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The targeted inactivation of polyketide synthase *mycAV* in the mycinamicin producer, *Micromonospora griseorubida*, and a complementation study

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Abstract

Mycinamicin is a 16-membered macrolide antibiotic produced by *Micromonospora griseorubida* A11725, which shows strong antimicrobial activity against gram-positive bacteria. Recently, the nucleotide sequences of the mycinamicn biosynthetic gene cluster in *M. griseorubida* have been completely determined. Mycinamicin non-producer M7A21 was isolated by *mycAV* inactivation, which encodes the module 7 of mycinamicin polyketide synthase (PKS) required for the biosynthesis of the mycinamicin biosynthetic intermediate protomycinolide-IV (PML-IV). When the bioconversion to mycinamicin II (M-II) from PML-IV was performed using M7A21 and the feeding culture method, the productivity of M-II was the same as that of M-II in wild-type strain A11725. p446M7 containing *mycAV* was constructed using the *Escherichia coli–Streptomyces* shuttle vector pGM446. The mycinamicin productivity of M7A21 was restored by the introduction of p446M7 into the M7A21 cell, but almost all p446M7 was integrated into the chromosome of M7A21 because the plasmid was unstable in M7A21. The feeding culture and the introduction of the complement gene for M7A21 would be powerful tools to perform combinatorial biosynthesis for the production of new macrolide antibiotics.

Keywords: *Micromonospora griseorubida*; Mycinamicin; Polyketide synthase; Intergeneric conjugation

1. Introduction

Modular-type I polyketide synthases (PKSs) catalyze the biosynthesis of macrolides and other macrocyclic polyketides, which have valuable medicinal properties, including antibiotic, antifungal, antiparasitic, antitumor, and immunosuppressive properties [1]. The linearity between the catalytic domains present and the structure of their polyketide products makes modular PKSs attractive systems for combinatorial biosynthesis [2,3]. The erythromycin PKS, or 6 deoxyerythronolide B synthase (DEBS), from *Saccharopolyspora erythraea*, has been the most widely used system for combinatorial biosynthesis based on modular PKSs. Moreover, the other modular PKS systems, i.e., pikromycin PKS, oleandomycin PKS, megalomicin PKS, tylosin PKS, rapamycin PKS, were also used with erythromycin PKS or DEBS to engineer novel compounds by manipulating the domains or modules of these PKSs. Using these PKS systems, many novel compounds were produced as 14-membered polyketides or their derivatives and there are few reports of other-membered polyketides.

Mycinamicin, a 16-membered macrolide antibiotic produced by *Micromonospora griseorubida*, consists of a polyketide macrolactone substituted with two different 6-deoxyhexose sugars, desosamine and mycinose [4–6].

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The mycinamicin PKS of *M. griseorubida* has several remarkable features that make it a powerful system for combinatorial biosynthesis. Mycinamicins have interesting features such as the presence of α,β-unsaturated lactone and a lack of aldehyde moiety in the polyketide macrolactone, and have shown higher antibacterial activity than other clinically used macrolide antibiotics. Recently, the nucleotide sequence of the mycinamicn biosynthetic gene cluster in *M. griseorubida*, which has 22 open reading frames (ORFs) including the self-resistance gene *myrB*, has been completely determined [7]. Here, we report the isolation of the mycinamicin non-producer, using the targeted inactivation of the polyketide synthase *mycAV*, and a complementation study of the non-producer using chemical and genetic methods. This genetic engineered strain would be useful to perform combinatorial biosynthesis for the production of new macrolide antibiotics. A new compound 9-deformyl-12,13-epoxydesmycosin has been produced from a derivative of 5-*O*-mycaminosyl-tylonolide, which is a tylosin biosynthetic intermediate, by this genetic engineered strain. The isolation of the new compound was described in detail in our report submitted to The Journal of Antibiotics [8].

