

## Thioesterases and the Premature Termination of Polyketide Chain Elongation in Rifamycin B Biosynthesis by *Amycolatopsis mediterranei* S699

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(Received for publication November 29, 1999)

The role of two thioesterase genes in the premature release of polyketide synthase intermediates during rifamycin biosynthesis in the *Amycolatopsis mediterranei* S699 strain was investigated. Creation of an in-frame deletion in the *rifR* gene led to a 30~60% decrease in the production of both rifamycin B by the S699 strain or a series of tetra- to decaketide shunt products of polyketide chain assembly by the *rifF* strain. Since a similar percentage decrease was seen in both genetic backgrounds, we conclude that the RifR thioesterase 2 is not involved in premature release of the carbon chain assembly intermediates. Similarly, fusion of the *Saccharopolyspora erythraea* DEBS3 thioesterase 1 domain to the C-terminus of the RifE PKS subunit did not result in a noticeable increase in the amount of the undecaketide intermediate formed nor in the amounts of the tetra- to decaketide shunt products. Hence, premature release of the carbon chain assembly intermediates is an unusual property of the Rif PKS itself.

Rifamycin B (**1**) is an ansamycin antibiotic produced by *Amycolatopsis mediterranei* and some of its derivatives are used clinically in the treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections<sup>1,2</sup>. This antibiotic is made from 3-amino-5-hydroxybenzoic acid (AHBA **2**, Fig. 1) plus two acetate and eight propionate-derived units that are added to AHBA by a modular polyketide synthase (PKS) to form proansamycin X (**3**), followed by various largely oxidative steps that result in rifamycin S, which is converted to rifamycin SV and then to **1** (Fig. 1)<sup>3</sup>. Chemical conversion of **1** to rifamycin SV followed by electrophilic substitution of the C-3 position has been used to make the important antibacterial drugs like rifampicin (rifampin) (Fig. 1) and rifapentine as well as numerous other derivatives<sup>2,4,5</sup>. Despite extensive structure activity studies of the rifamycins, as recently summarized by BACCHI *et al.*<sup>6</sup>, analogs with greatly improved utility over the current drugs have not been reported.

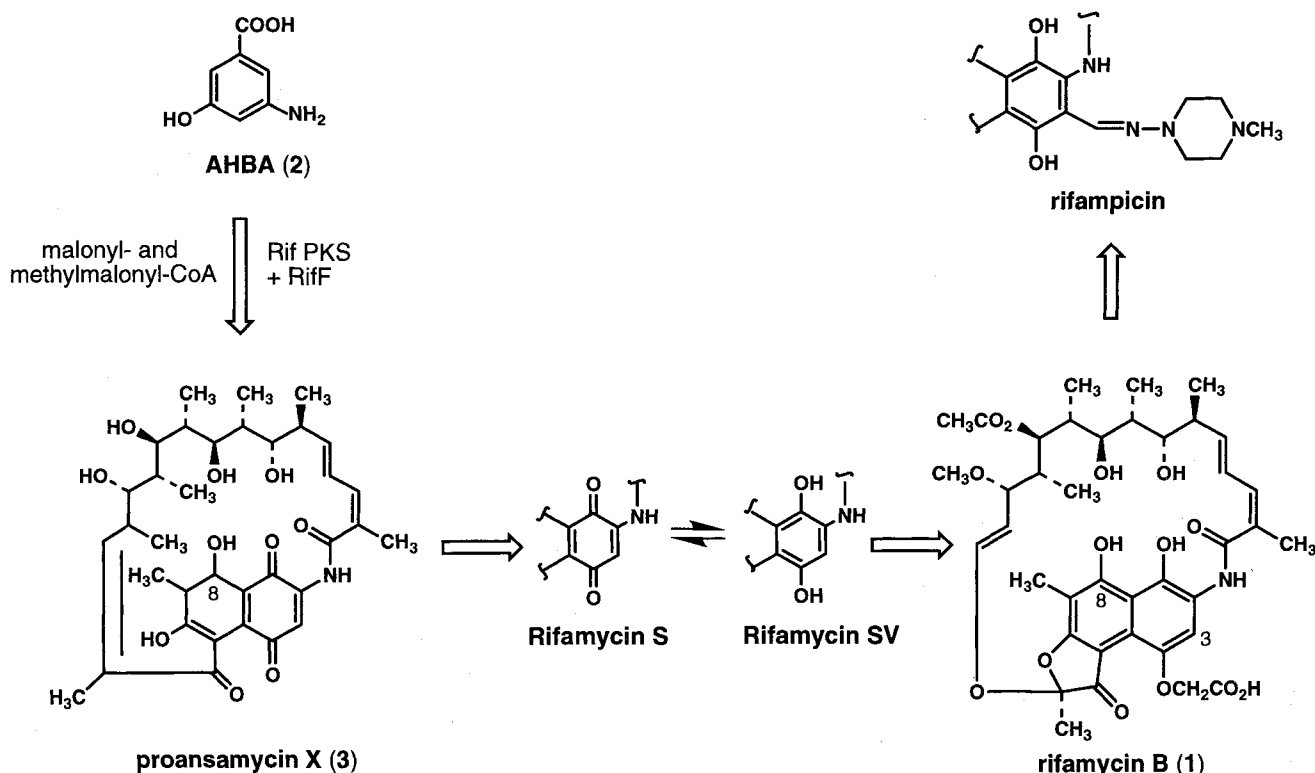
Our interest in applying the methods of combinatorial biosynthesis to the quest for better rifamycin-derived drugs led us to clone and characterize the gene cluster from *A. mediterranei* strain S699 that is responsible for the production of **1**<sup>7,8</sup>. This consists of approximately 40 genes that include five PKS genes, which were cloned and characterized independently by SCHUPP *et al.*<sup>9</sup>. The rifamycin PKS, composed of the RifA to RifE subunits, is responsible for the processive assembly of an undecaketide product (Fig. 2), which unlike typical macrolide PKSs<sup>10</sup>, is released from RifE and cyclized by the product of *rifF* acting as an amide synthase to form the ansamycin ring in proansamycin X (Fig. 2)<sup>11,12</sup>.

The *A. mediterranei* S699 wild-type and *rifF* mutant strains produce a series of acyclic polyketides ranging from tetra- to undecaketides (Fig. 2)<sup>11,12</sup>. All the ketides following formation of the tetraketide P8/1-OG<sup>13</sup> were isolated as their 8-deoxynaphthoquinones. We have

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Fig. 1. The principal steps of rifamycin B biosynthesis discussed in the text.

