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Mutational Analysis and Reconstituted Expression of the Biosynthetic Genes Involved in the Formation of 3-Amino-5-hydroxybenzoic Acid, the Starter Unit of Rifamycin Biosynthesis in *Amycolatopsis mediterranei* S699*

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To investigate a novel branch of the shikimate biosynthesis pathway operating in the formation of 3-amino-5-hydroxybenzoic acid (AHBA), the unique biosynthetic precursor of rifamycin and related ansamycins, a series of target-directed mutations and heterologous gene expressions were investigated in *Amycolatopsis mediterranei* and *Streptomyces coelicolor*. The genes involved in AHBA formation were inactivated individually, and the resulting mutants were further examined by incubating the cell-free extracts with known intermediates of the pathway and analyzing for AHBA formation. The *rifL*, *-M*, and *-N* genes were shown to be involved in the step(s) from either phosphoenolpyruvate/D-erythrose 4-phosphate or other precursors to 3,4-dideoxy-4-amino-D-*arabino*-heptulosonate 7-phosphate. The gene products of the *rifH*, *-G*, and *-J* genes resemble enzymes involved in the shikimate biosynthesis pathway (August, P. R., Tang, L., Yoon, Y. J., Ning, S., Müller, R., Yu, T.-W., Taylor, M., Hoffmann, D., Kim, C.-G., Zhang, X., Hutchinson, C. R., and Floss, H. G. (1998) *Chem. Biol.* 5, 69–79). Mutants of the *rifH* and *-J* genes produced rifamycin B at 1% and 10%, respectively, of the yields of the wild type; inactivation of the *rifG* gene did not affect rifamycin production significantly. Finally, coexpressing the *rifG–N* and *-J* genes in *S. coelicolor* YU105 under the control of the *act* promoter led to significant production of AHBA in the fermented cultures, confirming that seven of these genes are indeed necessary and sufficient for AHBA formation. The effects of deletion of individual genes from the heterologous expression cassette on AHBA formation duplicated the effects of the genomic *rifG–N* and *-J* mutations on rifamycin production, indicating that all these genes encode proteins with catalytic rather than regulatory functions in AHBA formation for rifamycin biosynthesis by *A. mediterranei*.

3-Amino-5-hydroxybenzoic acid (AHBA)[1] has been identified as the common starter unit (mC_7N unit) for the biosynthesis of ansamycins (1–3) and mitomycins (4). Ansamycins are a class of natural compounds produced by a variety of microorganisms and plants and are characterized by a macrocyclic structure consisting of an aromatic ring system connected to an aliphatic chain that forms an amide linkage to the amino group of the aromatic moiety (5). Based on the structure of the AHBA-derived aromatic moiety, this family of compounds can be further subdivided into a benzenic and a naphthalenic subgroup. The benzenic ansamycins, such as geldanamycin, ansatrienin A, and ansamitocin (Fig. 1), have been isolated from actinomycetes or higher plants and are mainly cytotoxic agents against eukaryotes (6–13). Naphthomycin, streptovaricin, rifamycin B, and tolypomycin Y (Fig. 1) represent the naphthalenic ansamycins and have antibacterial activity, particularly against Gram-positive bacteria and *Mycobacterium tuberculosis* (14–19).

The biosynthesis of AHBA has been studied in organisms producing various ansamycins and mitomycin C through the incorporation of ^{13}C- and ^{14}C-enriched glucose, glycerate, and other precursors. This work led to the hypothesis that the seven-carbon mC_7N unit is derived from the shikimate biosynthesis pathway (20–23). Genetic investigations on aromatic amino acid-deficient mutants of *Amycolatopsis mediterranei* N813 further revealed that the mC_7N unit of the rifamycin chromophore must be derived from early intermediates of the shikimate biosynthesis pathway (24–27). However, there has never been success in any attempts to obtain incorporation of labeled shikimic acid, quinic acid, or 3-dehydroquinic acid (DHQ) into the mC_7N unit (20, 23, 28–30).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF040570, AF040571, and AF335989.

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[1] The abbreviations used are: AHBA, 3-amino-5-hydroxybenzoic acid; DHQ, 3-dehydroquinic acid; DAHP, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate; aDAHP, 3,4-dideoxy-4-amino-D-*arabino*-heptulosonate 7-phosphate; aDHQ, 5-deoxy-5-amino-3-dehydroquinic acid; aDHS, 5-deoxy-5-amino-3-dehydroshikimic acid; kb, kilobase pair(s); bp, base pair(s); ORF, open reading frame; PCR, polymerase chain reaction; HPLC, high performance liquid chromatography; PLP, pyridoxal phosphate.

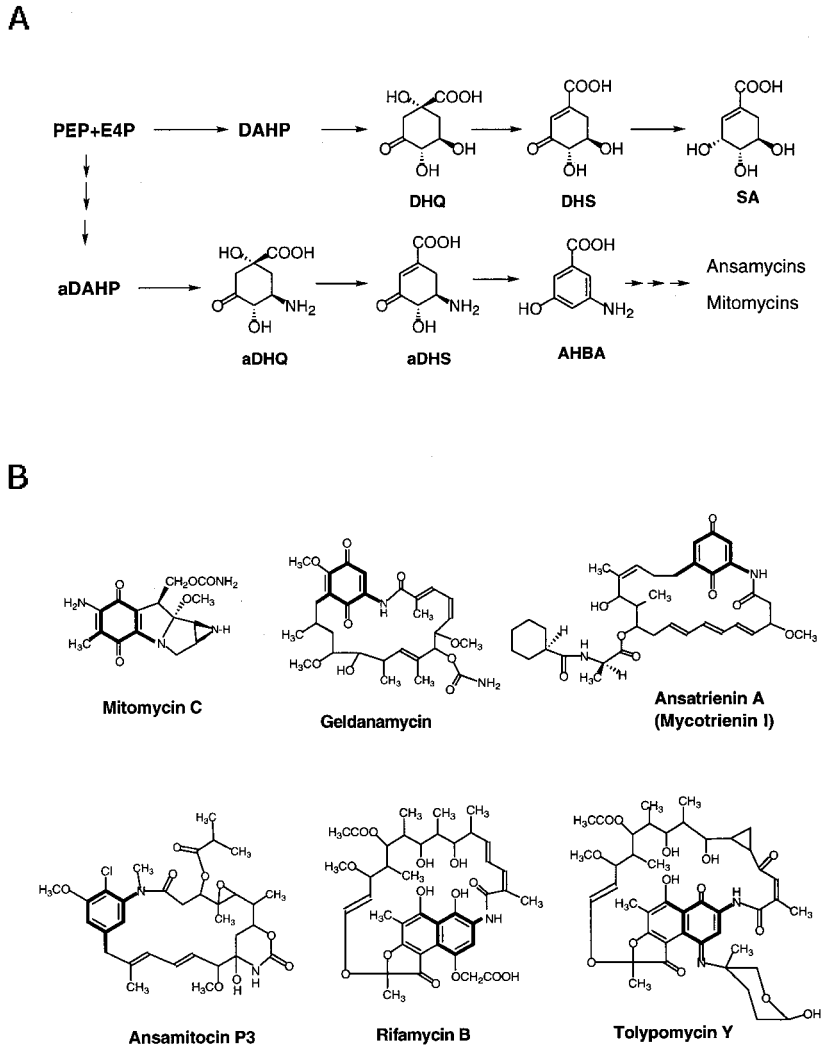

FIG. 1. **Comparison of early biosynthetic steps of the shikimate biosynthesis pathway with the proposed pathway for the formation of AHBA (32, 33) (*A*) and AHBA-derived antibiotics (*B*).**

Based on our previous studies (31–33), we have proposed a novel pathway for the formation of AHBA, which parallels the early stages of the shikimate biosynthesis pathway (Fig. 1). Nitrogen is introduced at the earliest biosynthetic step to form an amino analog of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP). Proposed intermediates such as 3,4-dideoxy-4-amino-D-*arabino*-heptulosonate 7-phosphate (aDAHP), 5-deoxy-5-amino-3-dehydroquinate (aDHQ), and 5-deoxy-5-amino-3-dehydroshikimate (aDHS) were synthesized and shown to be efficiently converted into AHBA in crude cell-free extracts of the rifamycin B producer, *A. mediterranei* S699, and the ansatrienin A producer, *Streptomyces collinus* Tü1892. However, the normal shikimate biosynthesis pathway intermediate, DAHP, did not seem to give rise to AHBA under the same conditions, although phosphoenolpyruvate plus erythrose 4-phosphate were indeed converted into aDAHP and AHBA, albeit in very low yield.