2. Materials and methods

2.1. Strains, media and culture conditions

The wild strain, *M. griseorubida* A11725, was kindly provided by Toyo Jozo, Japan (now Asahi Kasei Co., Japan). *M. griseorubida* was grown at 27 °C on MR0.1S medium [9] and in MR0.1S broth, which is MR0.1S medium without Bacto-agar. *Escherichia coli* JM109 was used as the general subcloning host. *E. coli* S17-1 [10], and *E. coli* ET12567/pUZ8002 [11] were used as donors in intergeneric conjugations. Media were supplemented with the appropriate antibiotics (100 μg ampicillin ml^{-1}, 50 μg apramycin ml^{-1}, 12.5 μg tetracyclin ml^{-1}, 25 μg chloramphenicol ml^{-1}, 50 μg kanamycin ml^{-1}, 10 μg thiostrepton ml^{-1} or 30 μg nalidixic acid ml^{-1}) as needed.

2.2. DNA manipulation and construction of plasmids

The procedure of genetic manipulation has been reported in our previous publication [7]. The DNA fragment containing *mycAV* was obtained from pMG10-1 [7] as 7.1 kb *Bst*XI–*Apa*LI fragment, treated with T4 DNA polymerase to create blunt ends, ligated *Eco*RI linker, and inserted into *Eco*RI-digested pLITMUS 38 (New England BioLabs) to create pM7AB. A 1.0-kb fragment containing the apramycin resistance gene (*apr*) was amplified from pKC505 [12] using oligo nucleotide primers AprF (5′-GGTTCATGTGCAGCTCCATC-OH-3′) and AprR (5′-TCGTTAGTCGGAGGCCAAAC-OH-3′), and cloned into pGEM-T Easy Vector (Promega) using the TA cloning system to create pGEMApr. The *Eco*RI fragment including *apr*, the Apr fragment, was obtained from pGEMApr, treated with T4 DNA polymerase, and inserted into *Not*I-digested and blunt-ended pM7AB to create pM7ABApr. The disrupted *mycAV* gene was obtained from pM7ABApr as 8.1 kb *Eco*RI fragment, and inserted into *Eco*RI-digested pDN18, which was the RK2-based cloning vector with *oriT* [13], to create pDNM7ABApr. As a complementation study of the *mycAV*-inactivated strain, a 7.1-kb *Eco*RI fragment, including the *mycAV* gene from pM7AB, was inserted into *Eco*RI-digested pGM446 to create p446M7.

2.3. Conjugation procedure

Intergeneric conjugation from *E. coli* S17-1 or *E. coli* ET12567/pUZ8002 into *M. griseorubida* was performed in a manner similar to the method of Rose and Steinbüchel [14] on MR0.1S medium. Overnight culture of *E. coli* donor strain was diluted into fresh medium and incubated for 3–5 h. The cells were harvested, washed twice and concentrated 10-fold in TS broth. *M. griseorubida* grown in MR0.1S broth for 5 days, was harvested by centrifugation, washed and re-suspended in a half volume of TS broth. *M. griseorubida* recipient cells were mixed with a half volume of *E. coli* donor cells and 150 μl were plated on MR0.1S medium. The plates were incubated at 27 °C for 20 h and then covered with 1 ml water containing 500 μg of nalidixic acid to inhibit further growth of *E. coli* and 1 mg apramycin or 500 μg thiostrepton to select *M. griseorubida* exconjugants. Incubation at 27 °C was continued for 2–3 weeks to allow outgrowth of the exconjugants.

2.4. Fermentation, feeding and HPLC conditions for mycinamicins

A seed culture was grown in 5 ml MR0.1S broth for 5 days, and 150 μl of seed culture was spread on 15 ml MR0.1S agar plates. Feeding of PML-IV, the precursor of mycinamicins, was performed for the *mycAV*-inactive strain of *M. griseorubida*. For the feeding examination, 1 ml of PML-IV (320 μg) in 30% dimethyl sulfoxide/water was overlaid on the agar plate-spread seed culture. After 8 days, the agar was homogenized and extracted with a 2-fold volume of EtOAc containing 1% of triethylamine at 50 °C. The organic layer was concentrated in vacuo and each residue was dissolved in MeOH for HPLC analysis [8]. PML-IV was obtained from the fermentation broth of *M. griseorubida* A11725 [15].

2.5. Biological activity assays

The microbiological activity of mycinamicin production, using the wild-type and *mycAV*-inactived strain,

was assayed against *Micrococcus luteus* ATCC 9431 by the conventional agar-piece method. Well-grown colonies on MR0.1S agar medium for 1–2 weeks at 27 °C were picked up with a cork borer, and then placed on HI soft agar (0.8% agar in heart infusion broth) containing freshly grown *M. luteus* as the test organism. After overnight incubation at 37 °C, the zone of target cell-growth inhibition by micinamicin production was detected on agar.