Rifamycin B (**1**) is oxidized back to rifamycin S and SV, then the latter compound is converted to the antibacterial drug rifampicin by formylation followed by imine formation.



suggested that the naphthoquinones **4**~**10** are shunt products arising by spontaneous dehydration of the various 8-hydroxydihydronaphthoquinone ketide intermediates<sup>11</sup> because the carboxyl oxygen of AHBA is retained during its incorporation into **1**<sup>11,13</sup>. Furthermore, we<sup>11</sup> and others<sup>14</sup> have been unable to observe the incorporation of the 8-deoxy metabolite, protorifamycin I (**11**, Fig. 2), into **1**. Thus, both protorifamycin I and its precursor proansamycin B (Fig. 2) appear to be shunt products of the rifamycin pathway.

The fact that the rifamycin PKS appears to shed its carbon chain assembly intermediates easily is unusual. Macrolide producing bacteria are not known to release the normal intermediates of polyketide chain assembly, except when one of the PKS genes has been mutated<sup>15,16</sup>, nor have such compounds been reported to be produced *in vitro* by the erythromycin PKS, 6-deoxyerythronolide B synthase (DEBS) (C. KHOSLA and R. MCDANIEL, personal communication). Therefore, we wondered whether the premature release of the ketides shown in Fig. 2 is catalyzed by a

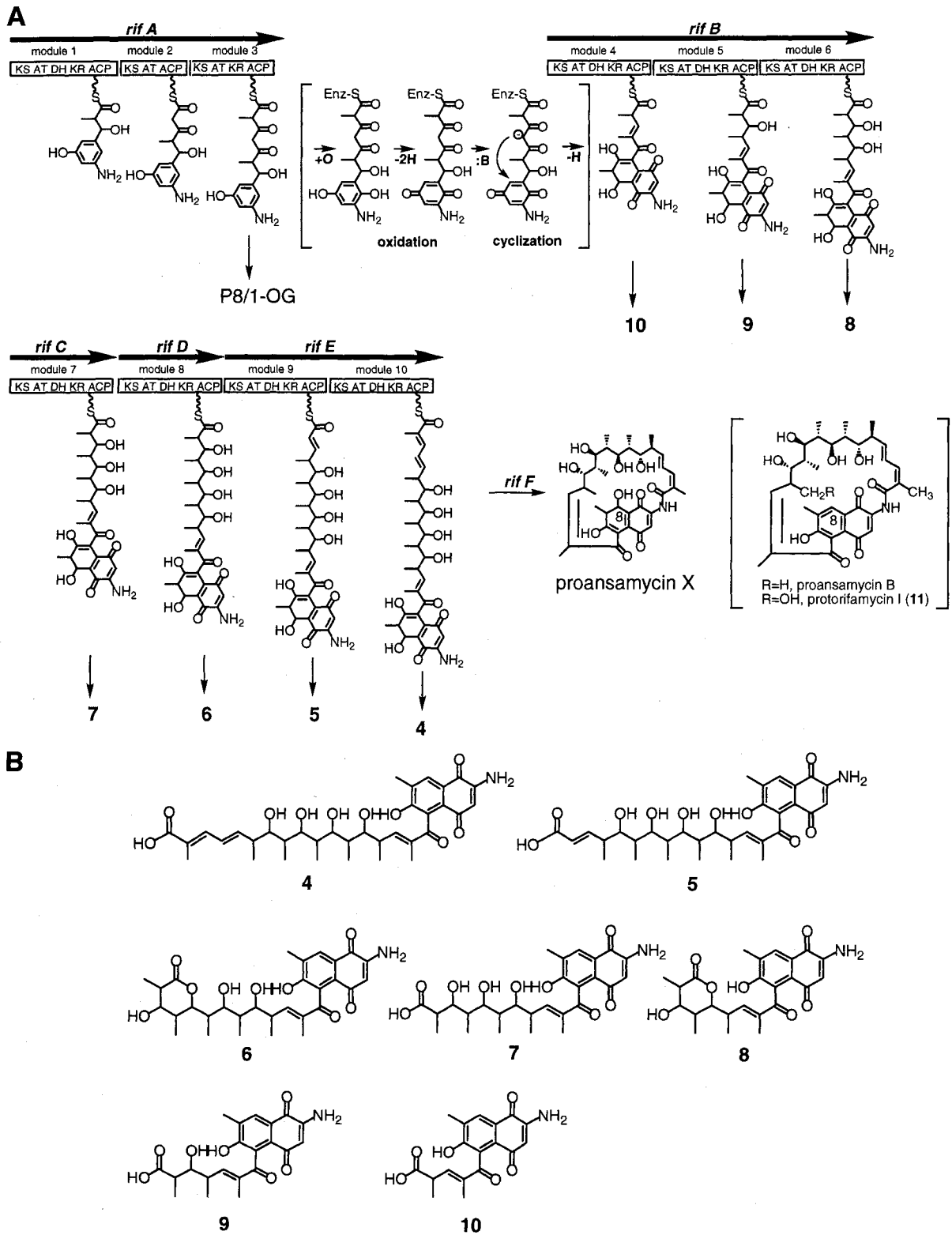
specific enzyme or is just a special feature of the rifamycin PKS itself. One candidate for an accessory enzyme is the thioesterase 2-like protein encoded by the *rifR* (formerly, *ORF12*) gene located approx. 23 kb downstream of the *rifA-rifF* genes<sup>7</sup>. Here we report that inactivation of the *rifR* gene in both the S699 wild-type and *rifF* backgrounds did not cause a notable decrease in the shedding frequency of the rifamycin PKS, when compared with the behavior of the S699 and *rifF* strains. Similarly, fusion of the DEBS3 thioesterase 1 domain to the C-terminus of the RifE protein in a *rifF* mutant background did not favor formation of the undecaketide **4** nor alter the amounts of the other ketides produced.

