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Premature Release of Polyketide Intermediates by Hybrid Polyketide Synthase in *Amycolatopsis mediterranei* S699

HONG, JAY SUNG JOONG[1], CHA YONG CHOI[1,2], AND YEO JOON YOON[3]*

[1]*Interdisciplinary Program of Biochemical Engineering and Biotechnology, Seoul National University, Seoul 151-742, Korea*
[2]*School of Chemical Engineering, College of Engineering, Seoul National University, Seoul 151-742, Korea*
[3]*School of Chemical Engineering and Bioengineering, University of Ulsan, Ulsan 680-749, Korea*

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Abstract The polyketide backbone of rifamycin B is assembled by the type I rifamycin polyketide synthase (PKS) encoded by the *rifA-rifE* genes. In order to produce novel analogs of rifamycin via engineering of the PKS genes, inactivation of the β-ketoacyl:acyl carrier protein reductase (KR) domain in module 8 of *rifD*, by site-specific mutagenesis of the NADPH binding site, was attempted. Module 8 contains a nonfunctional dehydratase (DH) domain and a functional KR domain that is involved in the reduction of the β-carbonyl group, resulting in the C-21 hydroxyl of rifamycin B. This mutant strain produced linear polyketides, from tetraketide to octaketide, which were also produced by a *rifD*-disruption mutant as a consequence of premature termination of the polyketide assembly. Another attempt to replace the DH domain of module 7, which has been considered nonfunctional, with a functional homolog derived from module 7 of rapamycin-producing PKS also resulted in the production of linear polyketides, including the heptaketide intermediate and its precursors. Premature release of the carbon chain assembly intermediates is an unusual property of the rifamycin PKS that is not seen in other PKSs such as the erythromycin PKS.

Key words: Rifamycin, polyketide synthase, combinatorial biosynthesis, *Amycolatopsis mediterranei*

Rifamycins [14], exemplified by rifamycin B, are one of the most notable naphthalenoic ansamycin antibiotics [15] produced by *Amycolatopsis mediterranei* [16]. Some of its derivatives have clinically been used in the treatment of tuberculosis, leprosy, and AIDS-related mycobacterial infections [18].

The development of resistance to antibiotics is an increasingly serious problem in the treatment of infectious diseases in humans, and researches on the development of antibiotics that are effective against drug resistant bacteria have recently been recognized as a critical need [3, 13, 22]. However, structure-activity research, clearly a difficult undertaking in view of the inherent synthetic complexities, has led to only a few derivatives different from rifampicin that show an improved activity against resistant bacteria [4, 17, 24]. Consequently, the development of alternative ways to manufacture novel rifamycins with potential activity against resistant bacteria, such as by genetic engineering of the biosynthetic pathway, remains as an important goal.

The biosynthesis of rifamycins involves the assembly of a polyketide through the 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) [11]. These multifunctional polypeptides consist of 10 modules that catalyze successive rounds of polyketide chain elongation to build an undecaketide [1, 21], which is then released as a macrocyclic lactam from the enzyme by the product of *rifF*, an amide synthase [19, 26]. The linearity between the catalytic domains present and the structure of the polyketide products produced makes modular PKSs attractive systems for combinatorial biosynthesis [7–9]. Hybrid PKSs have been generated via a number of genetic-engineering strategies, including (1) inactivation, deletion, insertion, and substitution of one or more catalytic domains, (2) deletion or exchange of complete modules, and (3) combining complete subunits from heterologous PKS clusters [8, 9, 12, 26].

The available information about the molecular mechanism of rifamycin resistance suggests that the C-21 and C-23 hydroxyls of rifamycin and its derivatives are essential for binding of rifamycin to RNA polymerase (RNAP) [2] but does not provide sufficient insight to guide the design of analogs that would bind tightly to a rifamycin-resistant RNAP β-subunit. In this study, analog biosynthesis was pursued initially by targeting modifications of the C-21

*Corresponding author
Phone: 82-52-259-2253; Fax: 82-52-259-1689;
E-mail: joonyoon@mail.ulsan.ac.kr