2.6. Southern hybridization

Hybridization followed the standard phototope-detection protocol (New England BioLabs) using the biotin-labeled probe. DNA fragments containing *apr* gene and ACP-TE regions, which were located in *mycAV*, were labeled with biotin using the NEBlot Phototope Kit (New England BioLabs). The Apr fragment was isolated from pGEMApr, and the 1.7 kb fragment containing ACP and TE regions, ACP-TE fragment, was amplified from pMG160 [7] using oligonucleotide primers KR7ACP7F (5′-ACGGCTTCCGGCCGCTCTCGGA-OH-3′) and TEmydDR (5′-AATGACACGCTGGTC-CACTAC-OH-3′).

3. Results and discussion

3.1. Inactivation of mycAV in M. griseorubida

The location of *mycAV* alongside a cluster of genes involved in polyketide biosynthesis (Fig. 1) raised the possibility that *mycAV* might encode one of the multifunctional mycinamicin PKS genes required during mycinamicin production. To investigate this possibility and create a hybrid biosynthesis system through the bioconversion of mycinamicin analogues, *mycAV* was inactivated by homologous recombination on the chromosomal DNA of *M. griseorubida* A11725. pDNM7ABApr, whose vector used pDN18 as the suicide vector for actinomycetes, was introduced into A11725 through conjugal mating with *E. coli* S17-1. MR0.1S medium was used for this study, because the frequency of exconjugant formation on MR0.1S medium was higher than that of other media examined (data not shown). Most of the apramycin-resistant exconjugants did not produce antibiotics, whose productivity was observed using agar plate bioassay with *Micrococcus luteu* (Fig. 2). M-II was not detected in the agar plate of the M7A21 strain, which was one strain of the apramycin-resistant exconjugants, whereas the productivity of M-II in A11725 was 30.2 μg/plate (Fig. 2). It was confirmed by Southern blot hybridization that the chromosomal copy of A11725 *mycAV* was inactivated by double cross-over (Fig. 3(a)). Two hybridized bands (2.5 and 6.7 kb) were observed in M7A21 using the Apr fragment as a probe, because the *apr* gene has a *Bst*XI site. Moreover, the 8.1 kb hybridized band in A11725 shifted to 6.7 kb in M7A21 using the ACP-TE fragment as a probe (Fig. 3(b)). In M-II productivity and the Southern hybridization pattern, the same results were obtained in other apramycin-resistant exconjugants, which produced no antibiotics (data not shown).