## Results

### Mutation of the *rifR* Gene in the S699 Wild-type and *rifF* Backgrounds by Gene Replacement

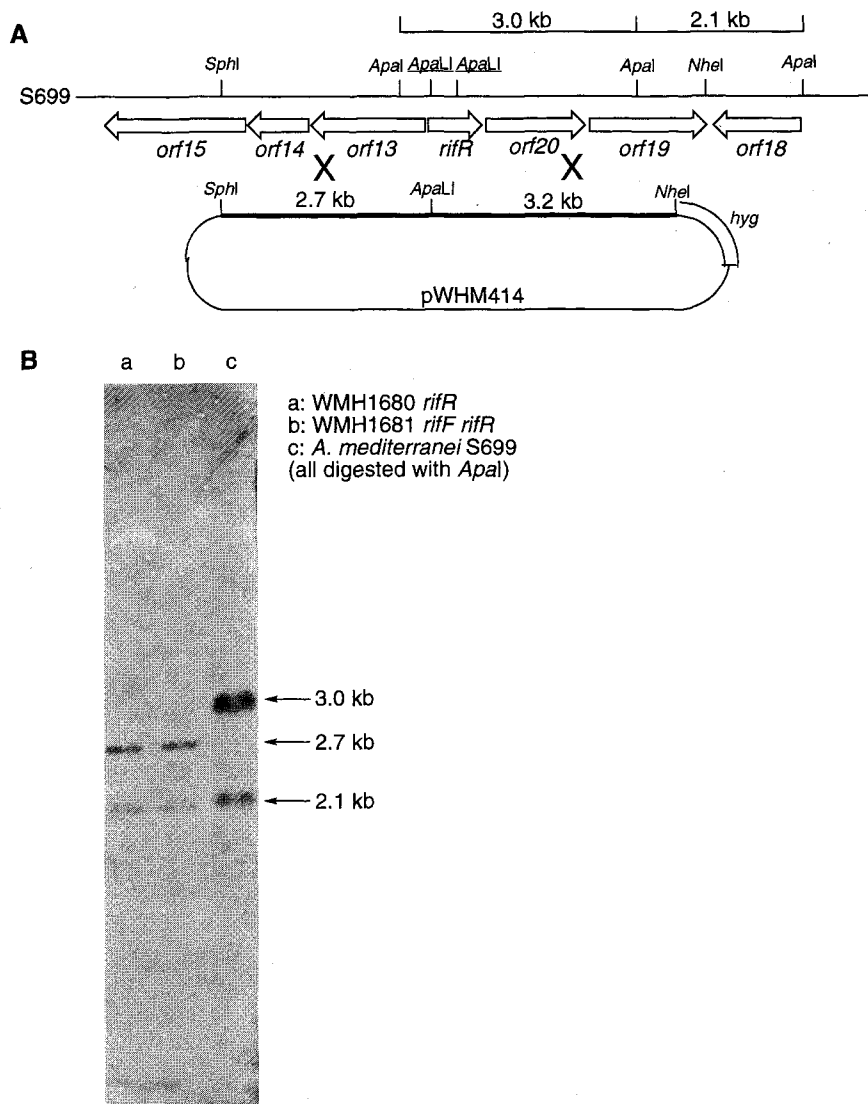
The *rifR* gene located between the *ORF13* and *ORF20*

Fig. 2. The hypothetical intermediates of carbon chain elongation in the biosynthesis of proansamycin X by the rifamycin PKS enzymes and the RifF amide synthase.



(A) Each intermediate is formed from the chain initiator unit AHBA (2) and the chain extender units methylmalonyl-CoA or 2-methylmalonyl-CoA. The oxidation and cyclization steps within the brackets are proposed to form the 8-hydroxy-7,8-dihydronaphthalene ring. Shunt products 4~10 may be formed by spontaneous dehydration to the 8-deoxynaphthoquinones followed by release from the PKS enzyme. Protorifamycin I (11) and proansamycin B, putative precursors of 1, are shown in brackets.

(B) The structures of the 8-deoxynaphthoquinones isolated from the wild-type and the *rifF* and *rifR* strains.

Fig. 3. Construction of the *rifR* mutants.

(A) Region surrounding the *rifR* gene in the cluster of rifamycin production genes in the S699 strain. The location of key restriction sites is indicated above open arrows that indicate the three genes flanking *rifR* on both sides. The two *Apa*LI sites flanking the region deleted in *rifR* are underlined, and the two DNA segments cloned in pWHM414 are indicated by the *Apa*LI, *Sph*I and *Nhe*I sites. X=the region where homologous recombination can occur.

(B) The results of Southern-blot hybridization for the three strains used in this work.

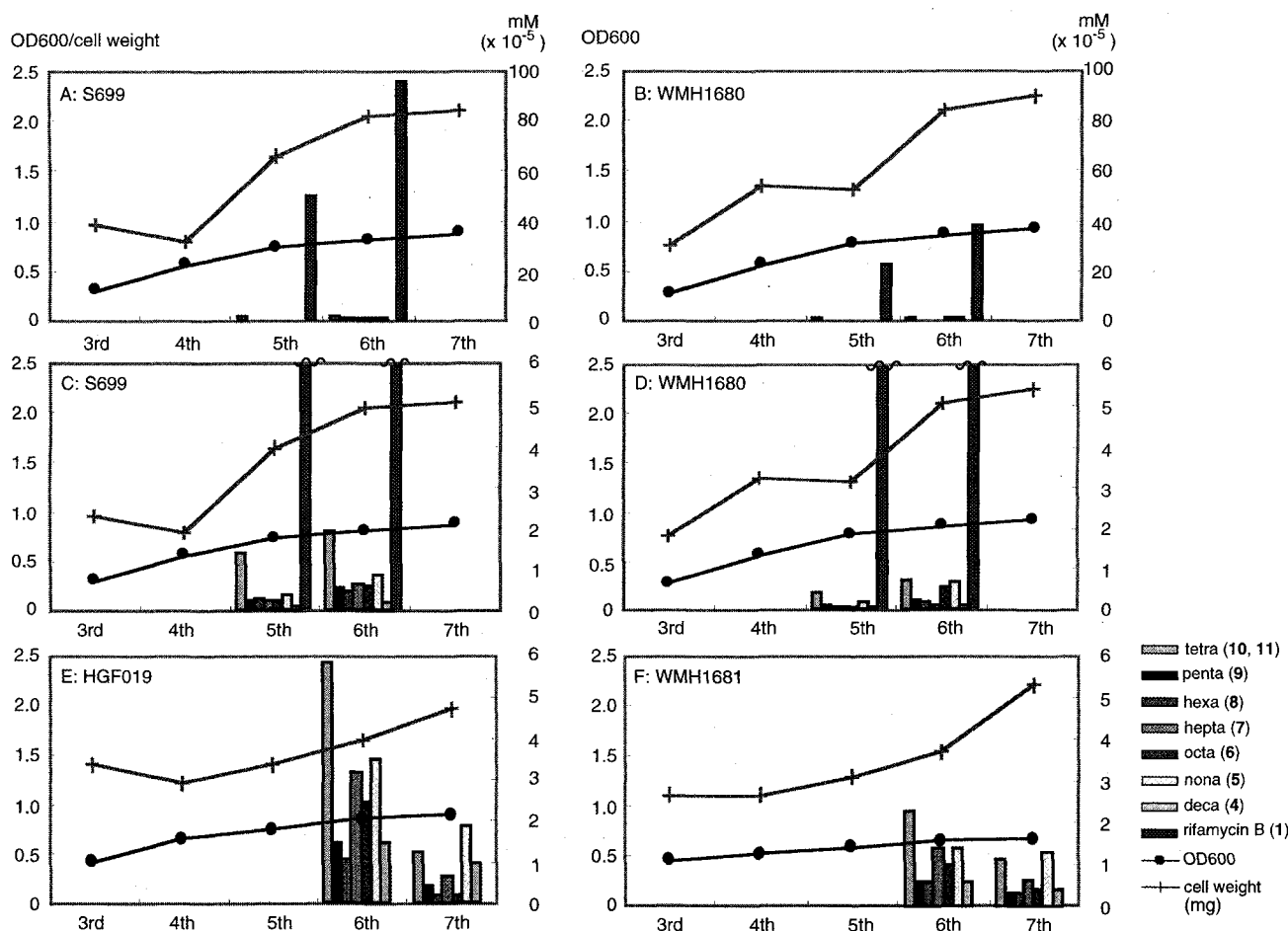
The two *Apa*I fragments used for Southern analysis are shown above the physical map of the genes.