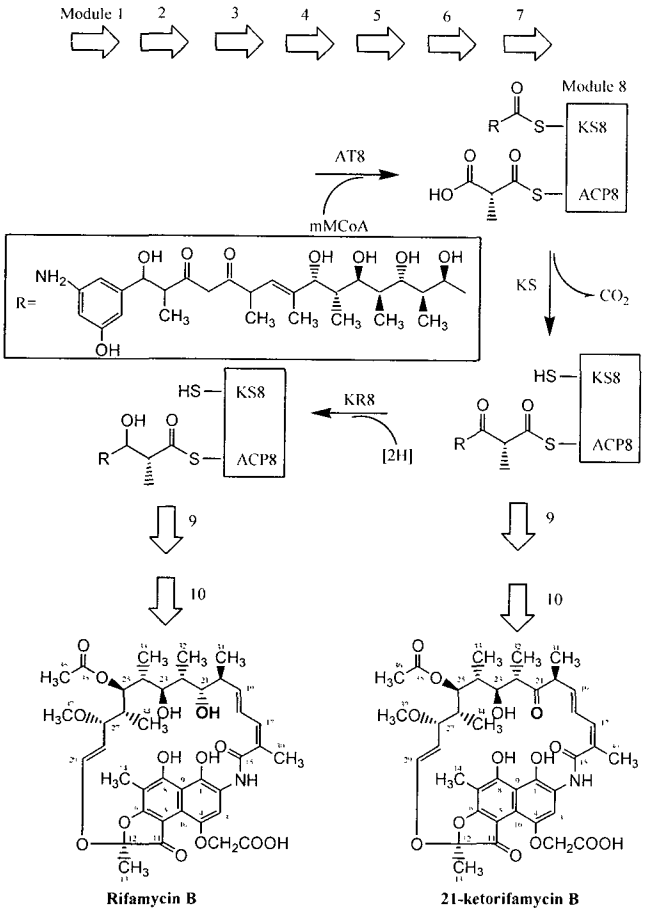

Fig. 1. Proposed role of module 8 in rifamycin biosynthesis.
The reactions catalyzed by the individual domains in parental module 8, and in a mutant domain in which the ketoreduction step is bypassed, are illustrated. The proposed 21-keto analog formed in the latter case is also shown along with rifamycin B.

and C-23 hydroxyl groups to produce 21-ketorifamycin and 22,23-dehydro-rifamycin B, respectively. Alteration of the C-21 hydroxyl to ketone was attempted by modifying the KR domain of module 8 by four amino acid substitutions and one amino acid deletion in the putative NADPH binding motif of the proposed KR domain encoded by *rifD* (Fig. 1). C-23 hydroxyl modification was attempted by replacing the nonfunctional DH domain of module 7 of the rifamycin PKS with a functional homolog from module 7 of rapamycin-producing PKS (Fig. 2).

Material and Methods

General Procedure

The *Escherichia coli* strain DH5α, XL-1 Blue (Stratagene, Kirkland, WA, U.S.A.), and JM110 were used in this work. *E. coli* plasmids pUC19, pGEM-3Zf(+), and pGEM-5Zf(+) (Promega, Madison, WI, U.S.A.), Litmus 28, and Litmus 38 (New England Biolabs. Inc., Beverly, MA, U.S.A.) were also used. Plasmid pANT841, a pUC18 derivative, was donated by Professor Chuck DeSanti of Ohio State University, and a hygromycin resistance gene was inserted in its *Sma*I site to produce plasmid pANT841H [4]. The *A. mediterranei* strain S699, which mainly produces rifamycin B, was a gift from Professor Giancarlo Lancini (Lepetit Research Laboratory, Geranzano, Italy). pKOS 002-14, SuperCos (Stratagene, Kirkland, WA, U.S.A.), a cosmid derivative containing the RAPS genes including module 3 through to module 10, was provided by Professor C. Khosla of Stanford University. Plasmids pGEM-3Zf(+) derivatives containing a segment of the rifamycin PKS genes, pP1, pS2, pS4, pP4, p151, and p15514 [1], were used as a template for PCR reactions and for the construction of mutagenic plasmids.

