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Identification of a Cytochrome P450 Hydroxylase Gene Involved in Rifamycin Biosynthesis by *Amycolatopsis mediterranei* S699

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Abstract In analyzing the region of the *Amycolatopsis mediterranei* S699 chromosome responsible for the biosynthesis of the ansamycin antibiotic rifamycin, we identified a gene, designated *orf0*, which is located immediately upstream of the rifamycin polyketide synthase (PKS). *Orf0* encodes a protein, on the basis of sequence-comparative analysis, that is similar to several cytochrome P450 monooxygenases from different sources. The rifamycin producer, *A. mediterranei*, predominantly produces rifamycin B from its macrocyclic intermediate, proansamycin X, through dehydrogenation and hydroxylation steps. However, an *A. mediterranei* strain, deleted in *orf0* by gene replacement, no longer produced rifamycin B. Furthermore, a versatile replicative vector in *A. mediterranei* was constructed and rifamycin B production was restored in a complementation experiment of *orf0* using this novel vector. These consecutive results verified that the Orf0 protein, which is a P450 hydroxylase, is required for the production of rifamycin B in *A. mediterranei*.

Key words: *Amycolatopsis mediterranei*, rifamycin, proansamycin X, replication vector, P450 monooxygenase

Rifamycins [13], exemplified by rifamycin B (Fig. 1), and some of rifamycin derivatives represent a major class of medically important ansamycin antibiotics produced by *Amycolatopsis mediterranei* [19]. Since isolated in 1959 from *A. mediterranei* [19], rifamycin has been clinically used in a synthetically modified form called rifampicin and is still one of the first-line effective antibiotics in the treatment of tuberculosis and other mycobacterial infections [20].

The biosynthesis of rifamycins involves the assembly of a polyketide through chain extension of an unusual starter unit, 3-amino-5-hydroxybenzoic acid (AHBA) [6], by two acetate and eight propionate units on a type I polyketide synthase (PKS) [8, 11]. This multifunctional enzyme consists of 10 modules that catalyze successive rounds of polyketide chain elongation to build an undecaketide [1, 23], which is then released from the enzyme as a macrocyclic lactam named proansamycin X [1, 22, 23, 25]. This initial cyclization product is then subjected to extensive downstream processings to give the final biologically active rifamycins. Among these post-polyketide modification steps, it shows that the hydroxylation at C-34a, followed by the dehydrogenation of C-7 and C-8 of proansamycin X, result in the formation of rifamycin B from the macrocyclic intermediate, proansamycin X (Fig. 1).

Several cytochrome P450 enzymes have been reported in different species of the industrially important antibiotic-producing actinomycetes [9, 12, 14]. Some of them have been reported to participate in the marcrolide biosynthesis [21, 24]. The rifamycin biosynthetic gene cluster is one of the best characterized ansamycin gene clusters so far [1, 18, 23] and its biosynthesis has been extensively studied [4, 5, 25]. But genes involved in downstream biosynthetic pathways after the formation of early macrocyclic intermediates remain to be characterized.

This study describes the participation of *orf0* encoding the cytochrome P450 monooxygenase involved in rifamycin B biosynthesis by means of gene deletion and a complementation experiment.