The enzyme which aromatizes aDHS to form AHBA has been purified from *A. mediterranei* S699 (33). The encoding gene, *rifK*, has been cloned, sequenced, and verified through gene inactivation to be essential for the biosynthesis of the mC_7N unit of rifamycin (33). Along with the *rifK* gene, a region of 95 kb of DNA (Fig. 2) has been isolated and sequenced (34). This revealed five large open reading frames (ORFs) coding for a modular type I polyketide synthase, various putative modifying and regulatory genes, and a subcluster of ORFs, the *rifG--N* genes, some of which are homologous to genes involved in the shikimate biosynthetic and quinate utilization pathways of plants, bacteria and fungi (34). The *rifG*, *-H*, and *-I* genes, encoding homologues of a DHQ synthase, a plant-type DAHP synthase, and a shikimate or quinate dehydrogenase, respectively, are located upstream of the *rifK* gene. Surprisingly, the *rifJ* gene, which appears to encode a type II DHQ dehydratase homologue, is located outside this subcluster about 26 kb downstream from the *rifK* gene. Presumably, the *rifH* product has a similar enzymatic activity as DAHP synthase to condense phosphoenolpyruvate and erythrose 4-phosphate in the formation of aDAHP. The formation of aDHQ and aDHS would be expected to involve the *rifG* and *-J* products to catalyze the cyclization and dehydration, respectively. The *rifK* product, AHBA synthase, then aromatizes aDHS to AHBA. The presence of the *rifI* gene is curious, as it suggests that the interconversion of aDHQ and 3-deoxy-3-aminoquinic acid or aDHS and 5-deoxy-5-aminoshikimic acid might play a role in AHBA formation.

The nitrogen of AHBA was originally proposed to originate from the amide nitrogen of glutamine by action of an amidohydrolase (31). However, a subsequent ^{15}N experiment did not support this notion (32), and no homology to known amidohydrolases was seen in the entire *rif* cluster. The only gene obviously related to nitrogen metabolism is *orf9*, located 11 kb downstream of the *rifK* gene, which encodes an aminotransferase predicted to catalyze amination of a keto group to an aminosugar (34). Curiously, there are four genes, *rifL*, *-M*, *-N*, and *-O*, located immediately downstream of *rifK*, which are not obviously shikimate pathway-related. *RifO* is homologous to

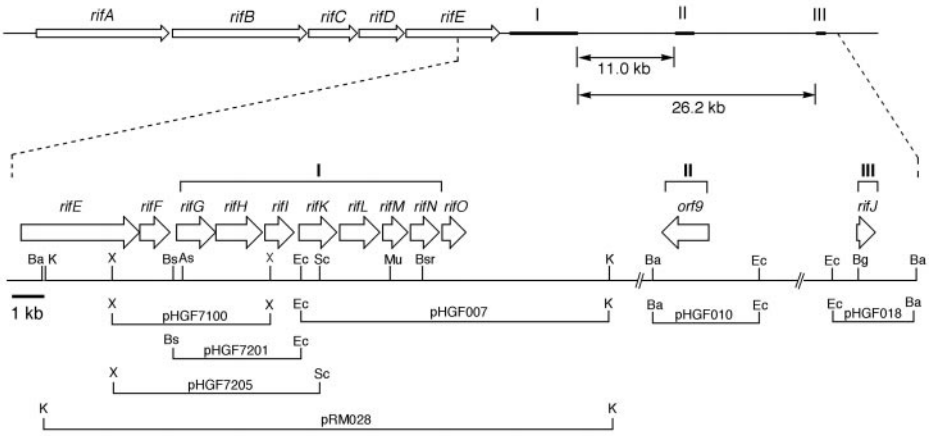

FIG. 2. **Genetic organization of the rifamycin biosynthetic gene cluster.** The cluster is shown at two scales; the *upper scale* shows the entire cluster (95 kb) and indicates five large ORFs coding for the modular type I polyketide synthase (*rifA–E*). Three regions (I–III) revealed coding sequences, which are either homologous to genes involved in the shikimate biosynthetic and quinate utilization pathways, or are implicated to be AHBA pathway-related components. The orientation of the *arrows* indicates the direction of each ORF. *Lines* indicate inserts of plasmid clones. *Ba*, *Bam*HI; *K*, *Kpn*I; *Bs*, *Bsi*WI; *As*, *Asc*I; *X*, *Xho*I; *Ec*, *Eco*RI; *Sc*, *Sac*I; *Mu*, *Mlu*I; *Bsr*, *Bsr*GI; *Bg*, *Bgl*II.

rif gene product	Sequence similarity	Proposed Function
RifG (*rifG*)	AroB; *E. coli* (AAC76414)	Aminodehydroquinate synthase
RifH (*rifH*)	AroG; *L. esculentum* (CAA79856)	AminoDAHP synthase
RifI (*rifI*)	AroE; *Synechocystis* sp. (BAA18699)	Aminoquinate dehydrogenase
RifJ (*rifJ*)	AroD; *A. pleuropneumoniae* (S41538)	Aminodehydroquinate dehydratase
RifK (*rifK*)	AHBAS; *S. collinus* (CAA90935)	AHBA synthase
RifL (*rifL*)	Pur10; *S. alboniger* (CAA63160)	Oxidoreductase
RifM (*rifM*)	CbbzP; *R. eutropha* (AAA20195)	Phosphatase
RifN (*rifN*)	XylR; *Synechocystis* sp. (BAA10031)	Kinase
RifO (*rifO*)	LmbE; *M. leprae* (AAA63037)	Putative regulator
Orf9 (*orf9*)	YokM; *B. subtilis* (AAB81154)	Aminotransferase

certain regulatory genes but its inactivation did not decrease rifamycin B formation.[2] The gene product of *rifL* is similar to a class of oxidoreductases that have been implicated in interconversions between hydroxyl and carbonyl groups, such as glucose-fructose oxidoreductase which oxidizes glucose to gluconolactone and reduces fructose to sorbitol in *Zymomonas mobilis* (37). The deduced peptide sequence of the *rifM* gene has considerable similarity with the CBBY family of phosphoglycolate phosphatases involved in glycolate oxidation (38). The *rifN* gene product shows a significant similarity with the glucose kinase from *Streptomyces coelicolor* and *Bacillus megaterium* involved in glucose repression (39, 40). In the context of the current model of rifamycin biosynthesis, the observed homologies of the gene products of *rifL*, *-M*, and *-N* do not clearly reveal their functions. However, the juxtaposition of the *rifK* and *rifL–N* genes suggests that there is an organized subcluster or a potential operon that may be responsible for AHBA biosynthesis.

In the present study we have examined the involvement of the *rifG–N*, *orf9*, and *rifJ* genes in AHBA formation. We describe inactivation experiments for each of these genes and the biochemical characterization of the mutants generated. The *rifG–N* and *-J* genes were further subcloned and coexpressed in *S. coelicolor* YU105.

MATERIALS AND METHODS

Strains and Culture Conditions

All strains and plasmids used in this work are listed in Table I. *A. mediterranei* S699 was a gift from Dr. G. C. Lancini (Lepetit S.A., Varese, Italy) and was grown as described previously (33). SM medium was used for sporulation. The *E. coli* strain XL-1 Blue (Stratagene) was routinely used as the host strain for DNA manipulations and for constructing the gene-inactivated suicide vectors. The strain was grown in LB medium supplemented with carbenicillin (100 μg/ml) or hygromycin (100 μg/ml). *S. coelicolor* YU105 served as the host for the coexpression of the *rifG–N* and *rifJ* genes and was cultured in modified R5 medium (without sucrose) (35).