genes (Fig. 3A) of the *A. mediterranei* S699 and HGF019 *rifF* strains<sup>11)</sup> was replaced with a mutated version by gene replacement. This was accomplished as described in the Experimental section by removing the 345 bp *Apa*LI segment between nts 2 and 346 of the *rifR* ORF, which creates a 115 aa in-frame deletion in the RifR protein, and introducing the resulting suicide plasmid pWHM414 (Fig. 3A) into the two strains by electroporation. Hygromycin

resistant transformants were transferred serially on solid media and the hygromycin sensitive clones isolated were screened by Southern analysis of their chromosomal DNA for ones that displayed the expected hybridization pattern (Fig. 3B). From the six *rifR* and two *rifF rifR* mutants isolated, a representative *rifR* mutant (WMH1680) and a *rifF rifR* double mutant (WMH1681) were chosen for metabolite analysis.

Fig. 4. Production of rifamycin B (1) and the shunt products shown in Fig. 2 by the *A. mediterranei* S699 wild-type, HGF019 *rifF*, WMH1680 *rifR* and WMH1681 *rifF rifR* mutants over a seven day period.

Graphs B, D and F show the average of the yields obtained with all six *rifR* and both *rifF rifR* mutants. The graphs in C and D are the same as in A and B except that the range of the mM ordinate has been decreased.



#### Production of Rifamycin B and Other Polyketides by the *A. mediterranei rifR* and *rifF rifR* Mutants

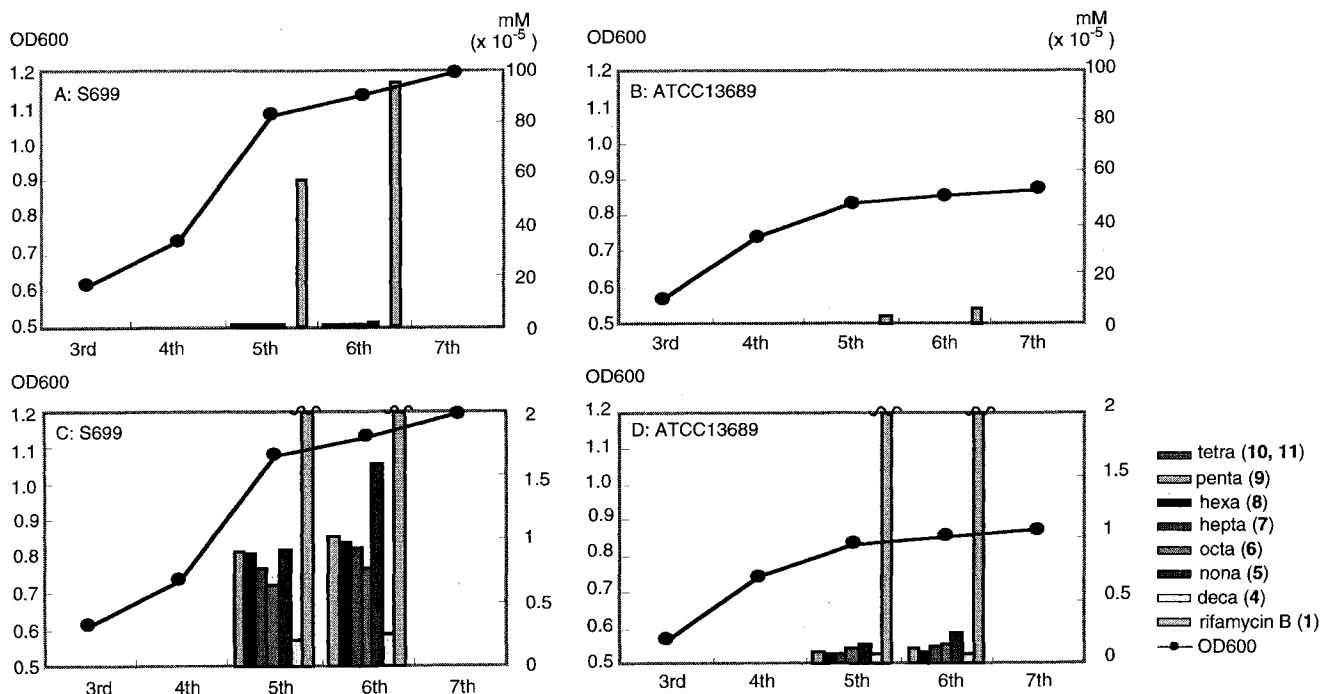
When the metabolite profiles of the S699 and HGF019 *rifF*<sup>11</sup> strains were compared with those of the *rifR* mutant and the *rifF rifR* double mutant, as determined by HPLC analysis at 320 nm of extracts of cultures grown in the YMG medium used by KIM *et al.*<sup>17</sup>, much less rifamycin B was found to be produced by the S699 *rifR* mutant than by the S699 strain at the end of the fifth and sixth days of growth at the stationary phase (Fig. 4A vs. 4B). The decrease in rifamycin B titer of the WMH1680 strain ranged from 40 to 60% of the values recorded for the S699 strain. This was accompanied by a similar decrease (40 to 60%) in the yield of the shunt metabolites arising from the

polyketide chain assembly intermediates (Fig. 4C vs. 4D). The WMH1681 *rifF rifR* double mutant, which does not make 1, also produced a decreased amount of these intermediates (Fig. 4F), and the percentage decrease (30 to 60%) compared with the amounts recorded for the *rifF* strain (Fig. 4E) was similar to that observed when the *rifR* gene was inactivated in the S699 strain.

Since the in-frame mutation in the *rifR* gene avoids the possibility of a polar effect on expression of the *ORF20* and *ORF19* genes downstream of it (Fig. 3A), we did not determine the metabolite profiles of the WMH1680 or WMH1681 strains after introduction of the wild-type *rifR* gene. The *ORF19* and *ORF20* genes are predicted to encode, respectively, proteins similar to the *Rhodococcus globerulus* HppA 3-(3-hydroxyphenyl)-propionate hydro-

Fig. 5. Production of rifamycin B (1) and the shunt products shown in Fig. 2B by the *A. mediterranei* S699 and ATCC 13689 wild-type strains over a seven day period.