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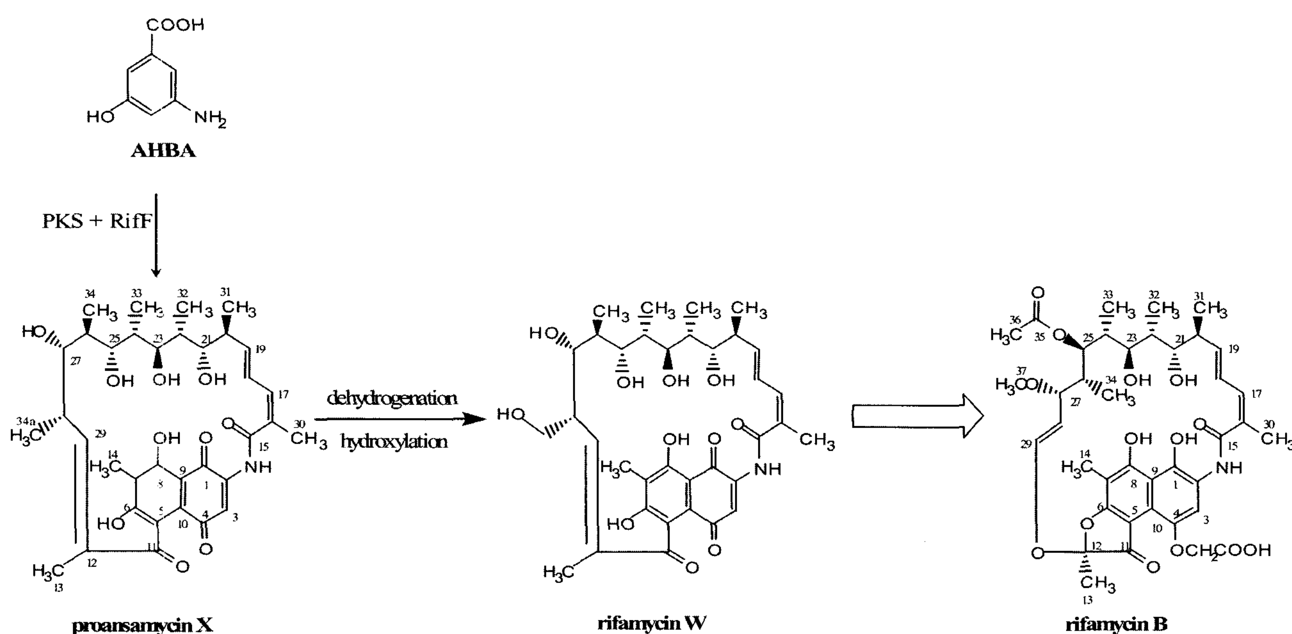

Fig. 1. Biosynthesis pathway to rifamycin B in *Amycolatopsis mediterranei.*

MATERIALS AND METHODS

Actinomycetes Strains and Plasmids

A. mediterranei strains and plasmids used in this study are listed in Table 1.

General Procedure

A. mediterranei strains were maintained on YMG agar and grown in YMG liquid medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose per liter of distilled water) for the preparation of protoplasts and seed inoculum for extractions. The *A. mediterranei* strain S699, which mainly produces rifamycin B, was a gift from Professor Giancarlo Lancini (Lepetit Research Laboratory, Geranzano, Italy).

A. mediterranei transformants were selected with hygromycin at 100 mg/ml in YMG medium. Reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or from EMD Chemical Co. (Gibbstown, NJ, U.S.A.).

The *Escherichia coli* strain DH5a was used for routine subcloning and *E. coli* plasmids pGEM-3Z, pGEM-3Zf(+) (Promega, Madison, WI, U.S.A.), and Litmus 28 (New England Biolabs. Inc., Beverly, MA, U.S.A.) were also used. The *E. coli* strain was grown in LB medium [16]. DNA isolation, restriction, and ligation were performed according to standard procedures [10, 16]. Restriction enzymes were obtained from New England Biolabs, Inc. (Beverly, MA, U.S.A.) and Promega (Madison, WI, U.S.A.). Southern analyses of genome DNA from

Table 1. The strains and plasmids used in this study.

Strains and plasmids	Description	References or source
Plasmids		
pANT841	cloning vector for *E. coli*	Chuck DeSanti
pANT841H	pANT841 derivative containing hygromycin resistant gene	This study
pANT841H/Δ*orf0*	pANT841H derivative containing part of *orf0* for in-frame deletion	This study
pULVK2	*Norcardia-E. coli* shuttle vector containing the replication origin of *A. mediterranei*	[3]
pXH106	vector containing hygromycin resistance gene	[7]
pAMR2	replicative vector in *A.mediterranei* for complementation experiment	This study
pAMR2-*orf0*	pAMR2 derivative containing *orf0*	this study
Strains		
A. mediterranei S699	Rifamycin B producer	Lancini
A. mediterranei Δ*orf0*	*A. mediterranei* S699 in which *orf0* was deleted by in-frame deletion	This study