DNA Manipulation and Analysis

The total genomic DNA of *A. mediterranei* was isolated as described (33). Southern blotting analysis was carried out as described (33) or using the DIG luminescent labeling and detection kit (Roche Molecular Biochemicals). Oligonucleotides were obtained from Life Technologies, Inc. PCR was carried out in a TEMPTRONIC thermal cycler (Thermolyne). General cloning procedures and manipulation of DNA were performed according to Sambrook *et al*. (36). The gene sequence of the rifamycin biosynthetic gene cluster, including *rifG–O*, has been deposited at GenBank℠ under accession numbers AF040570 and AF040571.

Mutagenesis in A. mediterranei

A 1.7-kb DNA fragment carrying the *hyg* gene for hygromycin resistance either from pIJ693 (41) or pIJ5607[3] was routinely used as the selection marker in the generation of the gene-inactivated constructs. The DNA fragments containing the targeted genes were subcloned into pUC119 (43) or pBluescript SK(−) (Stratagene). The DNA fragments containing the region to be mutated were as given below:

RifG Inactivation—A 3.8-kb *Xho*I fragment containing the *rifG* gene was cloned into pBluescript II SK(−), cut at the unique *Asc*I site on the N terminus of the *rifG* gene, blunt-ended, and religated to create pHGF7101. A new *Bss*HII site was generated and led to a frameshift mutation in the N terminus of the *rifG* gene. The replacement suicide vector pHGF7102 was created by insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pHGF7101.

RifG–I Inactivation—A 5.6-kb *Xho*I-*Sac*I fragment containing the C terminus of the *rifE* gene, the entire *rifF–I* genes and the N terminus of the *rifK* gene, was cloned into pBluescript SK(−), partially digested with *Not*I, and religated to create pHGF7206. A 2496-bp *Not*I fragment carrying the C terminus of the *rifG* gene, the entire *rifH* gene, and the N terminus of the *rifI* gene was deleted. The replacement suicide vector pHGF7207 was created by the insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pHGF7206.

RifI Inactivation—A 3.4-kb *Bsi*WI-*Eco*RI fragment containing the *rifI* gene was cloned into LITMUS 29 (New England Biolabs, New England), cut at the unique *Xho*I site on the N terminus of the *rifI* gene, blunt-ended, and religated to create pHGF7202. A new *Pvu*I site was generated and led to a frameshift mutation in the *rifI* gene. The replacement suicide vector pHGF7203 was created by the insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pHGF7202.

RifL Inactivation—The 1.6-kb *Eco*RI-*Xho*I and 1.65-kb *Xho*I-*Bam*HI fragments containing the N terminus and C terminus of the *rifL* gene, respectively, were ligated and cloned into pHGF008 to create pRM04. A 624-bp *Xho*I fragment was deleted in the *rifL* gene. The replacement

[2] R. Müller and M. Taylor, unpublished results.

[3] C. Khosla, personal communication.

TABLE I
Bacterial strains and plasmids used in this study

Strains or plasmids	Properties or product	Source or reference
Strains		
A. mediterranei S699	Wild type, rifamycin B	G.C. Lancini, Lepetit S.A.
HGF003	*rifK*::*hyg*	(33)
HGF005	*Xho*I site eliminated (filled-in) from *rifI*	This study
HGF008	*Asc*I site eliminated (filled-in) from *rifG*	This study
HGF009	2.5-kb *Not*I fragment (*rifG*, *rifH*, and *rifI*) deleted	This study
RM01	624-bp *Xho*I fragment deleted from *rifL*	This study
RM04	*Mlu*I site eliminated (filled-in) from *rifM*	This study
RM05	*Bsr*GI site eliminated (filled-in) from *rifN*	This study
MM01	897-bp *Pst*I fragment deleted from *orf9*	This study
XZ01	*Bgl*II site eliminated (filled-in) from *rifJ*	This study
S. lividans 1326	Wild type	(35)
S. coelicolor YU105		(42)
Plasmids		
pBluescript II SK(−)	Phagemid vector derived from pUC19	GenBank™ no. X52329
pUC119	Phagemid vector derived from pUC19	(43)
pCR-Script Amp SK(+)	Phagemid vector derived from pUC19	Stratagene
LITMUS 29	Multipurpose cloning/*in vitro* transcription phagemid vector	New England Biolabs
pNEB193	pUC19 derivative that carries single sites for *Asc*I, *Pac*I, and *Pme*I	New England Biolabs
pIJ963	*hyg* cassette in pUC18	(41)
pIJ4123	Thiostrepton-inducible His-tagged protein expression vector	(54)
pIJ5607	*hyg* cassette in pUC18	See Footnote 3
pIJ6021	Thiostrepton-inducible expression vector	(54)
pRM5	*act* expression vector	(44)
pRM004	1.6-kb *Eco*RI-*Xho*I and *Xho*I-*Bam*HI fragments containing the up- and downstream of the *rifL* DNA stretch ligated and cloned into pHGF008, and resulted in a 624-bp deletion in *rifL*.	This study
pRM005	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pRM004	This study
pRM028	15.8-kb *Kpn*I *rif* fragment cloned into pBluescript II SK(−)	This study
pRM029	*rifG* expression vector	This study
pRM031	*Mul*I site eliminated from *rifM* in pHGF007	This study
pRM032	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pRM031	This study
pRM035	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pRM036	This study
pRM036	*Bsr*GI site eliminated from *rifN* in pHGF007	This study
pRM041	1.1-kb PCR-generated fragment containing the N-terminal part of *rifG* cloned into *Srf*I site of pCRScript	This study
pRM043	11.4-kb *Asc*I-*Hin*dIII *rif* fragment from pRM028 cloned into pRM041	This study
pRM046	*Bgl*II site eliminated from *rifJ* in pHGF018	This study
pRM051	1.7-kb *Bgl*II *hyg* fragment from pIJ963 cloned into *Bam*HI site of pRM046	This study
pRM084	1.1-kb *Nde*I-*Eco*RI fragment cloned from pRM029 into pIJ4123	This study
pRM086	1.1-kb *Nde*I-*Eco*RI fragment cloned from pRM029 into pIJ6021	This study
pHGF007	8.6-kb *Eco*RI-*Kpn*I *rif* fragment cloned into pUC119	This study
pHGF008	*neo* cassette cloned into *Srf*I site of pCRScript	P.R. August, personal communication
pHGF010	3.8-kb *Bam*HI-*Eco*RI *rif* fragment cloned into pUC119	This study
pHGF018	2.5-kb *Eco*RI-*Bam*HI *rif* fragment cloned into pUC119	This study
pHGF102	897-bp *Pst*I fragment (*orf9*) deleted from pHGF010	This study
pHGFAT108	1.7-kb *Bgl*II *hyg* fragment from pIJ963 cloned into *Bam*HI site of pHGF102	This study
pHGF7100	3.8-kb *Xho*I *rif* fragment cloned into pBluescript II SK(−)	This study
pHGF7101	*Asc*I site eliminated from *rifG* in pHGF7100	This study
pHGF7102	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pHGF7101	This study
pHGF7201	3.4-kb *Bsi*WI-*Eco*RI *rif* fragment cloned into LITMUS 29	This study
pHGF7202	*Xho*I site eliminated from *rifI* in pHGF7201	This study
pHGF7203	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pHGF7202	This study
pHGF7205	5.6-kb *Xho*I-*Sac*I *rif* fragment cloned into pBluescript II SK(−)	This study
pHGF7206	2.5-kb *Not*I fragment deleted from *rif* in pHGF7205	This study
pHGF7207	1.7-kb *Kpn*I *hyg* fragment from pIJ5607 cloned into pHGF7206	This study
pHGF7406	*rifK* expression vector	(45)
pHGF7409	8.6-kb *Eco*RI-*Hin*dIII fragment cloned from pRM028 into pHGF7406	This study
pHGF7414	0.5-kb *Nco*I-*Hin*dIII fragment that was generated by PCR and carries the whole *rifJ*, cloned into pHGF7409 to replace the 0.84-, 2.3-, and 1.4-kb *Nco*I and *Nco*I-*Hin*dIII fragments	This study
pHGF7501	3.1-kb *Pac*I-*Hin*dIII fragment cloned from pRM5 into pNEB193	This study
pHGF7502	*Nde*I site eliminated from *lacZ* in pHGF7501	This study
pHGF7503	Described under "Materials and Methods"	This study
pHGF7504	4.5-kb *Nde*I-*Xba*I fragment cloned from pHGF7414 into pHGF7503	This study
pHGF7505	A pNEB193-based *act*II-*orf4*/*pact*III-*act*I cassette vector	This study
pHGF7603	3.4-kb *Pac*I-*Eco*RI fragment cloned from pRM043 into pRM5	This study
pHGF7604	6.0-kb *Pac*I-*Hin*dIII fragment cloned from pHGF7504 into pHGF7603	This study
pHGF7605	3.4-kb *Pac*I-*Bst*BI (*rifG*, *rifH*, and *rifI*) fragment deleted from pHGF7604	This study
pHGF7606	*Asc*I site eliminated from *rifG* in pHGF7604	This study
pHGF7607	624-bp *Xho*I fragment deleted from *rifL* in pHGF7604	This study
pHGF7608	224-bp *Apa*I fragment deleted from *rifM* in pHGF7604	This study
pHGF7609	832-bp *Nco*I-*Pml*I *rifN* containing fragment deleted in pHGF7604	This study
pHGF7610	505-bp *Nco*I-*Spe*I *rifJ* containing fragment deleted in pHGF7604	This study
pHGF7611	0.8-kb *Xho*I-*Eco*RI *rifI* containing fragment deleted in pHGF7605	This study
pHGF7612	0.8-kb *Xho*I-*Eco*RI *rifI* containing fragment deleted in pHGF7604	This study
pHGF7613	2.4-kb *Pac*I-*Bst*BI *rifG*, *rifH*, and *rifI* containing fragment deleted in pHGF7604	This study