For growth of *A. mediterranei* S699 either on plates or in liquid culture, YMG medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose per liter of distilled water) was used. For selection of hygromycin-resistant (Hyg^R) *A. mediterranei* strains, 100 mg of hygromycin/ml was used either on plates or in liquid culture. For sporulating of *A. mediterranei* strains, SM medium was used: One liter of SM medium contained 10 g of lactose, 2.6 g of $(NH_4)_2SO_4$, 2.4 g of KH_2PO_4, 4.3 g of K_2HPO_4, 0.49 g of $MgSO_4$, 2 ml of trace element solution [10], and 15 g of agar. 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.).

Construction of KR8 Inactivation Plasmid

The *rifD* sequence GAGVLGEIVA, encoding part of the KR domain, was converted to GAEGLGRH-A *in vitro* by replacing eight bases and deleting three bases to introduce an *Stu*I site (Fig. 3A).

The mutagenic plasmid was constructed in several steps (Fig. 3B). The *Kpn*I/*Pst*I fragment that was isolated from pP1 and the *Pst*I/*Bgl*II fragment isolated from pS2 were ligated simultaneously to *Kpn*I/*Bgl*II-digested pANT841H so as to generate plasmid pP1S2. The PCR-generated *Bgl*II-*Stu*I fragment, using primers 5'-TCCGGCGATCTG-GCGGGCCGGCGACGAGAT-3' and 5'-TTTTAGGCCT-TCGGCACCGGAGACGAGGACCG-3', carrying the *Stu*I site (underlined), was subcloned into LITMUS 28 to give plasmid pLT28/KR8/upstream. The *Bgl*II-*Stu*I fragment digested from pLT28/KR8/upstream was ligated into the same sites of pP1S2 to generate construct pANT841H/KR8/upstream. The PCR-generated *Stu*I-*Sst*I fragment using primers 5'-TTTTTAAGGCCTCGGCAGGCACGC-GCGGCACCTGGTCACC-3' and 5'-TTTTTGAGCTCG-TCACGCAGGTGCCGGGCGA-3', which also contains the *Stu*I site (underlined), was first subcloned into LITMUS 28 yielding plasmid pLT28/KR8/downstream, and it was then ligated to *Stu*I/*Apa*I-digested pANT841 with the

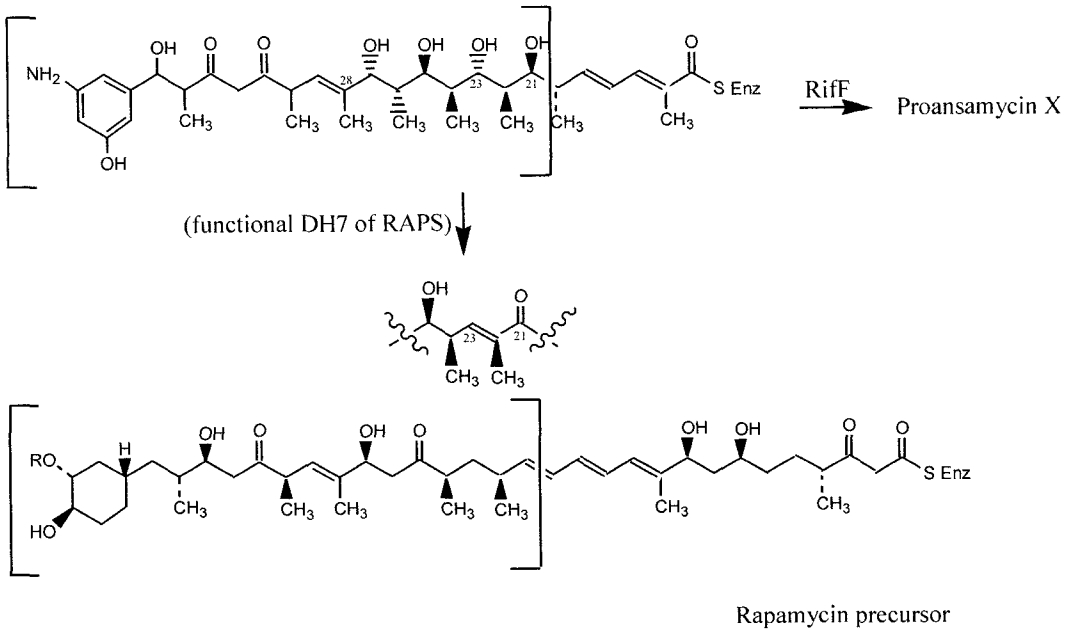

Fig. 2. The structures of precursors of rifamycin and rapamycin.
The structures in the bracket show the acyl chains bound to ACP7 of each polyketide synthase. The partial structure of the predicted compound formed by DH domain substitution is also shown.