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ATGACCGACGCAATATCCTTCGAGGTGCCGTGGGACCGGACCGACAAGTTCGACCCGCCC
M  T  D  A  I  S  F  E  V  P  W  D  R  T  D  K  F  D  P  P

GCGGTGTTCGACTCTCTGCGCGAAGAACGTCCGCTCGCGAAGATGGTTTACCCGGATGGG
A  V  F  D  S  L  R  E  E  R  P  L  A  K  M  V  Y  P  D  G
                                   NruI
CACGTCGGCTGGATCGTTTCCAGCTACGAGCTGGTCCGCGAGGTCCTCAGCGACCTGCGG
H  V  G  W  I  V  S  S  Y  E  L  V  R  E  V  L  S  D  L  R

TTCAGCCACAGCTGCGAAGTCGGCCACTTCCCGGTGACCCACCAGGGCCAGGTCATCCCG
F  S  H  S  C  E  V  G  H  F  P  V  T  H  Q  G  Q  V  I  P

ACCCACCCGCTGATCCCCGGCATGTTCATCCACATGGACCCGCCCGAGCACACGCGCTAC
T  H  P  L  I  P  G  M  F  I  H  M  D  P  P  E  H  T  R  Y

CGCAAGCTGCTGACCGGCGAGTTCACCGTCCGCCGCGCCAGCAGGCTGATCCCGCGGGCC
R  K  L  L  T  G  E  F  T  V  R  R  A  S  R  L  I  P  R  A

GAGGCCGTGGCCGCCGAGCAGATCGAGGTCATGCGGGCCAAGGGCGCCCCCGCGGACGTG
E  A  V  A  A  E  Q  I  E  V  M  R  A  K  G  A  P  A  D  V

GTCATGGACTTCGCCAAGCCGCTGGTGCTGCGGATGCTGGGCGAGCTCGTCGGCCTGCCC
V  M  D  F  A  K  P  L  V  L  R  M  L  G  E  L  V  G  L  P

TACGAGGAACGCGACCGGTACGTGCCCGCGGTGACCCTCCTGCACGACGCCGAAGCGGAC
Y  E  E  R  D  R  Y  V  P  A  V  T  L  L  H  D  A  E  A  D

CCGGCCGAGGCCGCGGCCGCCTACGAGGTGGCCGGGAAGTTCTTCGACGAGGTCATCGAG
P  A  E  A  A  A  A  Y  E  V  A  G  K  F  F  D  E  V  I  E
                                        XmnI
CGCCGCCGGCAGCGGCCCCAGGACGACCTCATCAGCTCGCTCGTCACCGAGGACCTGACC
R  R  R  Q  R  P  Q  D  D  L  I  S  S  L  V  T  E  D  L  T

CAGGAGGAGCTGCGCAACATCGTCACCCTGCTGCTGTTCGCCGGGTACGAGACCACCGAG
Q  E  E  L  R  N  I  V  T  L  L  L  F  A  G  Y  E  T  T  E
                                oxygen binding pocket
GGCGCGCTCGCCACCGGCGTCTTCGCGCTGCTGCACCACACCGATCAGCTGGCGGCACTG
G  A  L  A  T  G  V  F  A  L  L  H  H  T  D  Q  L  A  A  L