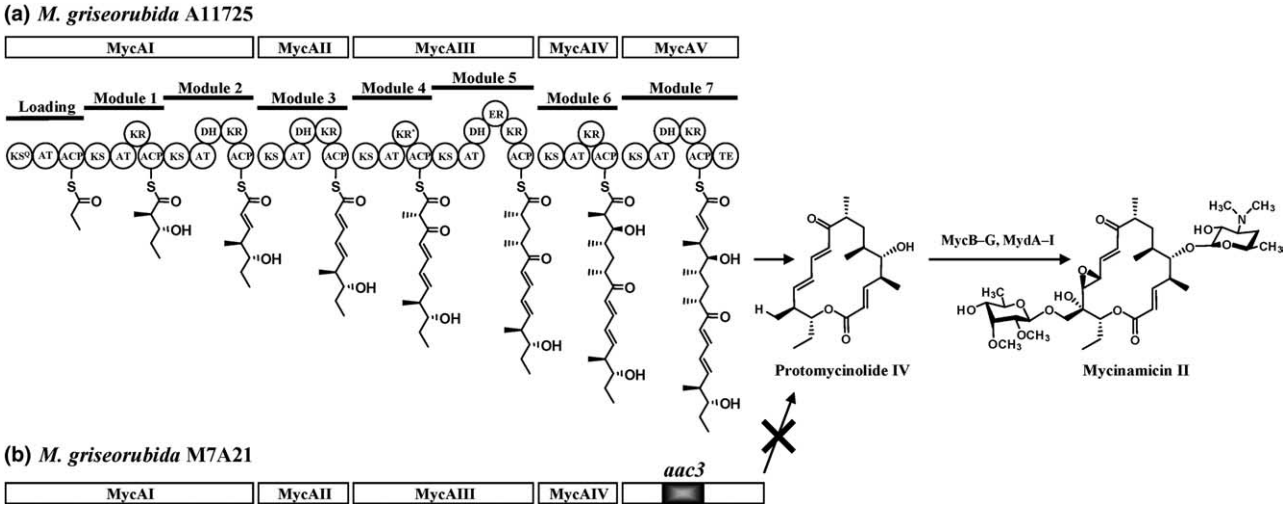

Fig. 1. Modular organization of mycinamicin polyketide synthase (mycinamicin PKS) in *Micromonospora griseorubida*. (a) Mycinamicin PKS (MycAI-V), MycB-G, and MydA-I are responsible for the production of mycinamicin II in wild-type *M. griseorubida* A11725. Each of the seven modules contains a ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP), as well as variable numbers of supplementary domains, such as ketoreductase (TE), dehydrase (DH), and enoylreductase (ER). The loading domain primer module 1 and a thioesterase (TE) domain cycle the octaketide and generate protomycinolide IV. (b) The formation of protomycinolide IV is blocked by inactive MycAV in *M. griseorubida* M7A21.

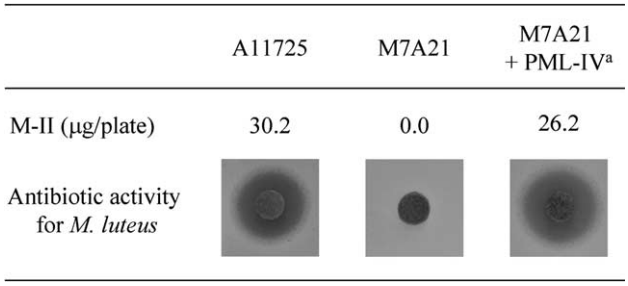

Fig. 2. Production of mycinamicin II in A11725 and M7A21, and bioconversion from protomycinolide IV to mycinamicin II in M7A21 on MR0.1S medium, and the antibiotic activity of these products for *M. luteus*. (a) 320 μg of PML-IV was fed into 15 ml MR0.1S medium.

3.2. Bioconversion of mycinamicin biosynthetic intermediates

M7A21 lost M-II productivity, because the *mycAV* gene was inactivated by double cross-over with pDNM7ABApr. The *mycAV* encoding module 7 of mycinamicin PKS was required for PML-IV biosynthesis. It was predicted that M7A21 only lacked productivity of mycinamicin biosynthetic intermediate, PML-IV, but the functions of deoxysugar biosynthesis by MydA to I, and the glycosylation and other modifications of PML-IV by MycB to G remained in this strain (Fig. 1). The characteristics of M7A21 may be similar to those of the non-produced strain C-34-10 [6], which was mutated by chemicals (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, NTG).

To confirm this, the bioconversion examination to M-II from PML-IV was performed with M7A21. When PML-IV (320 μg/plate) was fed into the MR0.1S agar plate spread with the culture broth of M7A21, M-II (26.2 μg/plate) was recognized in the 8-day culture plate (Fig. 2). The productivity of M-II with the bioconversion was the same as that of M-II in A11725, although the molecular ratio of bioconversion to M-II from PML-IV was about 4%. These results suggested that the bioconversion of macrolide intermediate using M7A21 might be suitable to produce new macrolide antibiotics. As a bioconversion product, desmycosin, which is the degradation product of tylosin, has been isolated by the feeding culture method from the tylosin biosynthetic intermediate 5-*O*-mycaminosyl-tylonolide (OMT) [8]. Furthermore, the hybrid compounds isolated from the cultured agar plate with 19-deformyl-5-*O*-mycaminosyl-tylonolide, which is a derivative of OMT, added to M7A21 were determined to be 19-deformyl-desmycosin [16] and a novel compound 19-deformyl-12,13-epoxydesmycosin [8], which was a further oxidation product similar to mycinamicin I. Therefore, the feeding culture with M7A21 would be powerful tools to perform combinatorial biosynthesis for the production of new macrolide antibiotics. The molecular ratio of bioconversion from the intermediate would be improved by optimization of the fermentation and feeding conditions.