The graphs C and D are the same as those in A and B except that the range of the mM ordinate has been decreased.



xylase (accession number U89712) and *Mycobacterium tuberculosis* Pap5 polyketide associated protein (accession number M95808) (T.-W.Y. and H.G.F. unpublished data).

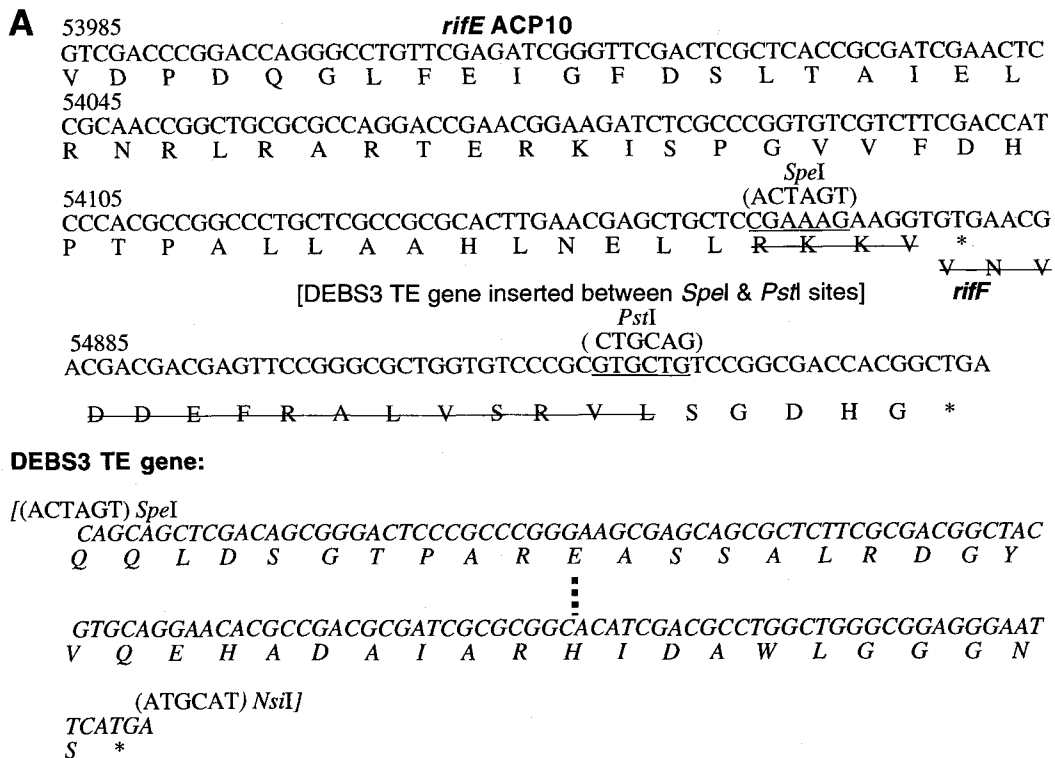
We were concerned that the S699 strain and the *rifF* and *rifR* mutants derived from it might be accumulating the shunt metabolites shown in Fig. 2 as a result of some mutation introduced into the S699 strain during yield improvement work carried out at Lepetit laboratories. Therefore, we obtained a culture of the original wild-type *A. mediterranei* ATCC 13689 strain from this company's successor (Biosearch Italia, S.p.a.) and determined the yields of 1 and the shunt metabolites. Although these yields were considerably lower than those typically obtained with the S699 strain, both 1 and many of the shunt metabolites arising from the polyketide chain assembly intermediates were still produced (Fig. 5).

#### Production of Polyketides by an *A. mediterranei* *rifE::TE rifF* Mutant

A notable advance in PKS biochemistry was the discovery of the functional independence of upstream

modules in a modular PKS from downstream ones, first discovered upon expression of the *eryAI* gene encoding the DEBS1 protein<sup>18</sup>). Placement of the thioesterase (TE) domain from module 6 of DEBS3 at the end of module 2 in DEBS1 increased the level of production of the polyketide product considerably<sup>18-20</sup>). Subsequent studies of chimeric DEBS::TE proteins have shown that the TE domain can act on a wide range of chain lengths among the natural acylthioester intermediates of carbon chain assembly. This domain can also hydrolyze artificial substrates of different chain lengths and functionality<sup>22-24</sup>), although it exhibits highest activity towards substrates whose stereochemistry and functionality are closest to those found at the alpha and beta positions of the natural acylthioester substrates<sup>23,24</sup>).

We investigated the behavior of the genetically engineered Rif PKS in which the DEBS3 TE domain was located immediately downstream of the ACP10 domain of the RifE protein to address the following two questions. (1) Can placement of the TE at the end of RifE accelerate product release so that the linear undecaketide precursor of proansamycin X would become the major product, and perhaps also suppress the amounts of the smaller ketides

Fig. 6. Construction of the *A. mediterranei* S699 *rifE::TE rifF* mutant strain.

(A) The C-terminal end of the *rifE* gene beginning with nt 53985 (GenBank accession number AF040570) followed by a short portion of the N-terminus of *rifF*.