CGCGCGGAGCCGGAAAAGCTCGACGCCGCGATCGAAGAGCTGCTGCGCTACCTGACCGTC
R  A  E  P  E  K  L  D  A  A  I  E  E  L  L  R  Y  L  T  V

AACCAGTACCACACCTACCGCACCGCGCTGGAGGACGTGAAGCTGGAGGGCGAGCTGATC
N  Q  Y  H  T  Y  R  T  A  L  E  D  V  K  L  E  G  E  L  I

AAGAAGGGCGACACGGTGACGGTGTCGCTGCCCGCGGCCAACCGCGACCCGGCCAAGTTC
K  K  G  D  T  V  T  V  S  L  P  A  A  N  R  D  P  A  K  F

GGCTGTCCCGCGGAGCTCGACATCGAGCGGGACACCTCCGGCCACGTCGCGTTCGGCTTC
G  C  P  A  E  L  D  I  E  R  D  T  S  G  H  V  A  F  G  F

GGCATCCACCAGTGCCTGGGCCAGAACCTGGCGCGCATCGAGCTGCGGGCCGGCTTCACG
G  I  H  Q  C  L  G  Q  N  L  A  R  I  E  L  R  A  G  F  T
               * heme-binding Cys
GCGCTCCTGCGGGCGTTCCCCGAGCTCCGGCTGGCCGTCCCGGCCGACGAGGTTCCGCTG
A  L  L  R  A  F  P  E  L  R  L  A  V  P  A  D  E  V  P  L

CGGCTGAAGGGTTCCGTCTTCTCGGTGAAGAAGCTGCCCGTCTCCTGGTGA
R  L  K  G  S  V  F  S  V  K  K  L  P  V  S  W  *
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Fig. 2. Nucleotide sequence and deduced protein sequence of *orf0*.

The deduced protein sequence is shown below the DNA sequence. Two restriction sites used for in-frame deletion are underlined. Two active site motifs are underlined on the protein sequence.

A. mediterranei strains were performed by standard methods [10, 16].

Nucleotide Sequence of *orf0*

The cloning, sequencing, and characterization of the *orf0* gene from *A. mediterranei* strain S699 were performed as previously described [1]. The sequence data of this study are shown in Fig. 2 and available from GenBank under the accession number AF040570.

In-frame Deletion of *orf0*

To construct an in-frame deletion mutant of *orf0*, a 483-bp in-frame deletion was introduced *in vitro* into the DNA segment of *orf0*, and the mutant allele was introduced into *A. mediterranei* S699 to replace the wild-type counterpart in the chromosome. It was anticipated that deletion of the 483-bp segment using two blunt end restriction sites, *Nru*I and *Xmn*I, would remove 161 amino acids from Orf0 but would leave the reading frame intact (Fig. 2). From a pGEM-3Zf(+) derivative plasmid containing the 7.5-kb DNA fragment of rifamycin biosynthetic gene clusters [1], the 1.5-kb *Pst*I/*Mlu*I, 1.2-kb *Mlu*I/*Nru*I, and 3.1-kb *Xmn*I/*Apa*I fragments were isolated (Fig. 3) and simultaneously ligated to the *Pst*I/*Apa*I-digested pANT841H in order to generate a pANT841H derivative containing the part of *orf0*, pANT841H/D *orf0* (Fig. 3B). To construct pANT841H [4], the hygromycin resistance gene (Hyg^R) of pXH106 [3] was excised by two restriction enzymes, *Sma*I and *Eco*RV and inserted into the *Sma*I site of pANT841 (Fig. 4). pANT841 *E.coli* cloning vector was donated by Professor Chuck DeSanti at Ohio State University.

The resulting plasmid, pANT841H/Δ*orf0*, was used for gene replacement in the *A. mediterranei* strain. The heat-denatured pANT841H/Δ*orf0* was introduced by electroporation into the *A. mediterranei* S699 strain, and hygromycin resistant transformants resulting from the integration of pANT841H/Δ*orf0* by a single-crossover homologous recombination were selected as previously described [25]. The initial hygromycin-resistant clone produced by single-crossover recombination was grown non-selectively to isolate the antibiotic sensitive recombinant resulting from a second crossover through which the vector was excised and the target gene was replaced with its disrupted copy. The deletion mutants were analyzed by Southern hybridization.

This resulting strain is designated *A. mediterranei* Δ*orf0* for further complementation experiment.