TABLE II
Oligonucleotide primers synthesized in this work

Primers	Oligonucleotides
M13 40	5′-GTTTTCCCAGTCACGAC-3′
T7 promoter	5′-TAATACGACTCACTATAGGGAGA-3′
TIN001	5′-GACCACGAGGCAGGGAGGGGAACATATGCATCTAGATTTAAATGACGCCGGCCGGTCCGTGAACGCGGTGGAGCC-3′
TIN004	5′-AGCTTAGATCTCGAGTTTAAACAGCT-3′
TIN005	5′-GTTTAAACTCGAGATCTA-3′
TIN006	5′-TGCTCTAGAGGTTCGAATGCATATGTTCCCCTCCCTGCCTCGTGGTC-3′
TIN007	5′-TACGCCAAGCTTAGATCTCGAGTTTAAACAGCTCGGATTCACGGTCGCGGTGG-3′
TIN010	5′-TCGGCGCCATGGAGGGTGAGATGGTCCAGGCCCCCTGCCGAACGAG-3′
TIN011	5′-TCGTCAAGCTTCTAGACTAGTCAGCGGAGCCCATTCGGCGATTCGG-3′
RM020	5′-GGAGGATCCCCATATGAGGACGACGATCCCGGTCCGCC-3′
RM021	5′-GCGAATTCCGGCTGCCGCTTCACGG-3′
RM024	5′-GTGCTTAATTAACGGAAGAAGGCGCCGTGAGGACGA-3′

suicide vector pRM05 was created by the insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pRM04.

RifM Inactivation—An 8.6-kb *Eco*RI-*Kpn*I fragment containing the *rifM* gene was cloned into pUC119, cut at the unique *Mlu*I site on the N terminus of the *rifM* gene, blunt-ended, and religated to create pRM031. A new *Bss*HII site was generated and led to a frameshift mutation in the *rifM* gene. The replacement suicide vector pRM032 was created by the insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pRM031.

RifN Inactivation—An 8.6-kb *Eco*RI-*Kpn*I fragment containing the *rifN* gene was cloned into pUC119, cut at the unique *Bsr*GI site on the N terminus of the *rifN* gene, blunt-ended, and religated to create pRM036. A new *Bsa*AI site was generated and led to a frameshift mutation in the *rifN* gene. The replacement suicide vector pRM035 was created by insertion of a 1.7-kb *Kpn*I fragment carrying the hygromycin resistance gene from pIJ5607 into *Kpn*I-treated pRM036.

RifJ Inactivation—A 2.5-kb *Bam*HI-*Eco*RI fragment containing the *rifJ* gene was cloned into pUC119, cut at the unique *Bgl*II site on the N terminus of the *rifJ* gene, blunt-ended, and religated to create pRM046. A new *Cla*I site was generated and led to a frameshift mutation in the *rifJ* gene. The replacement suicide vector pRM051 was created by the insertion of a 1.7-kb *Bgl*II fragment carrying the hygromycin resistance gene from pIJ963 into *Bgl*II-treated pRM046.

Orf9 Inactivation—A 3.8-kb *Bam*HI-*Eco*RI fragment containing the *orf9* gene, was cloned into pUC119, digested with *Pst*I, and religated to create pHGF102. An 897-bp *Pst*I fragment was deleted in the *orf9* gene. The replacement suicide vector pHGFAT108 was created by the insertion of a 1.7-kb *Bgl*II fragment carrying the hygromycin resistance gene from pIJ963 into *Bgl*II-treated pHGF102.

The mycelia of *A. mediterranei* S699 were cultured to an early log-phase in a 500-ml Erlenmeyer flask with springs containing 100 ml of YMG medium, harvested by centrifugation, and washed twice with ice-cold 10% glycerol. The prepared mycelia could be stored at −80 °C for at least 6 months. The DNA replacement vectors (~2 μg) were heat-denatured, immediately cooled on ice, and then used for transformation via electroporation. The transformed mycelia were transferred directly into 60 ml of YMG medium and grown at 28 °C for 16–36 h to increase the chance for homologous recombination between the introduced vectors and the chromosomal DNA in growing mycelia. Regenerated mycelia were then plated onto YMG agar plates containing hygromycin (100 μg/ml) and continually grown for 1–2 weeks. Integrated transformants start to appear as visible colonies after 3 days. The colonies resulting from the single crossover recombination were plated on nonselective SM medium and screened for hygromycin-sensitive recombinants derived from a second crossover event, through which the vector with the resistance marker is excised. A dilution series of the harvested spores was prepared and plated again on nonselective YMG agarose plates. After a sufficient growth, these colonies were replicated to YMG agarose plates containing hygromycin. Colonies growing on the nonselective, but not the selective, agar plates were isolated, and their total genomic DNA was prepared and analyzed by Southern blotting to determine whether the *rif* genes had been replaced with their corresponding inactivated versions or the colony was a revertant to the wild type. Typically, 10–20 transformants were picked in the initial gene disruption step. On average, 0.5–5% of gene replacement clones are obtained with this procedure depending on the length of DNA provided for homologous recombination.

AHBA Feeding and Rifamycin B Analysis

Cultures of the mutants were grown until they reached the early stationary stage, split equally into two portions, and then transferred into a new flask. AHBA (1–10 mg) was added to one of the cultures. The cultures were then grown for 4 more days. The culture broths were acidified with 1 N HCl to pH 2–3 and extracted twice with equal volumes of ethyl acetate. After drying and removal of the organic solvent in a vacuum, the residue was dissolved in 1 ml of methanol and analyzed by HPLC (System GOLD, Beckman). HPLC was performed on an RP-18 column (250 × 4.6 mm, gradient MeOH:0.05% HOAc at $t_{0\ min}$ = 30:70, at $t_{20\ min}$ = 100:0; 1.0 ml/min flow rate) with detection at λ = 256 and 425 nm. Rifamycin B t_{ret} = 12.5 min. The eluted rifamycin B samples were collected, and their identity was confirmed by electrospray-mass spectrometry analysis.