3.3. Complementation of the mycAV-inactive strain M7A21

For the complementation study of the inactivated *mycAV* gene, p446M7 was constructed using the *E. coli–Streptomyces* shuttle vector pGM446. p446M7 was introduced into M7A21 from *E. coli* ET12567/pUZ8002 with conjugation. The thiostrepton-resistant exconjugant of M7A21, the strain M7A21/p445M7, produced M-II with the same productivity as wild-type 11725A (data not shown). To confirm the presence of p446M7 in the exconjugant of M7A21, plasmid DNA was isolated and retransferred to *E. coli* JM109. The transformation frequency of *E. coli* JM109 by the plasmid was very low, and in about 20% of the transformants, the plasmid size differed from that of p446M7 (data not shown). To observe the p446M7 condition in M7A21/p446M7, Southern blot hybridization analysis was performed using the Apr fragment and ACP-TE fragment as probes (Fig. 3(b)). p446M7 digested with *Bst*XI, 3.1 and 11.7 kb hybridized bands, which had strong and weak signals, respectively, appeared using the Apr fragment, and an 11.7 kb hybridized band appeared using the ACP-TE fragment. There were no hybridized bands corresponding to those of p446M7 in the total DNA of M7A21/p446M7. Using the ACP-TE fragment as a probe, 8.6 kb and 9.7 kb hybridized bands were detected in the total DNA of M7A21/p446M7 digested with *Bst*XI, and, furthermore, two strong signals (3.1 and 9.7 kb) and two weak signals (2.5 and 8.6 kb) appeared using the Apr fragment. These results showed that there was little p446M7 in the cytoplasm of M7A21 for the instability of p446M7, and almost all p446M7 introduced into M7A21 was integrated into the inactivated *mycAV* region in the M7A21 chromosome by a single cross-over (Fig. 3(a)).

The *E. coli–M. griseorubida* shuttle cosmid vector pTYS507 (29.2 kb) was constructed by Inoue et al. [9,17]. However, the frequency of the transformant formation of *M. griseorubida* A11725 by pTYS507 prepared from *E. coli* was not high. We expected that it would be difficult to introduce >40 kb of plasmid, for example, pTYS507 cloned to about a 10–20 kb DNA fragment coding, such as the modular polyketide synthase, into the protoplasts of *M. griseorubida* with the transformation method. Therefore, intergeneric conjugation using pGM446 was performed to introduce a large DNA fragment including the mycinamicin PKS gene into *M. griseorubida*. pGM446 introduced from *E. coli* ET12567/pUZ8002 with conjugation was stable in 90% of *Micromonospora aurantiaca* exconjugants [14]. In this experiment, the mycinamicin productivity