Characterization of Metabolites Produced by *A. mediterranei* Δ*orf0* Strain

For HPLC analysis, cells were grown in shake flasks in YMG medium in the presence of hygromycin for 7 to 9 days at 30°C and then removed by centrifugation. The resulting supernatant was adjusted to pH 5.0 by the addition of 1 M of HCl and extracted twice with an equal volume of ethyl acetate. The organic phase, which contained the desired compounds, was dried in a rotary evaporator and redissolved in methanol to give an appropriate concentration for HPLC. Analyses were performed using Waters Model 510 pumps controlled by Waters Pump Control Module and Rheodyne injector with a Waters Photodiode Array detector 996 (Milford, MA, U.S.A.). Separations were performed at ambient temperature on a Waters Nova-Pak C18 column (3.9×150 mm). The mobile phase was a linear gradient from 100% of 0.1 M of sodium acetate (pH 4.5) to 100% of methanol over 20 min followed by isocratic elution for 10 min at a flow rate of 1 ml/min.

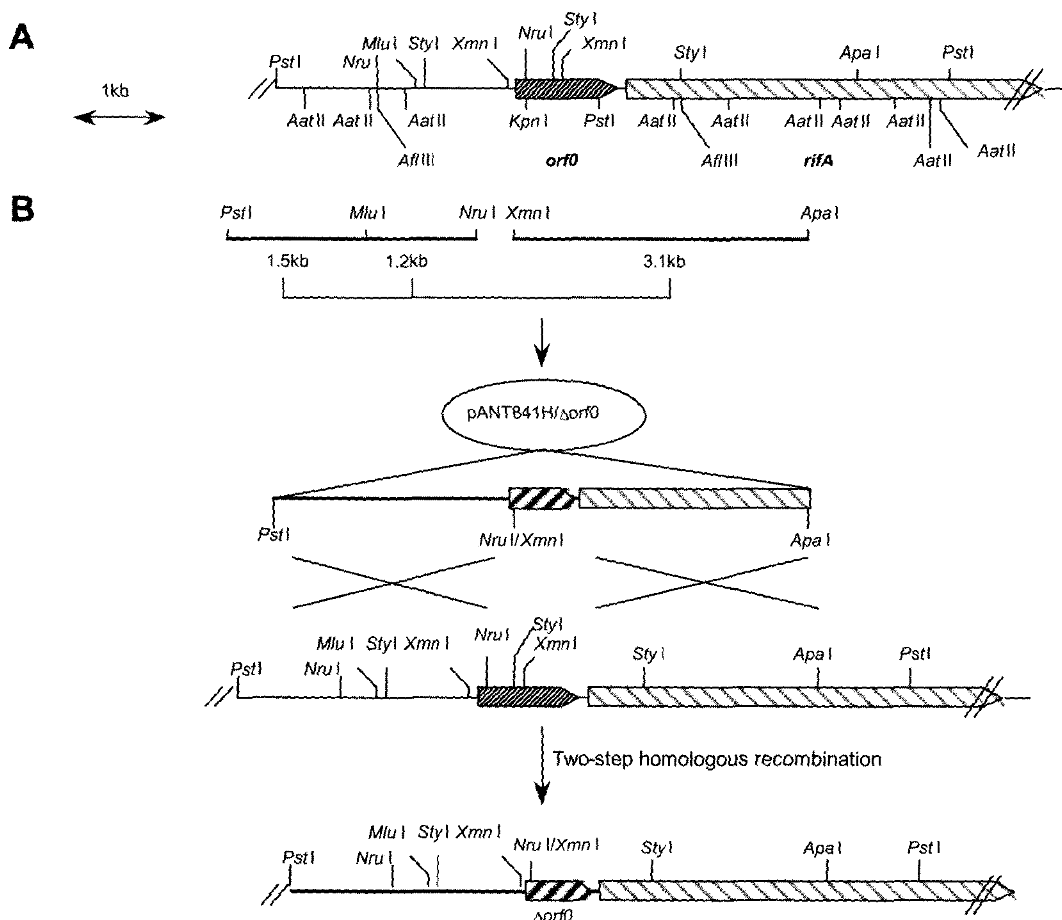

Fig. 3. Scheme for the deletion mutation of *orf0*.
(A) Restriction map of the *orf0-rifA* region of the *A. mediterranei* S699 chromosome. Shaded arrows indicate ORFs. (B) In-frame deletion of *orf0*.