The Vector for Expression of the rif Genes in S. coelicolor YU105

The actII-orf4/pactIII-actI Promoter Cassette—A 3.1-kb *Hin*dIII-*Pac*I fragment containing the divergent promoters (p*act*III-*actI*) and the regulatory gene (*act*II-*orf4*) from pRM5 (44) was cloned into pNEB193 (New England Biolabs). The unique *Nde*I site on the *lacZ* gene in pNEB193 was then cut, blunt-ended, and eliminated to generate pHGF7502. To introduce unique DNA cloning sites to increase the cloning capacity and to eliminate nonessential sequences, two further steps were carried out. First, a pair of oligomers, TIN004 and TIN005 (Table II), were annealed and cloned into *Hin*dIII-*Sac*I-treated pHGF7502 to replace the redundant *Hin*dIII-*Sac*I fragment and incorporate *Bgl*II and *Pme*I sites upstream of *act*II-*orf4* to generate pHGF7503. Further, using pHGF7503 as the DNA template, two separate PCR reactions were performed. The PCRI mixture contained oligomers TIN006 and M13–40 (Table II) as primers to obtain a 260-bp DNA fragment containing the p*act*III-*act*I promoter region. The PCRII mixture used oligomers TIN001 and TIN007 (Table II), resulting in a product with the 1.1-kb fragment carrying the whole *act*II-*orf4* gene. After treatment with *Xba*I, the products of PCRI and PCRII were assembled, digested with *Hin*dIII and *Eco*RI, and cloned into pHGF7503 to introduce the modified *act*II-*orf4*/p*act*III-*act*I DNA fragment, leading to pHGF7505. The sequence of the 1.4-kb *Hin*dIII-*Eco*RI assembled product (Fig. 3) was confirmed, using an Applied Biosystems model 377 sequencer.

Coexpressing rif Genes—An 8.6-kb *Eco*RI-*Hin*dIII fragment containing the C terminus of the *rifK* gene and the whole *rifL–N* genes from pRM028 (Table I) was cloned into pHGF7406 and replaced the 1.6-kb *Eco*RI-*Hin*dIII fragment (45) to create pHGF7409. Using pHGF018 (Table I) as the DNA template and primers TIN010 and TIN011, a 0.5-kb PCR product containing the whole *rifJ* gene was amplified and cloned into pHGF7409 to replace the 4.6-kb *Nco*I-*Hin*dIII DNA fragment located downstream of the *rifN* gene. The 4.5-kb *Nde*I-*Xba*I fragment carrying the whole *rifK–N* and *-J* genes from the resulting construct, pHGF7414, was further relocated to pHGF7505 to generate pHGF7504. To introduce the *Pac*I cloning site in front of the *rifG* gene, a 1.1-kb PCR product containing the N-terminal part of *rifG* was generated using the DNA template, pHGF7100 (Table I), and primers RM021 and RM024 (Table II). The resulting PCR product was subcloned into pCRScript digested with *Srf*I, and then the 0.5-kb *Asc*I-*Hin*dIII fragment was further replaced with the 11.4-kb *Asc*I-*Hin*dIII fragment from pRM028 (Table I) to create pRM043. The 3.4-kb *Pac*I-*Eco*RI fragment carrying the *rifG–I* genes from pRM043 and the 6.0-kb *Pac*I-*Hin*dIII fragment carrying the *rifK–N*, *rifJ*, *actII-orf4* genes and p*actI-III* promoters from pHGF7504 were sequentially cloned into

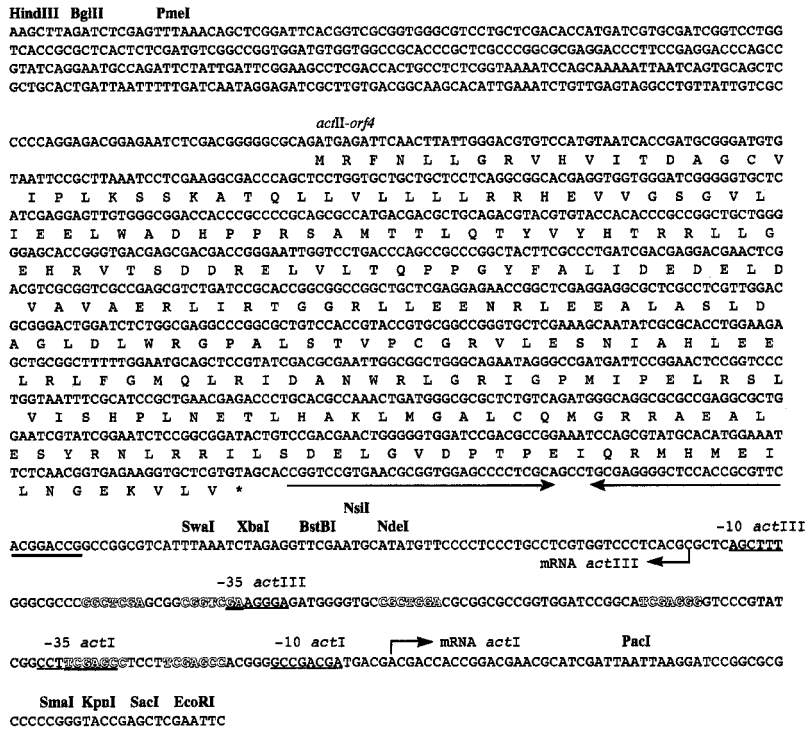

FIG. 3. **Nucleotide sequence of the 1486-bp *Hin*dIII-*Eco*RI insert carrying the entire regulatory gene, *actII-orf4*, and the modified p*act*III-*act*I promoters (accession no. AF335989) in pHGF7505.** The deduced *actII-orf4* gene product is indicated in *single-letter code* under the DNA sequence. *Long arrows* indicate sequences showing inverted repeat symmetry, which are putatively associated with termination or attenuation. The −10 and −35 regions of the identified p*act*III-*act*I promoters and the *act*II-*orf4* product binding sites (47) are *underlined* and *outlined*, respectively. The transcription start points of *act*I and *act*III are also indicated by *short arrows*.

pRM5 to replace the resident *act* genes. The resulting construct was designated pHGF7604 (Fig. 4).

pHGF7604-derived Constructs—To examine the functional activities of the *rif* genes in pHGF7604, the coding region of each individual gene was partially deleted or modified by the elimination of a unique restriction site. First, the unique *Asc*I site on the N terminus of the *rifG* gene in pHGF7604 was cut, blunt-ended, and religated to generate pHGF7606. This created a new *Bss*HII site and led to a frameshift mutation in the *rifG* gene. pHGF7607 and pHGF7608 were constructed directly from pHGF7604 by the deletion of a 624-bp *Xho*I or a 224-bp *Apa*I fragment located in the *rifL* and *rifM* coding regions, respectively. The 832-bp *Nco*I-*Pml*I fragment covering the C terminus of the *rifN* gene and the 505-bp *Nco*I-*Spe*I fragment containing the entire *rifJ* coding region, respectively, were deleted in pHGF7604 by double cutting with *Nco*I and *Pml*I, or *Spe*I, blunt-ending and religation to form pHGF7609 and pHGF7610. Using the same strategy, the 0.8-kb *Xho*I-*Eco*RI fragment containing the coding region of the *rifI* C terminus was removed from pHGF7604 and pHGF7605, to create pHGF7612 and pHGF7611, respectively. pHGF7613 was constructed from pHGF7604 by the elimination of the 2.4-kb *Pac*I-*Bst*BI fragment carrying the coding sequences of the *rifG–I* genes.

Assay for AHBA Production

The AHBA produced in the bacterial cultures was detected through the ability to restore rifamycin B production to the AHBA nonproducing *rifK* mutant, *A. mediterranei* HGF003 (33). First, *A. mediterranei* HGF003 and the test bacterial strains were grown separately in YMG medium and the modified R5 medium (without sucrose) (35). When the cultures had reached the stationary phase, the HGF003 and the test cultures were mixed in a 3 to 1 ratio and culturing was continued for 3 more days. The mixed cultures were then harvested and analyzed for rifamycin B production by HPLC.

AHBA production was assayed quantitatively by the inverse isotope-dilution procedure described by Kim *et al*. (32). In general, 40 μg of [7-^{13}C]AHBA (90% ^{13}C) was added to the crude *S. coelicolor* cell extract (2.5 ml), which was then silylated using 100 μl of SIGMA-SIL-A. The gas chromatography mass spectrometry analysis was carried out on a Hewlett Packard 5890 gas chromatograph connected to a Hewlett Packard 5971A mass selective detector under conditions as described previously (32). Enzymatic AHBA formation in cell-free extracts of *A. mediterranei* mutants was assayed as described by Kim *et al.* (32).

