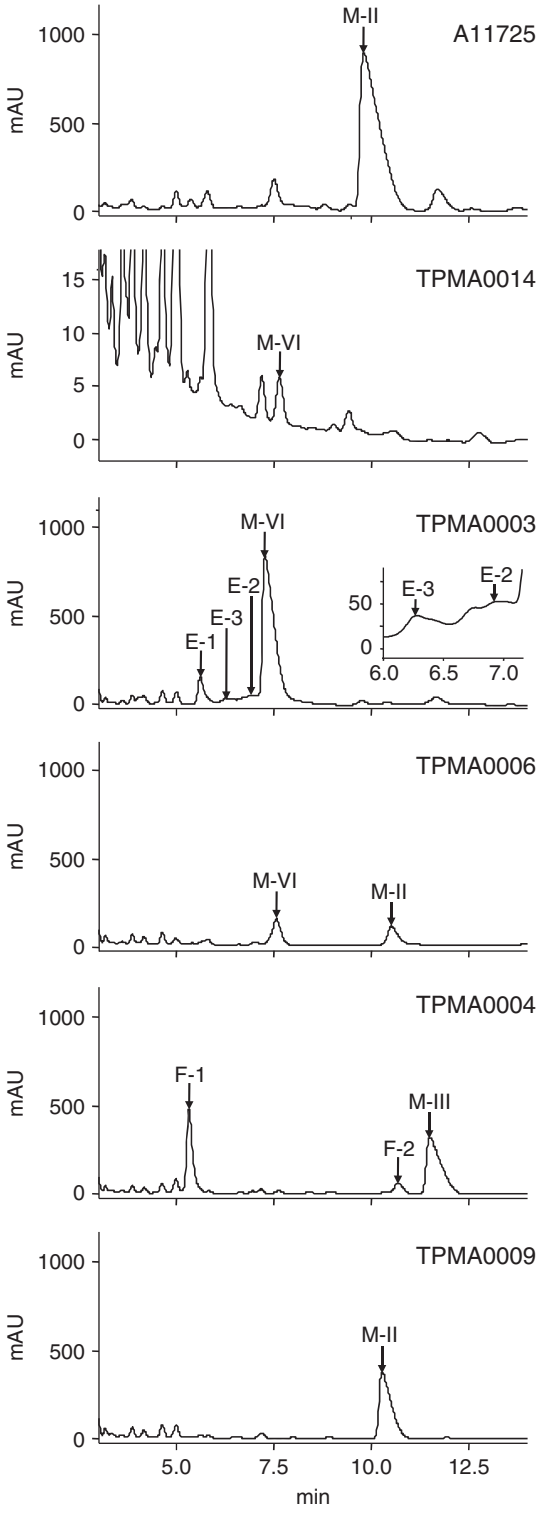


Fig. 3. HPLC analysis of the EtOAc extract from *Micromonospora griseorubida* culture broth. *Micromonospora griseorubida* A11725, TPMA0014, TPMA0003, and TPMA0004 were cultured in 5 mL of FMM broth at 27 °C for 10 days. *Micromonospora griseorubida* TPMA0006 and TPMA0009 were cultured in 5 mL of MR0.1S broth at 27 °C for 10 days. Chromatograms were scanned at 220 nm. UV λ_{max} nm of peaks: M-II, 218, 242 nm (sh); M-VI, 213, 284 nm; E-1, 216, 281 nm; E-2; 217, 242 nm (sh); E-3, 217, 281 nm; M-III, 215, 283 nm; F-1, 216, 281 nm; and F-2, 218, 242 nm (sh).