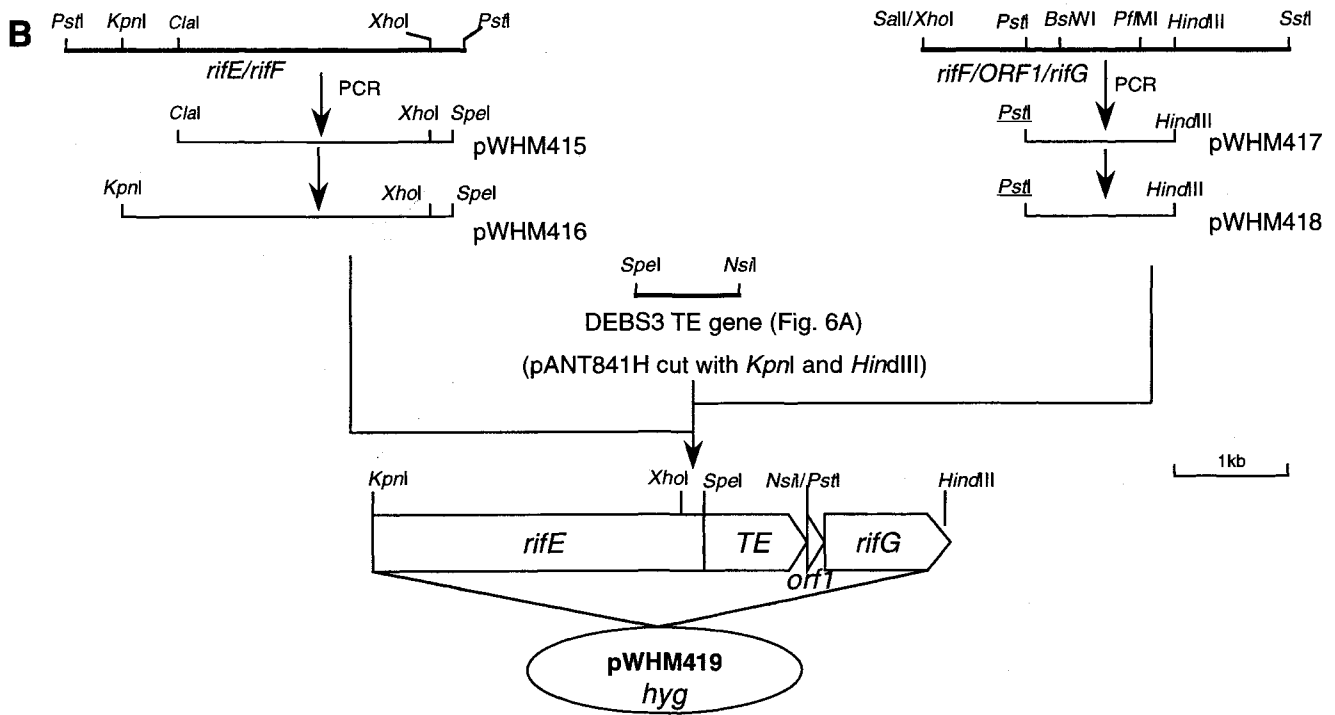
On the fourth line is a short portion of the C-terminus of *rifF* beginning with nt 54885. The sites are underlined where the *SpeI* and *PstI* sites, shown in parentheses, were engineered by PCR mutagenesis. The amino acids deleted from the C-terminus of RifE and the N-terminus of RifF upon insertion of the DEBS3 TE DNA are lined-out in the deduced protein sequence shown. The N- and C-termini of the *SpeI-NsiI* fragment carrying the *S. erythraea* DEBS3 TE gene are shown in italics below the *A. mediterranei* DNA sequence. The dashed vertical line indicates the intervening region of this gene that is not shown. This DNA was inserted into the *SpeI-PstI* sites of the *rifE/rifF* DNA as indicated.

formed by premature termination of carbon chain assembly? (2) Can the Rif PKS containing the RifE::TE protein produce new polyketides resulting from the action of the TE domain instead of the RifF amide synthase?

To test these ideas, we fused the TE domain of the DEBS3 PKS to the C-terminus of the chromosomal copy of the *rifE* gene. Since we could not predict whether the rate of polyketide release catalyzed by the TE domain would be competitive with the action of the RifF enzyme, we created the RifE::TE fusion in a *rifF* mutant background as follows. The RifE::TE fusion was engineered as shown in Figs. 6A and 6B (see Experimental section) to mirror the architecture of the DEBS3-TE interdomain region and the partial overlap of the C-terminus of RifE with the N-terminus of RifF as closely as possible (Fig. 6A).

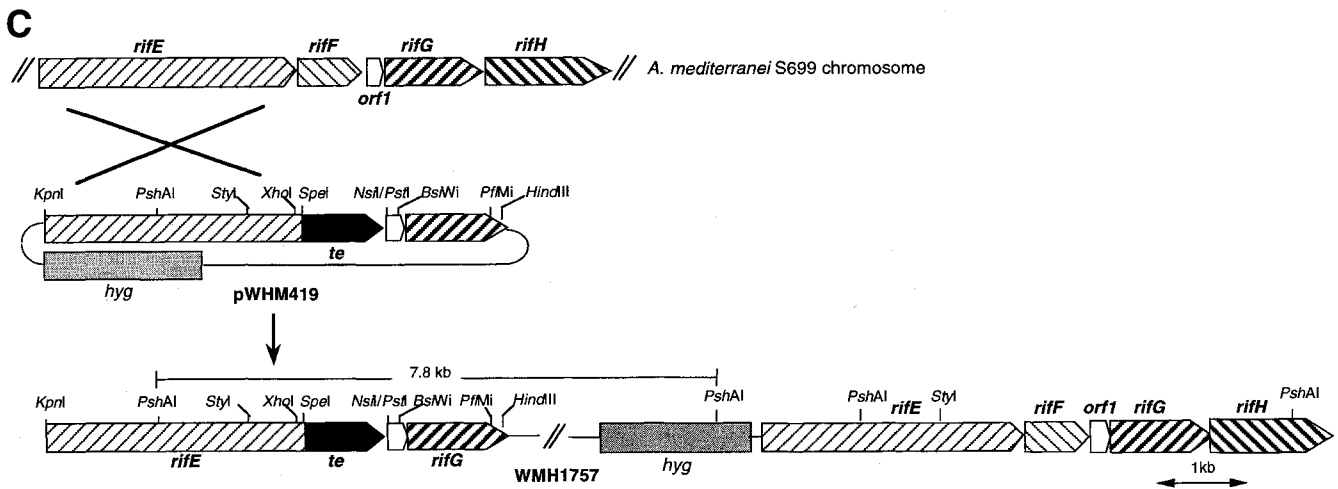
Introduction of this construct into the S699 strain by electroporation followed by selection for hygromycin resistant transformants resulted in a class of transformants where homologous recombination had taken place between the 2.3 kb C-terminal portion of the *rifE* gene in pWHM419 and the chromosomal *rifE* gene, resulting in the WMH1757 strain (Fig. 6C). Here, the intact *rifA-rifE::TE* PKS,  $\Delta$ *rifF*, ORF1 and *rifG* genes are separated by vector DNA from the N-terminally truncated *rifE* gene followed by *rifF*, ORF1, etc., as found in the native *rif* gene cluster (Fig. 6C). This organization was deduced from the results of Southern analysis of DNA isolated from the WMH1757 strain (Fig. 7). Since the *rifA* to *rifF* genes are thought to be cotranscribed, the intact copy of *rifF* should not be expressed in this recombinant but the *rifA* to *rifE::TE* genes

Fig. 6. (continued)



(B) The *rifE/rifF* and *rifF/ORF1/rifG* templates from the *rif* gene cluster<sup>7)</sup> are shown at the top left and right corners, respectively.

pWHM416 and pWHM418 were derived from these templates by PCR mutagenesis and subcloning as explained in the Experimental section. Plasmid pANT841H is a pANT841 derivative containing the hygromycin resistance (*hyg*) gene from plasmid pXH106<sup>32)</sup> as a 1.7 kb EcoRV-*Sma*I fragment and inserted at *Ecl*136II site of pANT841. A three piece ligation of pWHM416 and pWHM418 with the *Spe*I-*Nsi*I fragment containing the DEBS3 TE gene into the *Kpn*I-*Hind*III sites of pANT841H gave pWHM419.