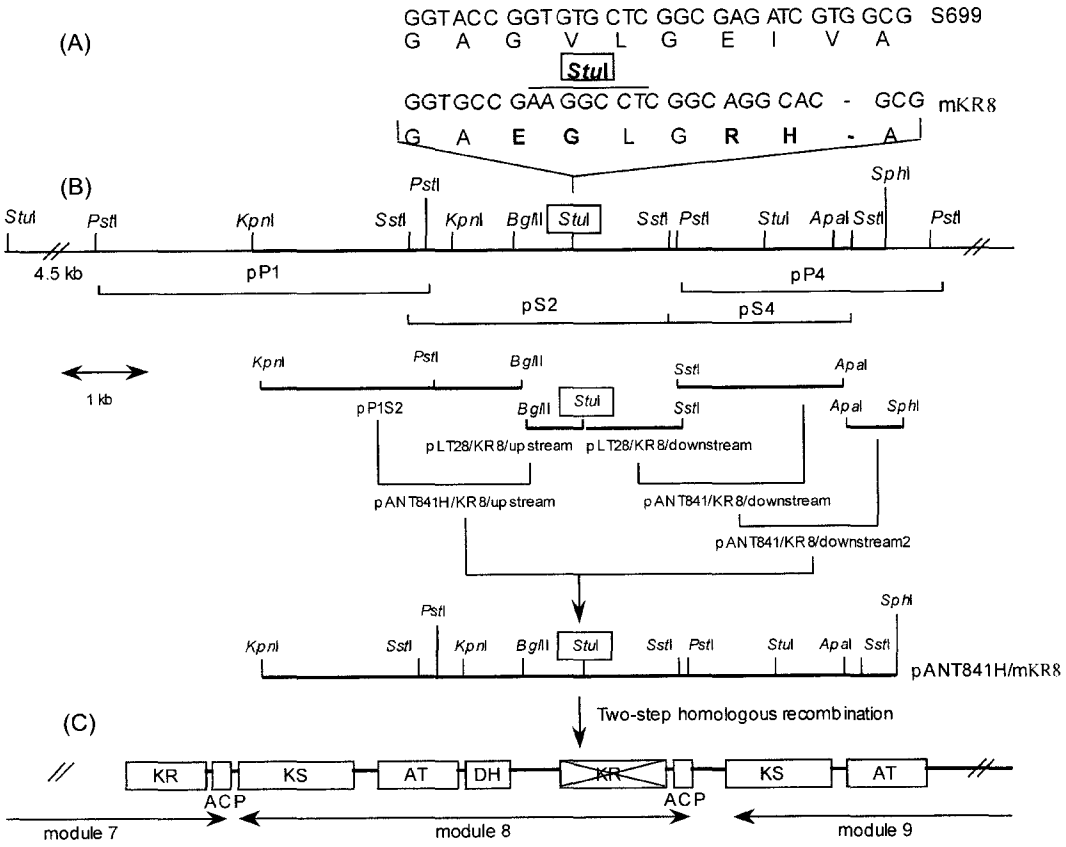

Fig. 3. Construction of strain *A. mediterranei* mKR8.
(A) Parent and mutant sequences. Changed nucleotides are underlined and corresponding changed amino acids are in bold type. Engineered *Stu*I site is boxed. (B) Scheme showing the construction of plasmid to inactivate the KR domain of module 8 (see text for details). (C) Mutant module 8 and the domain it encodes, including the inactivated KR domain.

SstI/ApaI DNA fragment isolated from pP4, so as to give plasmid pANT841/KR8/downstream. The *ApaI/SphI* fragment was isolated from pP4 and subcloned into the same sites of pANT841/KR8/downstream to yield plasmid pANT841/KR8/downstream2. The 3.5-kb downstream fragment was isolated from pANT841/KR8/downsteam2 by partial digestion with *StuI* and *SphI* and then subcloned in pANT841H/KR/upstream to generate the final integrative plasmid pANT841H/mKR8.