RESULTS

Effects of rifG, rifH, and rifI Genes on Rifamycin B Production—Based on DNA and deduced peptide sequence analysis,

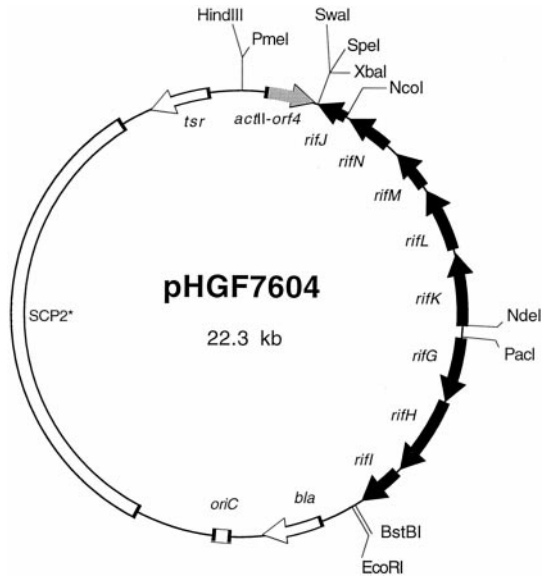

FIG. 4. **The vector pHGF7604.** The plasmid is bifunctional with the ColE1 replication origin (*oriC*) and β-lactamase (*bla*) gene for selection in *E. coli*, and SCP2* origin of replication and thiostrepton resistance gene (*tsr*) for selection in *Streptomyces*. The cloned AHBA biosynthetic genes to be expressed in *S. coelicolor* YU105 are inserted downstream of p*act*III (*rifK–N*, *rifJ*) and p*act*I (*rifG–I*), respectively, which are transcriptionally activated by the product of the *act*II-*orf4* gene. The detailed procedure for this construction is described under "Materials and Methods."

the products of *rifG* and *rifH* are homologous to a DHQ synthase and a plant-type DAHP synthase involved in the shikimate biosynthetic pathway, respectively. The *rifI* gene product is similar to a shikimate or quinate dehydrogenase in the shikimate biosynthesis and quinate utilization pathways in bacteria, plants, and fungi. These three genes are located side by side and immediately upstream of *rifK*, the AHBA synthase gene. To relate their functions to AHBA biosynthesis, we constructed a mutant of *A. mediterranei*, HGF009, in which a 2496-bp *Not*I DNA fragment carrying the C terminus of *rifG*,

TABLE III
Characters of A. mediterranei S699-derived rif mutants and their effects on rifamycin productivity

Strains	Gene(s) inactivated	Rifamycin production[a]	Restored by AHBA supplementation
		%	
HGF003	*rifK*	0	+
HGF005	*rifI*	100	Not tested
HGF008	*rifG*	100	Not tested
HGF009	*rifG*, *rifH*, *rifI*	1–2	+
RM01	*rifL*	0	+
RM04	*rifM*	0	+
RM05	*rifN*	0	+
MM01	*orf9*	100	Not tested
XZ01	*rifJ*	10	+

[a] The relative rifamycin production levels were compared with the yield obtained from fermented cultures of *A. mediterranei* S699 wild-type. 100% indicates that the yield is the same as or close to the wild-type levels, and 0 stands for not detectable.

the entire *rifH*, and the N terminus of *rifI* had been deleted from the genome. The mutant construction showed that there is an endogenous promoter located upstream of *rifG* since all isolated primary tranformants that arose by the expected single-crossover integration either upstream of *rifG* or downstream of *rifI* had maintained the ability to produce a normal amount of rifamycin B (data not shown). HGF009 is able to grow on SM medium and produces rifamycin B, but HPLC analysis showed that the yield has decreased to 1–2% of that of the wild type strain S699 (Table III). Full rifamycin B productivity can be restored by supplementation of the culture with AHBA.

To further examine the functional role of each of the three genes in rifamycin biosynthesis individually, *A. mediterranei* mutants HGF005 and HGF008 were constructed. In both HGF005 and HGF008, four additional nucleotides were introduced either at the unique *Xho*I or *Asc*I site, resulting in frameshift mutations at the N terminus of the *rifI* and *rifG* genes, respectively (see "Materials and Methods"). Both mutants show a similar growth rate, either in SM or YMG medium, as the wild type strain S699. Neither the truncation of the *rifI* gene in HGF005 nor that of the *rifG* gene in HFG008 causes a significant change, if any, in the production of rifamycin B compared with the wild type strain S699 (Table III). Thus, the reduced rifamycin production by mutant HGF009 must be due largely to the absence of *rifH*.

The Products of rifL, rifM, and rifN Genes Are Essential for AHBA Formation—Located downstream of the *rifK* gene are three other genes, *rifL*, *rifM*, and *rifN*, that form a subcluster and potentially an operon sharing a transcription unit starting at *rifK* or possibly earlier (33, 34). The *rifL* gene encodes a 359-amino acid protein (37.91 kDa), and its initiation codon appears to be located 73 bases downstream of the *rifK* gene. To probe the function of the *rifL* product in AHBA and rifamycin B biosynthesis, the replacement vector pRM05 (see "Materials and Methods") was transformed and integrated into *A. mediterranei* S699. Mutant RM01, in which a 624-bp *Xho*I DNA fragment has been deleted from the *rifL* gene, was isolated after a serial screening for the loss of resistance to hygromycin. RM01 is unable to produce rifamycin B, but production can be restored to wild type levels by supplementation of the culture with AHBA (Table III).

The *rifM* gene encodes a 232-amino acid protein (24.84 kDa) and is located 14 bases downstream of *rifL*, and 34 bases upstream of the *rifN* gene, which codes for a 293-amino acid protein (29.78 kDa). The mutants RM04 and RM05 carry the inactivated *rifM* and *rifN* genes, respectively, in which four additional nucleotides were introduced at the unique *Mlu*I or *Bsr*GI site, leading to a frameshift mutation in the N terminus of *rifM* and *rifN*, respectively. As observed above with RM01, there is no detectable rifamycin B production in either mutant and AHBA supplementation restores rifamycin B productivity to wild type levels (Table III). Incubation of the crude cell-free extracts of the mutants with the known pathway intermediates, aDAHP, aDHQ, or aDHS, showed that both the RM04 and RM05 strains were able to convert aDAHP to AHBA with efficiency comparable to that of the wild-type strain S699 (Table III). However, cell-free extracts of strain RM01 failed to convert aDAHP or aDHQ into AHBA, and gave only poor conversion of aDHS, 10% compared with about 90% for the wild-type strain (32).

The rifJ Gene Is Involved in Rifamycin B Biosynthesis—The *rifJ* gene is located 26 kb downstream of the *rifK* gene (Fig. 2). The deduced peptide it encodes is closely related to type II DHQ dehydratases found in the biosynthetic shikimate and the quinate utilization pathways. *rifJ* is likely to be part of the rifamycin biosynthetic gene cluster, since *rpoB* and *rpoC*, genes coding for the DNA-dependent RNA polymerase β- and β′-subunits respectively, have been located 2.3 kb downstream of the *rifJ* gene (34). The product of the *rpoB* gene was shown to confer resistance to high doses of rifamycin B or rifampicin when transferred to *E. coli* and *Mycobacteria*.[4] As antibiotic biosynthetic gene clusters in Actinomycetes typically include one or more genes that confer resistance to the antibiotic produced, the *rif* gene cluster is assumed to extend to the *rpo* genes, and thus include *rifJ*. The *rifJ* gene product may catalyze the conversion of aDHQ to aDHS through the elimination of a water molecule.

To probe for the involvement of *rifJ* in AHBA formation, the *rifJ*-inactivated suicide vector pRM051 (see "Materials and Methods") was introduced and integrated into *A. mediterranei* S699. After serial propagation in hygromycin-free YMG medium, the *rifJ*-inactivated mutant XZ01 was isolated. In it the functional *rifJ* gene has been replaced with a mutated version carrying four additional bases, GATC, at the unique *Bgl*II site, which causes a frameshift mutation at the N terminus. The elimination of *rifJ* does not abolish antibiotic production in the XZ01 strain completely, but results in a reduction to ~10% of the wild type level (Table III). Full rifamycin B production can be restored by supplementation with AHBA (Table III). Mutant XZ01 shows similar growth rates in SM and YMG media as the wild type strain S699.