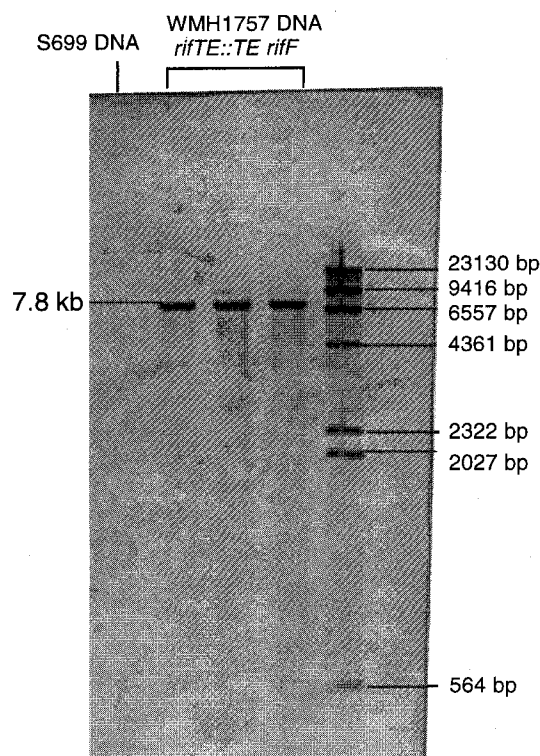


(C) The region of the *rif* gene cluster undergoing recombination with pWHM419 is shown at the top.

Homology driven recombination by a single crossover between the two *rifE* regions, as indicated by the 'X', gives the WMH1757 hygromycin resistant *rifE::TE rifF* strain. The 7.8 kb *Psh*AT fragment shown in Fig. 7 is indicated by the line above the WMH1757 genotype.



Fig. 7. Genotype of the *A. mediterranei* S699 *rifE::TE rifF* mutant strain.



Total DNA from the S699 and three *rifE::TE rifF* mutant strains was digested with *PshAI*, separated by electrophoresis in a 0.8% agarose gel, transferred to a nylon membrane, and probed with DEBS3 TE DNA fragment as described above for the analysis of the *rifF* mutant. Molecular weight markers are shown on the right hand side.

should, to form the engineered Rif PKS. The genes governing AHBA biosynthesis lying downstream of the *ORF1* gene were also expected to be expressed, based on the behavior of strains with disrupted *rifD* and *rifE* PKS genes<sup>11</sup>.

The metabolite profile of the WMH1757 strain, when cultured in the same way as the *rifR* and *rifR rifF* mutant strains, showed the presence of the same mixture of tetra- to decaketide shunt products as in the latter two strains (data not shown). Preferential formation of the undecaketide nor any of the other, smaller ketides was not observed. We did not compare the relative amounts of the shunt products isolated from the WMH1757 *rifE::TE rifF* strain with those produced by the *rifF* or *rifR rifF* mutants.

## Discussion

Many of the gene clusters that have been cloned from macrolide and nonribosomal peptide producing bacteria contain homologous genes for thioesterase 2-like proteins. In the case of pikromycin<sup>25</sup> and tylosin<sup>26</sup>, inactivation of these genes resulted in a major decrease in antibiotic yield. Similar results have been reported for a strain that makes the non-ribosomal peptide antibiotic, surfactin<sup>27</sup>. This has led to the suggestion that the thioesterase 2 enzymes act as editing enzymes, able to detect and release abnormal substrates affixed to modular PKS or nonribosomal peptide synthetase enzymes, to protect them from becoming inactivated<sup>11,26,27</sup>. HEATHCOTE *et al.*<sup>28</sup> have recently shown that the *Streptomyces fradiae* Orf5 thioesterase 2, when purified from an *E. coli* strain expressing the *tyl ORF5* gene from the cluster of genes governing tylosin biosynthesis, can efficiently hydrolyze *p*-nitrophenyl and *N*-acetyl-cysteamine thioesters of acetate, propionate, butyrate as well as several other acylthioesters<sup>28</sup>. The highest activity was towards the propionate esters. These observations lend support to the idea<sup>28</sup> that one role of such enzymes is to remove propionate groups from the PKS that have resulted from decarboxylation of enzyme-bound 2-methylmalonate groups, the carbon chain extension substrate, before they can react with the acylthioester bound to the KS domain. Without their removal, the propionate groups would block further extension of the carbon chain by the PKS.

Since we had found that the rifamycin PKS appears to release its carbon chain assembly intermediates prematurely, we investigated whether this is catalyzed by a specific enzyme. Although these are normal intermediates while attached to the PKS in their 8-hydroxydihydro-naphthoquinone forms, if spontaneous dehydration were to occur while they are still attached to the PKS enzymes, the resulting 8-deoxynaphthoquinones might block further extension of the carbon chain. The RifR protein might catalyze the release of such intermediates since it is a member of the thioesterase 2 like protein family. If this were so, then we expected to see a large decrease in the amounts of these shunt products accumulated by the S699 and *rifF* strains when the *rifR* gene is inactivated. However, this is not the case since the *rifR* mutation caused about the same percentage decrease in the titer of both **1** and the shunt products in the wild-type background and of the shunt products in the *rifF* genetic background. As has been seen for the other antibiotics noted above, the *rifR* mutant produced considerably less rifamycin B and thus lack of the RifR protein clearly has a negative effect on some step of antibiotic biosynthesis. This step must be associated with

the polyketide assembly process, since the yields of the PKS shunt products were decreased as much as that of 1. Although the available information does not allow us to deduce the exact role of RifR, an editing activity is the most reasonable function and lack of this would tie up a certain fraction of the PKS enzymes to result in a lowered overall yield of their product(s).

In contrast, the expectation that the addition of the DEBS3 TE domain to the C-terminal end of the RifE PKS subunit would result in enhanced production of the undecaketide precursor of proansamycin X, or less likely, one or more of the ketide intermediates accumulated by the S699 and *rifF* mutants, was not borne out by our results. Hence, it appears that the substrate recognition properties of the RifE::TE enzyme are not broad enough to encompass the proximal region of the undecaketide intermediate, even though the basic properties of the novel Rif PKS were maintained, as reflected in the formation of the tetra- to decaketide shunt products.

Together, these two sets of results show that premature release of its carbon chain assembly intermediates is an unusual property of the Rif PKS itself.

## Experimental

### Bacterial Strains and Plasmids

The *A. mediterranei* S699 strain was obtained from GIANCARLO LANCINI at the former Lepetit laboratories, Geranzano, Italy, and the ATCC 13689 strain from STEFANO DONADIO at Biosearch S.p.a, Italy. *E. coli* strain DH5 $\alpha$ <sup>29</sup> was used for routine subcloning. Plasmid pANT841 was obtained from W. R. STROHL, Ohio State University.