KR8 Inactivated Strain of *A. mediterranei* S669

The KR8 domain of *rifD* in *A. mediterranei* S699 was inactivated by using the above construct in a two-step procedure that involved homology-based integration of the recombinant plasmid into the chromosomal copy of *A. mediterranei* S699, followed by homology-driven resolution of the integrants to obtain the desired replacement (Fig. 3C). After transformation with the plasmid pANT841H/mKR8, several Hyg^R integrative transformants were obtained. Integration of the plasmid into the chromosome was verified by Southern hybridization using the KR8 sequence as a probe (data not shown). One integrant was then used for resolution of the integration event. Ten hygromycin-sensitive resolvants were obtained and examined for their genotype by Southern hybridization. Two independent isolates lacked the 10.2-kb wild-type *StuI* band and exhibited two novel bands of 9.1 and 2.1 kb, indicating the presence of a mutant allele (data not shown). One of these isolates was selected for further studies and designated *A. mediterranei* mKR8.

Construction of the DH Replacement Plasmid

The boundaries of the rifamycin and rapamycin DH domains were defined by amino acid sequence alignment of all known modular PKS DH domains [19], and the boundaries chosen for construction of the hybrid *rif/rap* PKS encompassed the entire interdomain linker between the DH and KR domains as well as the DH domain. The