Construction of Replication Vector

The pA-rep, DNA fragment required for plasmid replication in *A. mediterranei* was originated from pULVK2 [7], which is a versatile bifunctional *Norcardia-E.coli* shuttle vector developed by Kumar *et al.* [ref]. To add several restriction enzyme sites with high GC contents and hygromycin resistance gene into pULVK2, 1.5 kb *Pvu*II and *Hin*dIII fragments of pANT841H were ligated with large fragments of *Sma*I and *Hin*dIII digests of pULVK2 (Fig. 4). The resulting vector named pAMR2 was used for the complementation experiment of P450 monooxygenase in *A. mediterranei*.

Complementation of *orf0*

The gene corresponding to P450 monooxygenase was obtained from genomic DNA by PCR using two primers, one of 5'-TCTAGAATGACCGACGCAATATCCTTCGAG-3 for the upstream and the other of 5-AAGCTTCTCG-AGGTCGCCGTAGGTGACCGT-3' for the downstream. These primers were designed to have the start codon and 160 nucleotides downstream from the stop codon of the *orf0* gene, respectively. The 1.3-kb PCR fragments were cloned into *Hin*dIII and *Xba*I sites in pGEM-3Z vector, resulting in pGEM-*orf0*. No errors in PCR were confirmed by DNA sequencing. For the efficient expression of *orf0* in *A. mediterranei*, a strong promoter *ermE** [2,17] was employed. The *ermE** was digested with *Eco*RI and *Xba*I and ligated into *Eco*RI-*Hin*dIII sites of Litmus28 together with the *orf0* gene obtained from pGEM-*orf0* by digesting with *Hin*dIII and *Xba*I. In the resulting vector, the *orf0* gene was under the control of *ermE** promoter. Finally, *ermE** promoter and *orf0* gene were ligated into *Bgl*II and *Hin*dIII sites of pAMR2. The final replicative expression vector for *orf0* was designated as pAMR2-*orf0* and used for the transformation of the *A. mediterranei orf0* deletion mutant.

Electroporation of pAMR2-*orf0* into *A. mediterranei orf0* was performed as previously described [25]. The transformants were selected on the YMG agar plate containing hygromycin. They were easily distinguished from non-transformants since the transformants exhibited a yellow color. The organic extract of the pAMR2-*orf0* transformant was analyzed by HPLC.