### Media, Growth Conditions, and Fermentations

*A. mediterranei* strains were maintained on YMG agar and grown in YMG liquid medium<sup>17)</sup> for preparation of protoplasts and seed inoculum for fermentations. Seed inoculum was also grown in YMG medium. *A. mediterranei* transformants were selected with hygromycin at 100  $\mu$ g/ml in solid medium and 100  $\mu$ g/ml in liquid medium. *E. coli* strains were grown in LB medium<sup>29)</sup> supplemented with ampicillin (100  $\mu$ g/ml), apramycin (25  $\mu$ g/ml) or tetracycline (15  $\mu$ g/ml) for the selection of plasmids.

### General DNA Manipulations

DNA isolation, restriction and ligation were performed according to standard techniques<sup>29)</sup>. Qiaex resin (Qiagen, Chatsworth, CA) was used routinely to purify DNA

fragments from gel slices. Southern analysis of genomic DNA from the *A. mediterranei* strains was performed by standard methods<sup>29,30)</sup> using the *ApaI* segment of the *rifR* gene as the probe DNA after labeling it with the digoxigenin-dUTP DNA labeling and detection kit from Boehringer, Mannheim, Germany.

### Mutation of the *A. mediterranei* S699 *rifR* Gene

The *rifR* gene was replaced by first creating the suicide plasmid pWHM414 by insertion of the 2.7 kb *SphI*-*ApaI* DNA segment from *ORF13* and the 3.2 kb *ApaI*-*NheI* DNA segment from *rifR* and *ORF20* (Fig. 3), cloned from pFKN108-E2 (T.-W. YU & H.G.F, unpublished), into the *SphI*-*NheI* site of pANT841, a pUC18 derivative that also carries a 1.7 kb hygromycin resistance gene inserted in its *SmaI* site<sup>11)</sup>. This resulted in pWHM414 carrying a 345 bp in-frame deletion at the N-terminal end of the *rifR* ORF. The heat-denatured pWHM414 was introduced by electroporation into the *A. mediterranei* S699 strain and the HGF019 *rifF* mutant strain as described by YU *et al.*<sup>11)</sup>, and hygromycin resistant transformants resulting from integration of pWHM414 by a single-crossover homologous recombination were selected. Putative double crossover mutant strains were obtained by serial transfer of the hygromycin resistant transformants on solid medium and screening for hygromycin sensitive clones. The WMH1680 *rifR* mutant and WMH1681 *rifF rifR* double mutants were chosen from clones exhibiting the expected genotype (Fig. 3B).

### Construction of the *A. mediterranei* S699 *rifE::TE rifF* Mutant Strain

To construct pWHM419 (Fig. 6B), *SpeI* and *NsiI* sites were introduced at the C-terminal ends of *rifE* and *rifF* as follows. Pairs of oligonucleotide primers were designed for PCR subcloning for the two regions of DNA that flank *rifF* so that a *SpeI* site could be introduced in the *ClaI*-*SpeI* fragment obtained from the *rifE/rifF* template (Fig. 6B). The *ClaI*-*XhoI* segment of the resulting *ClaI*-*SpeI* fragment (pWHM415) was replaced with the *KpnI*-*XhoI* segment of the template to give pWHM416. The internal *BsiWI*-*PflMI* segment of the PCR fragment pWHM417 from the *rifF/ORF11/rifG* template (Fig. 6B) was also replaced with the same DNA from the template to give pWHM418. The DNA sequences of the remaining portions of pWHM416 and pWHM418 were verified by DNA sequencing. The 848 bp *SpeI*-*NsiI* fragment from plasmid pKAO98, containing the DEBS3 TE domain (Fig. 6A), was cloned with pWHM416 and pWHM418 in a three-piece ligation to give pWHM419 (Fig. 6B), in which the last 4 amino acids of the

*rifE* ACP10 domain and all but the final five amino acids of the *rifF* ORF are replaced with the DEBS3 TE domain (Fig. 6A).

The *A. mediterranei rifE::TE rifF* mutant was constructed by introducing pWHM419 into the S699 strain by transformation, isolating hygromycin resistant transformants, and analyzing their genomic DNA by Southern-blot hybridization as described above. Three independent transformants were analyzed and all contained the unique 7.8 kb *PshAI* fragment (Fig. 7) with the *rifE::TE* and  $\Delta$ *rifF* genes expected for the genomic arrangement of the WHM1757 strain shown in Fig. 6C.

#### Determination of the Metabolic Profile of the *A. mediterranei* S699 and ATCC 13689 Strains and the *rifR*, *rifF rifR* and *rifE::TE rifF* Mutants

The S699 and *rif* mutant strains were cultured in 50 ml YMG (g/liter) (yeast extract, 4; malt extract, 10; glucose 4) in 250 ml baffled flasks with 4 g of 3 mm diameter glass beads for 3 days at 300 rpm and 30°C. A 100  $\mu$ l portion of this seed culture (OD<sub>600</sub> 0.5) was inoculated into 50 ml YMG medium in 250 ml baffled Erlenmeyer flasks and the cultures were grown as before for 7 days. A 0.5 ml portion of the culture was removed, diluted to 10% with distilled water, and the OD<sub>600</sub> checked from day 3 to 7. These culture samples were separated from mycelium by centrifugation at 14,000 rpm in an Eppendorf tube, then the mycelia were washed with 0.5 ml distilled water and dried in a vacuum oven at 40°C overnight prior to weighing. The culture broth remaining on day 5 and 6 was acidified with 10  $\mu$ l acetic acid and extracted once with 0.5 ml ethyl acetate. The ethyl acetate solution was dried *in vacuo* at less than 40°C and the residue was dissolved in 50  $\mu$ l methanol. A 20  $\mu$ l portion of this methanol solution was analyzed by HPLC<sup>31)</sup> using a Nova-Pak C<sub>18</sub> column (Waters, Milford, MA; 3.9  $\times$  150 mm; 4  $\mu$ m particle size) at a flow rate of 1 ml/minute. The mobile phase consisted of a linear gradient from 0.1 M sodium acetate buffer, pH 4.5 (adjusted with acetic acid), to 100% methanol over 20 minutes, followed by 10 minutes at 100% methanol and then immediate return to the initial conditions for a 5 minutes re-equilibration period. The eluent was monitored at 320 nm with the 996 Photodiode Array Detector (Waters). All the calculations of metabolite concentrations and preparation of tables were performed using the Millennium Chromatography Manager (Waters). The calibrations for determining the amounts of tetraketides and penta- to decaketides, and 1 for each set of analyses were generated by comparison of the peak-area ratio using authentic compounds<sup>11)</sup> as reference standards.

#### Acknowledgments

This work was supported by National Institutes of Health grants AI 20264 to H.G.F and AI 38947 to C.R.H.

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