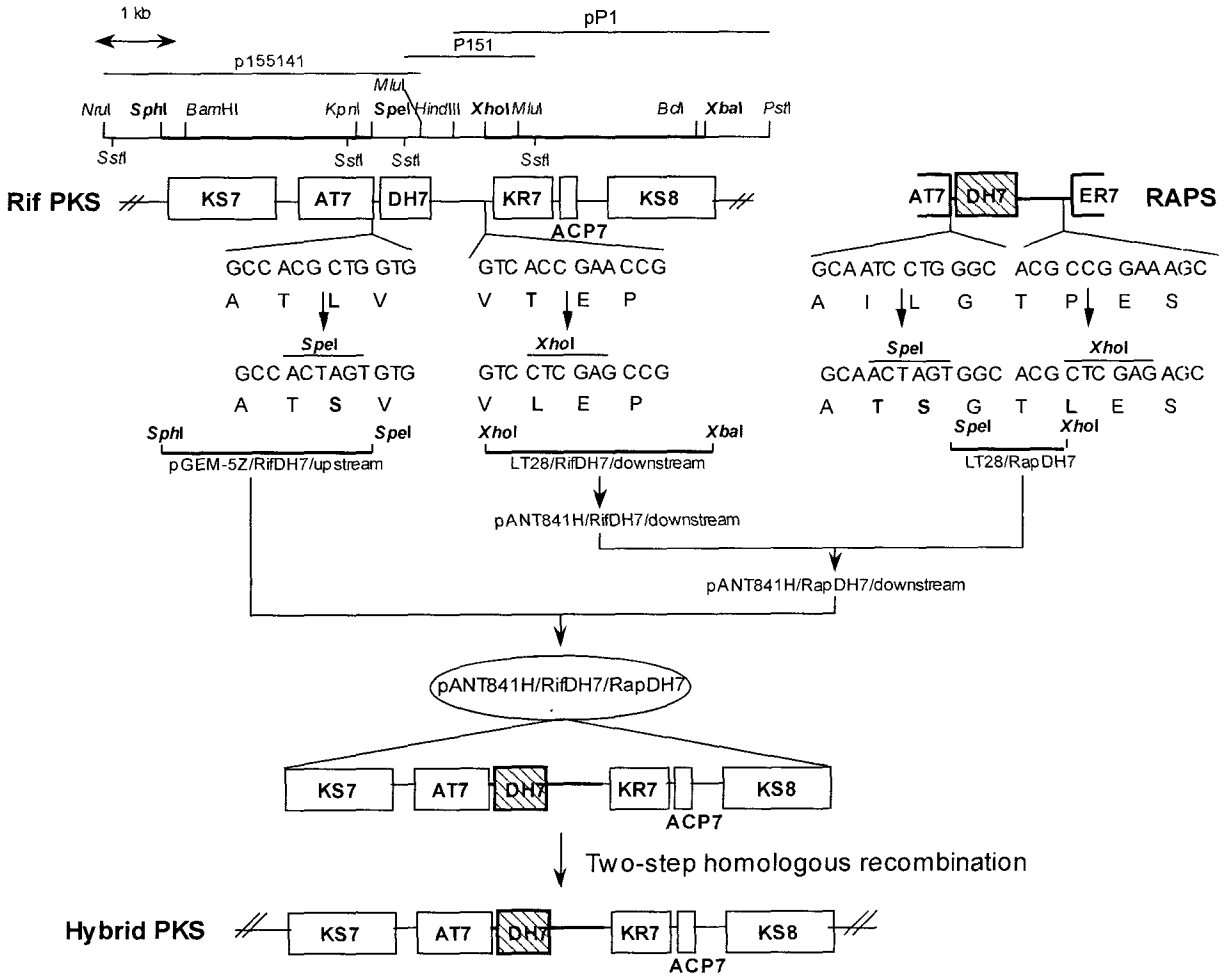

Fig. 4. Scheme of the construction of pANT841H/RifDH7/RapDH7 and the *A. mediterranei* RifDH7/RapDH7 strain.
The newly introduced restriction sites and modified amino acids are in bold type. The DH domain from rapamycin-producing polyketide synthase is indicated by cross hatching.

boundaries were chosen to minimize changes in the original amino acid sequence (Fig. 4). A *Spe*I site was engineered immediately downstream of the *rif* AT7 domain, and a *Xho*I site was engineered immediately upstream of the *rif* KR7 domain.

The mutagenic plasmid was constructed in several steps (Fig 4). The downstream flank was generated by PCR, using primers (cloning sites underlined) 5'-TTTTTCTCGAGCCGGCGGAAGCCCCGCTGACGTTCC-3' and 5'-TTTTTTCTAGAGGCCGACCAGTCGACTTCGGTGGAC-3', and subcloned into LITMUS28, resulting in plasmid pLT28/RifDH7/downstream. The *Xho*I-*Xba*I fragment was digested from pLT28/RifDH7/downstream and inserted into the same sites of pANT841H to give plasmid pANT841H/RifDH7/downstream. The PCR-generated DNA fragment carrying DH7 of RAPS using primers (cloning sites underlined) 5'-TTTTTACTAGTGGCACCGCCACGACACGGGTACCGG-3' and 5'-TTTTTCTCGAGCGTATTCGCCCGCGCCAGCCGCGGT-3' was first subcloned into LITMUS 28 to yield plasmid pLT28/RapDH7. The *Spe*I-*Xho*I fragment was digested from pLT28/RapDH7 and then inserted into pANT841/RifDH7/downstream to generate the construct pANT841H/RapDH7/downstream. The upstream flank was amplified by PCR using primers (cloning sites underlined) 5'-TTTTTGCATGCCGACCTCGTGCAACTGGCTTTCGGC-3' and 5'-TTTTTACTAGTGGCCAGTCGACGCGAACACCGCGG-3', and the PCR product was ligated into pGEM-5Zf(+) to give plasmid pGEM-5Z/RifDH7/downsteam. The *Sph*I-*Spe*I fragment from pGEM-5Z/RifDH7/downsteam was inserted into the same sites of pANT841H and excised by digestion with *Hin*dIII and *Spe*I so as to introduce a *Hin*dIII site, and then the resulting *Hin*dIII/*Spe*I upstream flank was subcloned into the same sites of pANT841H/RapDH7/downstream to generate the final construct pANT841H/RifDH7/RapDH7.

DH Replacement Strain of *A. mediterranei*

The pANT841H/RifDH7/RapDH7 plasmid was used to replace the chromosomal counterpart in *A. mediterranei* S699 by *in vivo* recombination as described for the KR inactivation strain. Integration of the recombinant plasmid into the chromosome was followed by screening for a second recombination event to yield a strain designated *A. mediterranei* RifDH7/RapDH7, in which the DH7 of rifamycin PKS was replaced with the corresponding homolog from rapamycin-producing PKS (Fig 4). Isolates were analyzed by Southern hybridization using the heterologous DH sequence as a probe to detect a novel band of DH7 of RAPS, which is absent in the parental strain (data not shown).