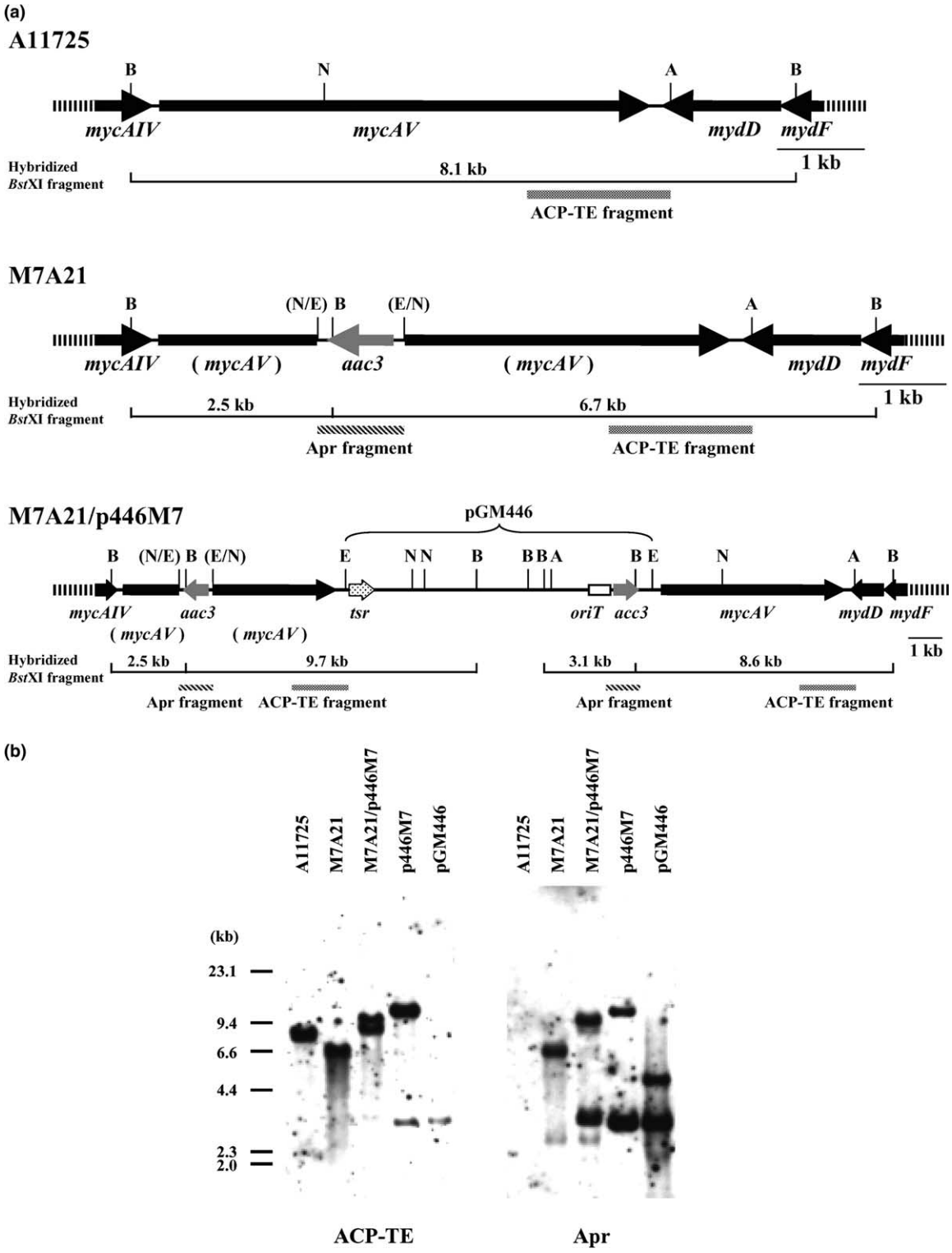

Fig. 3. (a) Physical maps of chromosomal DNA comprising wild-type *M. griseorubida* A11725 *mycAV*, the inactivated *mycAV* of *M. griseorubida* M7A21, and the inactivated *mycAV* region, into which p446M7 was integrated by a single cross-over of *M. griseorubida* M7A21. The gray arrow, the stippled arrow, and the open box represent the apramycin-resistant gene (*aac3*), thiostrepton-resistant gene (*tsr*), and *oriT*, respectively. The localization of hybridized probes (Apr and ACP-TE fragments) is shown in the maps of each chromosomal DNA. The relevant restriction sites (A; *Apa*LI, B; *Bst*XI, E; *Eco*RI, N; *Not*I) are indicated. (b) Southern hybridization analysis of chromosomal DNA from A11725 (wild-type), M7A21 (*mycAV*-inactivated strain), and M7A21/p446M7 (complemented strain). DNA (1 μg) from the corresponding strain and plasmid DNA (0.01 μg) used in this experiment was digested with *Bst*XI, separated by electrophoresis in 0.6% (w/v) agarose gel, and transferred on Hybond N (Amersham). Hybridization followed the standard phototope-detection protocol (New England BioLabs) using the biotin-labeled probe. *Hin*dIII-digested λ DNA was used as the standard size. The 3.1-kb hybridized bands of p446M7 and pMG446 appeared as non-specific bands using the ACP-TE fragment as a probe.

of M7A21 was restored by the introduction of p446M7 into the M7A21 cell, but almost all p446M7, which was unstable in M7A21, was integrated into the M7A21 chromosome.

Furthermore, we obtained the thiostrepton-resistant exconjugant with a plasmid, which introduced the *mycAV* and thiostrepton-resistant gene into the site-specific integration vector pSET152 [18], and the exconjugant produced M-II with the same productivity as wild-type 11725A. This may be suitable to introduce a large DNA fragment into the cells of *M. griseorubida* using pSET152, but if a large DNA fragment was necessary *in trans*, a new plasmid vector should be constructed. As described above, these genetic approaches would be also a powerful tool in combinatorial biosynthesis.

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