No Detectable Effect of the orf9 Gene on Rifamycin B Production—The gene product of the *orf9* gene is related to a dNTP-hexose aminotransferase and has been proposed to be responsible for the formation of an aminodeoxyhexose nucleotide (34). However, since no glycosylated rifamycin has been identified in the cultures of *A. mediterranei* S699, one cannot exclude the possibility that this gene product might play a role in the introduction of the amino group in the AHBA biosynthetic pathway. Thus, the *orf9*-inactivated suicide vector pHGFAT108 was constructed (see "Materials and Methods") and introduced into *A. mediterranei* S699. After serial culturing, the *orf9* gene was then replaced through a second homologous recombination with the truncated version in which an 897-bp *Pst*I DNA fragment has been deleted. The resulting mutant MM01 showed no significant phenotypic change in either the growth pattern or rifamycin B production (Table III).

Production of AHBA in S. coelicolor—The *act*II-*orf4* gene product has been characterized as a DNA-binding protein that positively regulates the transcription of the actinorhodin biosynthetic genes in *S. coelicolor* (46, 47). The *act*III-*act*I intergenic region, as the regulatory target for the *act*II-*orf4* protein,

[4] E. Pogosova-Agadjanyan and T.-W. Yu, unpublished data.

TABLE IV
Accumulation of AHBA in S. coelicolor YU105 harboring AHBA biosynthetic genes

Plasmids	Relevant genes								AHBA yield[a]
	rifG	*rifH*	*rifI*	*rifK*	*rifL*	*rifM*	*rifN*	*rifJ*	
									mg/liter
pHGF7604	+	+	+	+	+	+	+	+	350–400
pHGF7612	+	+	−	+	+	+	+	+	450–500
pHGF7606	−	+	+	+	+	+	+	+	30–40
pHGF7611	−	+	−	+	+	+	+	+	150–200
pHGF7613	+	−	−	+	+	+	+	+	<1
pHGF7605	−	−	−	+	+	+	+	+	0[b]
pHGF7607	+	+	+	+	−	+	+	+	0[b]
pHGF7608	+	+	+	+	+	−	+	+	0[b]
pHGF7609	+	+	+	+	+	+	−	+	0[b]
pHGF7610	+	+	+	+	+	+	+	−	20–30

[a] Cultures were grown in 60 ml of modified R5 medium for 1 week and worked up as described under "Materials and Methods." The values are typical of those obtained in five different fermentations.
[b] Not detectable.

carries a pair of divergently arranged promoters for the early biosynthetic steps to build the polyketide backbone of actinorhodin. The development of a regulatory expression system employing the transcription regulator (the *act*II-*orf4* gene product) and the pathway-specific promoters (p*act*III-*act*I) has already served to generate many novel products of aromatic and macrolide polyketide synthases (44, 48, 49). To verify the functional specificity for each candidate gene in the AHBA biosynthetic pathway, a pNEB193-based *act*II-*orf4*/p*act*III-*act*I cassette vector, pHGF7505, was constructed (see "Materials and Methods"). This *act*II-*orf4*/p*act*III-*act*I promoter cassette (Fig. 4) with different multiple cloning sites allows for cloning of the target genes to be regulated by a p*act*I or p*act*III promoter for tight transcriptional control. The putative AHBA biosynthetic genes defined as described above are located at two separate regions of the *rif* gene cluster and may be organized into three separate transcription units (Fig. 2). The 0.5-kb *Nco*I-*Hin*dIII PCR-amplified DNA fragment carrying the entire *rifJ* gene and a putative ribosome binding site, GGAGG, was connected to the *Nco*I site, 64 bases downstream of the stop codon of the *rifN* gene. As described under "Materials and Methods," the fused 4.5-kb *Nde*I-*Xba*I DNA fragment containing the *rifK–N* and *rifJ* genes, and the 3.4-kb *Pac*I-*Eco*RI DNA fragment carrying the *rifG–I* genes, were relocated into the *act*II-*orf4*/p*act*III-*act*I promoter cassette at the corresponding restriction sites. The resulting *E. coli-Streptomyces* shuttle vector pHGF7604, which carries the *act*II-*orf4* regulatory gene and eight *rif* genes (*rifG–N* and *-J*) under the control of the p*act*III-*act*I promoters, was then transformed into *S. coelicolor* YU105. Unlike *S. coelicolor* YU105, which is unable to produce AHBA or AHBA-derived ansamycins, *S. coelicolor* YU105/pHGF7604 transformants can restore rifamycin B production in the AHBA nonproducing *rifK* mutant, *A. mediterranei* HGF003, and they produce a significant amount of AHBA (350–400 mg/liter of culture) (Table IV).

AHBA Production Is Repressed by the Presence of the rifI Gene—The specific functional roles of the *rif* genes in pHGF7604 in AHBA formation were further examined in *S. coelicolor* YU105. First, individual *rif* genes were removed from pHGF7604 by deleting a region of the DNA fragment or by eliminating restriction sites. The resulting nine new constructs, pHGF7605 to pHGF7613 (Table I), were then transformed into *S. coelicolor* YU105 to assay for AHBA production. Table IV summarizes the *rif* gene compositions and the AHBA yields of pHGF7604 and the nine pHGF7604-derived constructs in *S. coelicolor* YU105. There was no AHBA detectable in the three cultures of *S. coelicolor* YU105 transformed with pHGF7607, pHGF7608, or pHGF7609. These results are consistent with those from the corresponding three genomic mutants of *A. mediterranei*, rifamycin-deficient strains RM01, -04, and -05, and confirm that the *rifL*, *-M*, and *-N* gene products are indeed absolutely essential for the formation of AHBA.

As mentioned above, *A. mediterranei* HGF008 and HGF005, the *rifG*- and *rifI*-knockout mutants, did not show a significant change in rifamycin B production compared with the wild type. However, the absence of these genes from the AHBA gene cassette clearly changes the AHBA production profile in *S. coelicolor* YU105. Reproducibly, 20–25% more AHBA is detected in the cultures transformed with pHGF7612, lacking the *rifI* gene, than in those carrying pHGF7604. Removal of the product of the *rifI* gene is not detrimental but seems to enhance AHBA production. In contrast, the product of the *rifG* gene plays a significant role in maintaining the AHBA productivity in *S. coelicolor* YU105, since the yield decreased to only 10% in the cultures of pHGF7606 transformants. These opposing effects are further confirmed by the moderate yields detected in the cultures of transformants with pHGF7611, in which both the *rifG* and *rifI* genes have been removed.

Deletion of the *rifH* gene causes a similarly severe effect on AHBA production in *S. coelicolor* YU105 as on the production level of rifamycin B in *A. mediterranei* seen in mutant HGF009. The pHGF7605 transformants, in which both the *rifH* and *rifI* genes have been deleted, produce less than 0.2% of the amount of AHBA of the pHGF7612 transformants which lack only the *rifI* gene. When the *rifG* gene is also removed, AHBA production is completely abolished in cultures of the pHGF7605 transformants.

Eliminating the product of the *rifJ* gene decreases the yield of AHBA to 6–8% in the cultures of the pHGF7610 transformants. This result is comparable to the one observed with the genomic *rifJ* mutant, *A. mediterranei* XZ01, which lost about 90% of its rifamycin B productivity.

DISCUSSION

The biosynthesis of AHBA has been investigated through target-directed mutagenesis in *A. mediterranei* S699 and heterologous expression in *S. coelicolor* YU105 of specific genes from the rifamycin biosynthetic gene cluster. Three genes homologous to shikimate biosynthesis pathway genes, the *rifG*, *-H*, and *-J* genes, and four not shikimate pathway-related genes, the *rifK* (33), *-L*, *-M*, and *-N* genes, were identified as being necessary and sufficient for AHBA formation. The observation that all these genes were required for AHBA production in the heterologous host when expressed under the control of an external promoter/regulator system suggests that none of these genes have a regulatory function in AHBA formation by the natural producer, *A. mediterranei*. Rather, all of them should encode proteins with catalytic functions in the biosyn-

thetic pathway. The requirement for seven proteins for AHBA formation, particularly the involvement of the *rifL, -M*, and *-N* gene products, points to a degree of complexity of the pathway not predicted by our previous hypothesis.