Characterization of Rifamycin Derivatives Produced by Recombinant *A. mediterranei*

For high-pressure liquid chromatography (HPLC) analysis, cells were grown in shake flasks in YMG 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 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. Analysis was performed using Waters Model 510 pumps controlled by Waters Pump Control Module, and a Rheodyne injector with a Waters Photodiode Array detector 996 (Waters, Milford, MA, U.S.A.). Separations were performed at the ambient temperature in a Waters Nova-Pak 3.9×150 mm C18 column (Waters, Milford, MA, U.S.A.). The mobile phase used was a linear gradient from 100% 0.1 M sodium acetate (pH 4.5) to 100% methanol over 20 min, followed by isocratic elution for 10 min at a flow rate of 1 ml/min.

Results and Discussion

Inactivation of KR Domain in Module 8

According to the model for rifamycin biosynthesis [1, 21], module 8 governs the eighth chain elongation cycle, which requires processing of the β-carbonyl through the action of the KR domain, yielding the hydroxyl at the C-21 position of rifamycin B (Fig. 1). Thus, inactivation of the KR8 domain was expected to result in the formation of 21-keto analogs of rifamycin B, if the downstream modules could process the intermediate assembled by the KR inactivated module 8. One possible strategy to inactivate the KR domain in the module 8 of rifamycin PKS, in the absence of structural information, would be to modify the sequence GAGVLGEIVA, which is the NADPH binding motif of the KR domain in module 8, to GAEGLGRH-A. Since the latter amino acid substitutions are encountered in the supposedly inactive KR domain in module 3 of the same multienzyme, it is reasonable to expect that this modification of the KR8 domain would not affect the activity of the "core" domains (i.e., the KS, the AT, and the ACP). The *A. mediterranei* S699 with the KR8 domain of *rifD*, which was inactivated by the procedure described above, was designated *A. mediterranei* mKR8.

HPLC analysis of an ethyl acetate extract of the fermentation broth of the *A. mediterranei* mKR8 strain indicated that the phenotype of this mutant was similar to that of the *rifD* disrupted mutant (Fig. 5). Disruption of the *rifD* module 8 led to the production of linear polyketides, ranging from tetraketide to octaketide products, but mainly the heptaketide and octaketide [24]. This result was confirmed by co-injection of the authentic linear polyketides, obtained from Professor H. Floss of University of Washington, with the extract of *A. mediterranei* mKR8. A rifamycin B-like compound was not detected by HPLC, when the peaks observed were scanned for the characteristic UV chromophore and retention time.

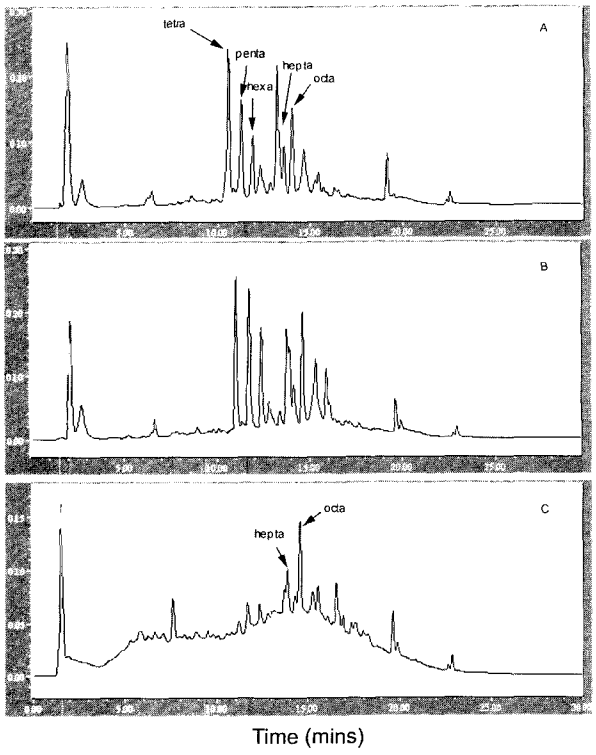

Fig. 5. HPLC chromatogram of *A. mediterranei* mKR8.
(A) The extract of *A. mediterranei* mKR8, (B) the extract of *A. mediterranei* mKR8 spiked with standard, and (C) the extract of module 8 disrupted mutant.