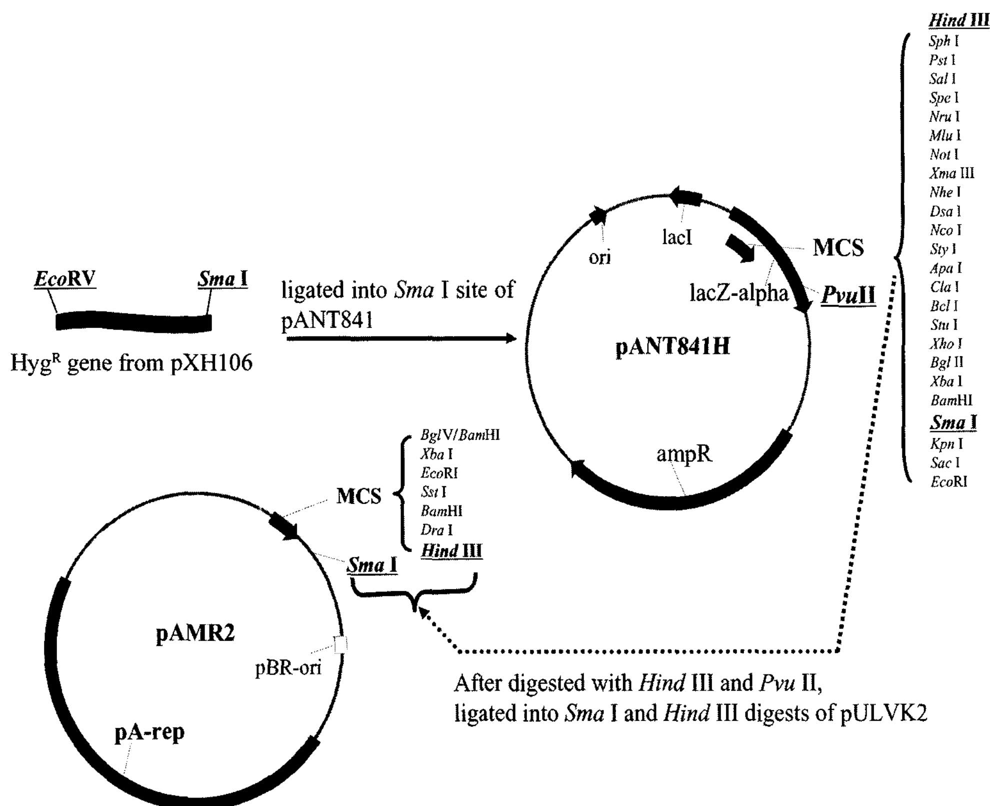

Fig. 4. Construction of replication vector, pAMR2.
Note that pAMR2 was obtained by combining the hygromycin resistance gene (Hyg^R), multiple cloning sites of pANT841H, and pA-rep, a DNA fragment required for plasmid replication in *A. mediterranei*, of pULVK2. The restriction enzyme sites used in this study are in bold and underlined.

RESULTS AND DISCUSSION

Cloning of the Upstream DNA Region of Rifamycin PKS and Sequencing

In order to identify PKS genes for rifamycin biosynthesis by Southern hybridization, the upstream region of *A. mediterranei* genes was analyzed for AHBA synthesis. One of the plasmids thus isolated was found to contain *orf0* and three other open reading frames (ORFs) lying immediately upstream of the five PKS genes [1]. A search in the public databases revealed that the deduced sequence of the protein encoded by *orf0* showed a high degree of similarity to members of the P450 family of monooxygenases. The Orf0 protein contains a highly conserved segment similar to the O_2 binding pocket and the motif around the cysteine residue known as a ligand for the heme prosthetic groups of all cytochrome P450 enzymes (Fig. 2) [15].

In-frame Deletion Mutant of *orf0* and Analysis of its Metabolites

In order to establish the role of *orf0* in rifamycin B biosynthesis, this gene was deleted by gene replacement. The plasmid pANT841H/Δ*orf0*, which contains a DNA fragment lacking the 483-bp segment of *orf0*, was constructed to transform *A. mediterranei* S699. A 483-bp in-frame deleted *orf0* gene was ligated into the plasmid pANT841H, a pANT841 derivative containing a hygromycin resistance gene.

The resulting plasmid, pANT841H/Δ*orf0*, has a hygromycin resistance gene as a selection marker and in-frame deleted *orf0* segment lacking the function of hydroxylation.

Two-step homologous recombination by the deleted *orf0* segment from pANT841H/Δ*orf0* was incorporated into the chromosome of the wild-type *A. mediterranei* S699. Replacement of the deleted *orf0* segment with the corresponding region of chromosome in the wild-type *A. mediterranei* S699 strain was confirmed by Southern hybridization. This *orf0* deleted strain exhibited a 2.3-kb *Sty*I band due to deletion of one *Sty*I site between *Nru*I and *Xmn*I sites (Fig. 3) and thus lacked 1.1-kb and 1.2-kb bands of the parental strain (data not shown). Southern analysis of the hygromycin-sensitive colonies confirmed the desired genotype of the resolvants. This deletion mutant was designated strain *A. mediterranei* Δ*orf0* and used in a complementation experiment.