Functions of the rifG, rifH, and rifJ Gene Products—The products of the *rifG*, *-H*, and *-J* genes have been shown to be functionally active and, as predicted by our original hypothesis, to play a key role in providing the substrate aDHS for AHBA formation in the rifamycin producer, *A. mediterranei* S699. It is worth noting that all three mutants, HGF005, -8, and -9, showed no detectable growth defects in both the YMG and SM media. This implies that the products of the *rifG*, *-H*, and *-J* genes are probably not directly involved in any other, essential shikimate pathway-related metabolic processes in the cell. The fact that all three mutants kept producing rifamycin B at some level suggests that the defects in rifamycin production caused by the gene inactivations can be at least in part functionally compensated by the presence of the corresponding shikimate pathway isoenzymes in the strain, due to some overlap in substrate specificity. For example, the DHQ synthase from *E. coli* can cyclize aDAHP to produce aDHQ (32). The heterologously expressed RifG protein from *S. lividans* 1326 catalyzes the cyclization of DAHP as well as aDAHP as substrate.[5] We have detected and partially purified a second type II DHQ dehydratase, a homologue of the *rifJ* gene product, from *A. mediterranei* S699 (50). Recently, two additional DAHP synthase isoenzymes and their encoding genes have been identified and cloned from *A. mediterranei*.[6] Interestingly, one of the deduced peptide sequences is closely related to the product of the *rifH* gene and to type II DAHP synthases from higher plants.

The functional substitution and cross-talk between the products of the *rifG*, *-H*, and *-J* genes, and their corresponding normal shikimate pathway homologues also occurred in the expression of the *rif* genes to form AHBA in *S. coelicolor* YU105. However, judging from the amounts of AHBA and rifamycin B produced (Tables III and IV), it is evident that the isoenzymes from *S. coelicolor* YU105 and from *A. mediterrainei* S699 display different levels of competence to replace the functions of the *rifG*, *-H*, and *-J* gene products. Notably, and perhaps significantly, in both systems, the complementation of the *rifH* mutation by endogenous DAHP synthases is less efficient. It is not clear whether the greater competence for complementation observed in the *A. mediterranei* mutants is accidental or has evolved due to a long term molecular adaptation between substrates and the enzymes exposed to the rifamycin-producing environment.

Functional Role of the rifI Product—Although its functional role is not obvious, the presence of the *rifI* gene in the *rif* gene cluster of *A. mediterranei* S699 is unlikely to be an evolutionary accident. First, the recombinant protein expressed from the *rifI* gene in *E. coli* had the ability to catalyze the 3-dehydrogenation of shikimate, aminoshikimate, and aminoquinate, but not quinate (51). The present work showed that AHBA production in *S. coelicolor* YU105 was significantly repressed by the presence of the *rifI* gene in the expression vectors, pHGF7604 and -6 (Table IV). Since the *rifI* gene is part of the same transcription unit as the *rifG* and *rifH* genes, it should be functionally expressed and produce enzymatically active protein in *A. mediterranei* S699; yet, its disruption had no effect on rifamycin B production. This result recalls the previous observation (32) that there is no increase in the production of rifamycin B by supplementation of wild-type *A. mediterranei* S699 with AHBA, suggesting that rifamycin B synthesis in the cell is not limited by the production of AHBA.

The earlier AHBA feeding experiments had indicated that AHBA is very stable in the cells of *A. mediterranei* and is maintained throughout the whole fermentation process without substantial degradation. It is not known whether the accumulation of AHBA could lead to inhibition or suppression of regular shikimate pathway-related enzyme functions or cause any other cellular toxicity, but this possibility might provide an explanation for the presence of the *rifI* gene. The product of the *rifI* gene may act in the conversion of aDHQ and/or aDHS to 3-deoxy-3-aminoquinic acid and/or 5-deoxy-5-aminoshikimic acid, and closely regulate an AHBA-precursor reservoir to prevent the uncontrolled accumulation of AHBA in the cell.

Functions of the Products of the rifL, rifM, and rifN Genes—As the enzyme AHBA synthase (the product of the *rifK* gene) described previously (33), the products of the *rifL*, *-M*, and *-N* genes are also absolutely essential for the biosynthesis of AHBA, yet they are not related to any shikimate pathway enzymes. It is noteworthy that the location and arrangement of all AHBA synthase genes identified relative to other genes involved in AHBA biosynthesis in their respective gene clusters varies (34, 52, 53).[7] However, the close association between the AHBA synthase gene and the *rifL* and *-M* gene homologues is conserved in all the AHBA biosynthesis gene clusters analyzed. This suggests that there are functional interactions between these gene products. The results of the cell-free experiments with the *rifL, -M*, and *-N* mutants suggest that the *rifM* and *-N* gene products act at an early stage of AHBA biosynthesis, probably in or prior to the formation of aDAHP. The product of the *rifL* gene may also be related to the formation of aDAHP, but it could also or additionally affect the enzymatic activity of AHBA synthase.

The Nitrogen Source in aDAHP Formation—The available data do not support the original suggestion that the nitrogen of AHBA originates from the amide nitrogen of glutamine through the action of an amidohydrolase, which acts in concert with a DAHP synthase to generate aDAHP. Therefore, a different source and mode of introduction of the nitrogen must be identified. The product of the *orf9* gene is homologous to pyridoxal phosphate (PLP)-dependent transaminases and was suspected to carry out this function of introducing the nitrogen into a precursor of AHBA. This has been ruled out by the mutagenesis experiment, which showed no effect of the inactivation of *orf9* on rifamycin B production. The fact that the coexpression of *rifG–H*, *-K–N*, and *-J* resulted in production of AHBA in *S. coelicolor* YU105 further suggests that the nitrogen-introducing activity must reside on these seven *rif* genes, although we cannot exclude the possibility that a protein from *S. coelicolor* YU105 has taken over this function. It is worth noting that the deduced peptide sequence of the AHBA synthase gene (RifK) shows homology to the products of a series of genes implicated primarily in dehydration/deoxygenation as well as transamination reactions in deoxysugar biosynthesis (33). The recombinant AHBA synthase can bind the cofactor pyridoxamine phosphate as well as PLP, and the PLP form of the enzyme can be converted to the pyridoxamine phosphate form by incubation with the amino donor, glutamate.[8] Based on these observations, it is speculated that the AHBA synthase, possibly together with the *rifL*, *-M*, and/or *-N* gene products, may have an additional function to introduce the nitrogen into an intermediate in AHBA biosynthesis. Consistent with this hypothesis, no accumulation of aDHS, aDHQ, or their likely

[5] R. Müller and H. G. Floss, unpublished results.
[6] L.-Y. Kuan and T.-W. Yu, unpublished results.
[7] T.-W. Yu, D. Hoffmann, D. Clade, E. Zeistner, and H. G. Floss, unpublished results.
[8] T.-W. Yu and C.-G. Kim, unpublished results.

aromatization product, protocatechuic acid, was observed in the *rifK* mutant of *A. mediterranei* (33).[9] Plausible scenarios for such a second function of *rifK* could involve its joint action with *rifL* either to convert a keto group into the corresponding imine by transamination (RifK) and oxidation (RifL), or to replace a hydroxy with an amino group by oxidation to the ketone (RifL) and transamination (RifK).

Pathway of AHBA Formation—The results of this study define which of the *rif* biosynthetic genes are necessary for formation of the AHBA starter unit of rifamycin. This sets the stage for the further definition of the AHBA biosynthetic pathway at the enzymatic level. The transfer of the ability to produce AHBA to the heterologous host, *S. coelicolor*, through the expression cassette built into vector pHGF7604 also provides an important tool both for further analysis of the AHBA pathway and for the heterologous expression of other genes from the biosynthetic gene clusters of AHBA-derived antibiotics.

The demonstrated involvement of *rifG*, *-H*, *-J*, and *-K* in AHBA formation is consistent with and lends credence to the originally proposed sequence of reactions leading from aDAHP via aDHQ and aDHS to AHBA (Fig. 1). However, the part of the original hypothesis dealing with the formation of aDAHP may require some modification to accommodate a different mode of nitrogen introduction as well as the requirement for three additional genes, *rifL*, *-M*, and *-N*, in the pathway. The formation of aDAHP may involve a very different way of generating a nitrogen-containing precursor, presumably the imine of erythrose 4-phosphate or erythrose, for the condensation reaction catalyzed by the *rifH* gene product, one that is mediated by the action of the RifK–N proteins. Alternatively, the nitrogen may be introduced at a later stage, again by a process that involves the action of the *rifK–N* gene products.

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