Gene replacement to change the five amino acid residues in the putative NADPH binding site of the KR8 domain of rifamycin PKS resulted in the failure of the host to produce a detectable amount of the desired compound. There are several successful reports demonstrating that inactivation of reducing domains can be readily accomplished in a variety of ways and that in at least some cases the intermediates can be efficiently processed by downstream modules. It is of interest to point out that in these reports many changes were introduced into the ER domain of DEBS, from deletions of large segments down to one or two amino acid replacements in different locations of the domain. However, there is only one report where the mutant strain produced any identifiable polyketide [8, 23]. Moreover, attempts to produce an altered polyketide through inactivation of the dehydratase function of the DH4 domain of DEBS by one amino acid change have not been successful either in P. F. Leadlay's laboratory at Cambridge University or at Abbot Laboratories [8]. Therefore, it is currently difficult to predict whether the substitution of a small number of amino acid residues in the NADPH binding site or replacement of an entire KR domain would result in a functional chimeric PKS and manufacture of the desired altered polyketide. This indicates a major difficulty which the researchers of this study have encountered with all of the strains containing hybrid rifamycin PKSs: the presence of small amounts of numerous products formed in engineered strains, where polyketide chain assembly is not disrupted simply at one particular step, as in the *rifD* and *rifE* gene disruption mutants described by Yu *et al.* [26]. Thus, determination of the structures in order to conclude about the success or failure of the experiment took considerable time and effort, even though the formation of putative rifamycin B analogs could be assessed rather easily by UV and HPLC/MS analysis. These analogs of rifamycin may not have been produced by any of the mutants currently being examined. Until the structure-function relationships of the *rif* PKSs are understood, the effect of amino acid substitution in the particular reducing function cannot be predicted. Consequently, it is important to study the behavior of the native rifamycin PKS to learn whether this difficulty can be circumvented.

Replacement of DH Domain in Module 7

The DH domain from rapamycin module 7, which contains reductive segments required for a full cycle of β-carbonyl reduction, was targeted, since the substrate accepted by this module was likely to be most related in the structure in the corresponding substrate bound to ACP7 of rifamycin PKS (Fig. 2). The desired hydrated structure was energy minimized using Macro Model Siani (KOSAN Biosciences Inc., Hayward, CA, U.S.A.) (data not shown). The new structure produced by introducing a double bond between C-22 and C-23 is likely not to have prohibitive strain energy.

Polyketide compounds produced by the DH replacement strain of *A. mediterranei* were extracted and characterized by HPLC and HPLC/MS, as described above. The DH replacement appeared to produce linear polyketides ranging from the tetra- to the heptaketide, based on HPLC and HPLC/MS analyses (data not shown). These results indicate that *A. mediterranei* RifDH7/RapDH7 behaves like a *rifC* disrupted mutant [26]. The presence of numerous chain assembly intermediates formed in small amounts in the DH replacement strain was also observed, similar to the *rifD*, *rifE* gene disruption mutant [24] and KR8 inactivated mutant. Consequently, in order to establish the basis for rapid construction and evaluation of strains containing engineered rifamycin PKS genes, it is important to study the behavior of the native rifamycin PKS and to develop a way to express the native rifamycin PKS gene under conditions that lessen premature release of chain assembly intermediates.

An unexpected property of the rifamycin PKS was discovered that might complicate further work and, as expected from the outset, it was found that *A. mediterranei* was not a convenient host for rapid genetic manipulation. Thus, the ways to overcome the possible limitations of these features and to enhance the yield of modified rifamycins

must be addressed in the future. Study of rifamycin PKS and its production of rifamycin analogs might lead to important discoveries about modular PKSs and the genetics of antibiotic production. This knowledge would help to advance the emerging field of combinatorial biosynthesis and understand microbial secondary metabolism for its use in the production of novel rifamycin analogs.

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