HPLC analysis of an organic extract of the metabolites produced by the *A. mediterranei* Δ*orf0* strain revealed that no rifamycin B was synthesized. However, its macrocyclic intermediate, proansamycin X, was detected and this was

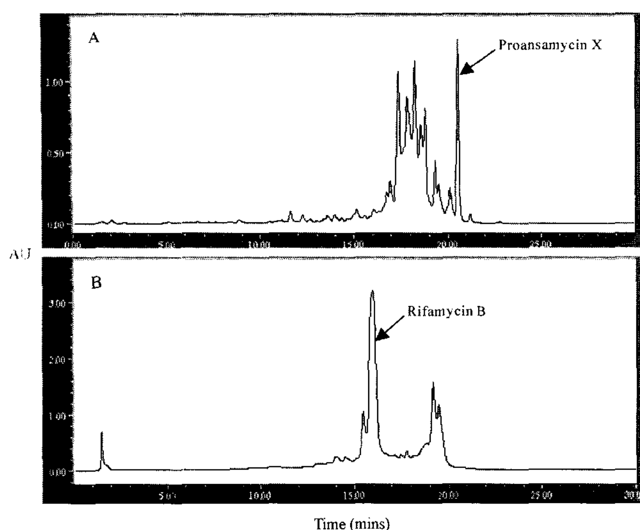

Fig. 5. HPLC chromatogram of the extract of *A. mediterranei* Δ*orf0* (A), and the extract of pAMR2-*orf0* transformant (B).

confirmed by HPLC analysis (Fig. 5A) using authentic proansamycin X (kindly donated by C.R. Hutchinson at University of Wisconsin-Madison). Because the *orf0* gene was deleted in this mutant strain, proansamycin X accumulated without further processing.

This study of gene replacement strongly suggests that *orf0* acts as a monooxygenase after the formation of proansamycin X, and thus the deletion of *orf0* gene blocks the conversion of proansamycin X to rifamycin B via rifamycin W.

Construction of Replication Vector for *A. mediterranei*

The replicative vector with useful restriction enzyme sites and an effective selection marker is a prerequisite for overcoming the limitation of genetic tools in *A. mediterranei*. The components employed to construct an improved replicative vector in *A. mediterranei* were the replication origin of *A. mediterranei* (pA-rep), hygromycin resistance gene as a selection marker, and several multiple cloning sites with high GC contents. The resulting plasmid, pAMR2, was obtained by combining the hygromycin resistance gene (Hyg^R) and multiple cloning sites from pANT841H, and pA-rep of pULVK2 (Fig. 4) [3]. We improved a versatile shuttle vector for *A. mediterranei* and *E. coli*, pULVK2, by introducing Hyg^R carrying a good-selective marker and useful restriction enzyme sites more easily to handle the high GC DNA of actinomycetes. Therefore, pAMR2 can be useful for the functional study of the gene including the complementation experiment in *A. mediterranei*.

Construction of pAMR2-*orf0* and the Complementation of *orf0*

The gene *orf0* was ligated into pAMR2, resulting in pAMR2-*orf0*. This final replcative expression vector was used for the transformation of the *A. mediterranei* Δ*orf0* strain.

The recombinant colonies obtained by transforming pAMR2-*orf0* exhibited a yellow color on the YMG agar plate containing hygromycin, whereas the *orf0* deleted mutant named *A. mediterranei* Δ*orf0* and pAMR2 transformants showed a brown color on the same plate. HPLC analysis of the organic extract revealed that the pAMR2-*orf0* transformant produced a significant amount of rifamycin B in the growth media (Fig. 5B). In this complementation experiment, the plasmid including Orf0 P450 monooxygenase of *A. mediterranei* could convert the proansamycin X to the final product, rifamycin B.

This complementation experiment indicates that *orf0* encodes a protein responsible for the C-34a hydroxylation of proansamycin X and is envolved in the production of rifamycin B. However, the full biosynthetic pathway next to the biosynthesis of proansamycin X, through rifamycin W to rifamycin B, has not been elucidated yet. In particular, the unknown genes encoding dehydrogenase which catalyze the dehydrogenation at C-7 and C-8 of proansamycin X must be identified.

Acknowlegments

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