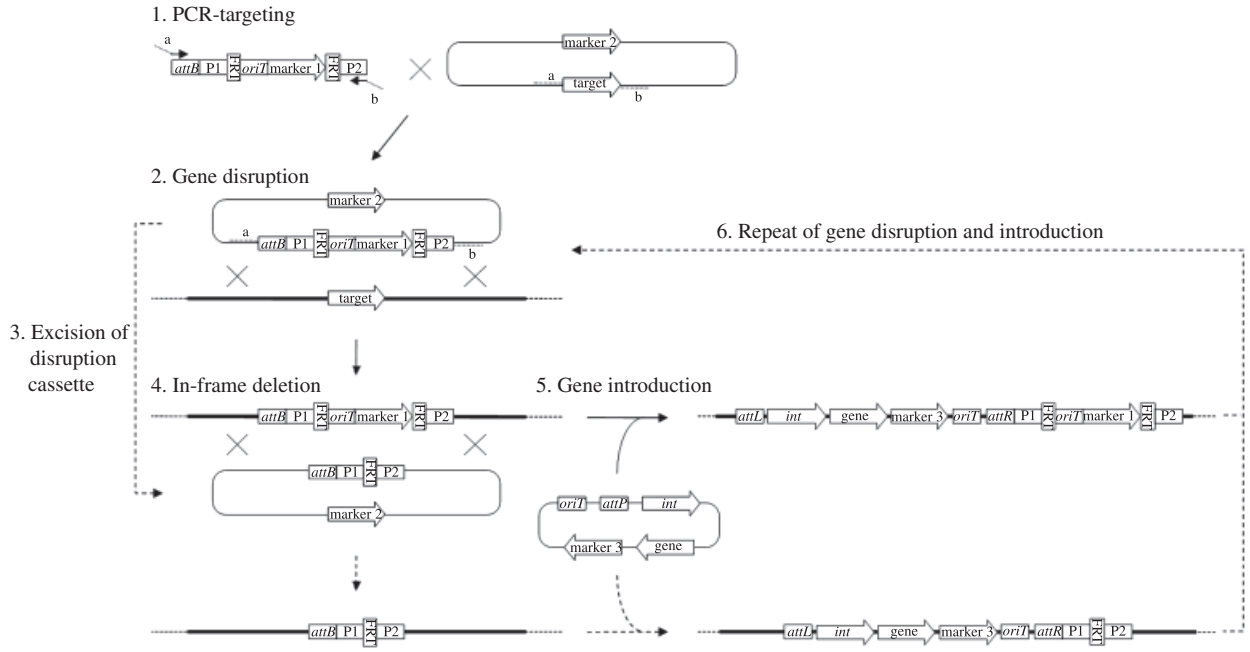


Fig. 4. Scheme of the multiple gene disruption and complementation method using the disruption cassette FRT-marker-*oriT*-FRT-*attB*. Step 1, PCR targeting: a disruption template containing marker 1, *oriT*, P1, P2, two FRT sites, and *attB* is amplified by PCR using primers containing a 39-bp 5′ homology region flanking the target gene (dotted lines a and b) and a 20-bp priming site (*attB* and P2 site). Then, the PCR product is transformed to *Escherichia coli* BW25113/pIJ790 (expressing the λ-Red recombination function) containing the plasmid with the cloned target gene. Step 2, Gene disruption: the recombinant plasmid, whose target gene is replaced with the disruption cassette marker 1-*oriT*-*attB*, is transferred by intergeneric conjugation from *E. coli* to the actinomycete strain. Selected actinomycete exconjugants are screened for resistance and susceptibility for two antibiotics corresponding to marker 1 and marker 2, respectively. The double cross-over allelic exchange is confirmed by PCR. Step 3, Excision of the disruption cassette: *E. coli* DH5α containing pCP20 was transformed with the recombinant plasmid, and FLP synthesis was induced. Step 4, In-frame deletion: the recombinant plasmid, with loss of the disruption marker, is transferred to the gene-disrupted actinomycete mutant by intergeneric conjugation. Exconjugants, resistant for an antibiotic corresponding to marker 2 and arising from single cross-over events, were restreaked nonselectively and screened for the loss of resistance for the two antibiotics corresponding to marker 1 and marker 2, indicating a successful replacement by in-frame deletion. The resulting exconjugants possess *attB*, P1, P2, and one FRT site, which is located at the target gene on the chromosome of the actinomycete strain. Step 5, Gene introduction: the transconjugation plasmid, possessing the ϕC31 *int* gene and an *attP* site with marker 3 and the extra and/or complementation gene(s), is transferred to disruption mutants obtained in stages 2 and 4. Selected actinomycete exconjugants are screened for resistance to an antibiotic corresponding to marker 3, and site-specific recombination between *attB* and *attP* sites is confirmed by PCR. Step 6, Repeat of gene disruption and introduction: additional gene disruption and complementation studies can be performed with the same or other resistant markers using the same scheme. The steps shown with the dashed line were not demonstrated in this study.

C12/13-epoxyl-M-VI has never been reported. Moreover, TPMA0003 possesses the activity of methylation at the C3″-OH group of javose because the *mycF* gene was not disrupted in this mutant. Accordingly, the MycF protein would be able to recognize M-VI as its substrate and methylate the C3″-OH group of 6-deoxyallose on M-VI. The compound E-3 was estimated to be hydroxylated and methylated M-VI; these M-VI derivatives have never been reported. Therefore, we should determine their molecular structures in our future studies. Two new minor peaks F-1 and F-2 were detected in the EtOAc extract from the culture broth of TPMA0004. The overexpressed MycG protein also recognized M-III as its substrate (Anzai *et al.*, 2008). C14-hydroxy-M-III has already been reported as mycinamicin IX by Kinoshita *et al.* (1992), and C12/13-epoxyl-M-III has also been reported by Mierzwa *et al.* (1985). Therefore, the compounds of F-1 and F-2 were estimated to be C14-hydroxy-M-III (M-IX) and C12/13-epoxy-M-III, respectively.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Southern-blot analysis (a) of total DNA from wild-strain *Micromonospora griseorubida*, *mycE* and *myc*F disruption mutants, and the complementation strains, and physical maps (b) of the region including *mycE*, *mycF*, and those flanking the genes.

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