Modification of Rifamycin Polyketide Backbone Leads to Improved Drug Activity Against Rifampicin-Resistant *Mycobacterium tuberculosis*

Aeshna Nigam[1,§], Khaled H. Almabruk[2,§], Anjali Saxena[1], Jongtae Yang[2], Udita Mukherjee[1], Hardeep Kaur[1], Puneet Kohli[1], Rashmi Kumari[1], Priya Singh[1], Lev N. Zakharov[3], Yogendra Singh[4], Taifo Mahmud[2,3] and Rup Lal[1]

[1]From the Department of Zoology, University of Delhi, Delhi-110007, India

[2]Department of Pharmaceutical Sciences and [3]Department of Chemistry, Oregon State University, Corvallis, OR 97331-3507, U.S.A

[4]Institute of Genomics & Integrative Biology (CSIR-IGIB), Delhi-110007, India

Supplemental Data

CAGGCGCTCGCCCGGCACCTGCGCGACGAACTCGGTGGTGCGGCCCAGACGCCGGTGACC
ACAGCGGCCGCGAAGGCCGACCTCGACGAGCCGATCGCCATCGTCGGGATGGCGTGCCGC
TTGCCGGGCGGGGTCGCCGGGCCCGAGGACCTCTGGCGGCTGGTCGCCGAGGGCCGGGAC
GCGGTGTCGAGCTTCCCGACCGACCGCGGCTGGGACACCGACAGCCTGTACGACCCCGAT
CCGGCCCGCCCGGGCAAGACCTACACCCGGCACGGCGGCTTCCTGCACGAAGCCGGGCTC
TTCGACGCGGGCTTCTTCGGGATCTCGCCACGCGAGGCCGTCGCCATGGACCCGCAGCAG
CGGCTGCTGCTGGAGGCCTCCTGGGAGGCCATGGAAGACGCCGGGGTCGACCCACTTTCG
CTGAAGGGCAACGACGTCGGCGTGTTCACCGGCATGTTCGGCCAGGGTTACGTCGCTCCC
GGGGACAGCGTCGTCACGCCGGAGCTGGAGGGTTTCGCGGGCACGGGCGGGTCGTCGAGT
GTCGCGTCCGGCCGCGTGTCGTACGTGTTCGGGTTCGAAGGCCCGGCCGTGACGATCGAC
TCGGCGTGCTCGTCCTCGCTGGTCGCGATGCACCTCGCCGCGCAGTCGCTGCGGCAGGGC
GAGTGCTCGATGGCCTTGGCCGGCGGCGCGACGGTGATGGCGAACCCCGGCGCATTCGTG
GAGTTCTCGCGGCAGCGGGGCCTCGCCGTCGACGGTCGCTGCAAGGCGTTCGCCGCCGCG
GCCGACGGCACCGGCTGGGCCGAGGGCGTCGGTGTGGTCATCCTCGAGCGGCTGTCGGTG
GCGCGGGAACGCGGCCACCGGATCCTGGCCGTGCTGCGCGGCAGCGCGGTCAACCAGGAC
GGCGCCTCGAACGGCCTGACCGCGCCGAACGGGCCGTCGCAGCAGCGGGTGATCCGCCGG
GCGCTGGTGAGCGCCGGGCTGGCACCGTCCGATGTGGACGTCGTCGAGGCGCACGGCACC
GGGACCACGCTGGGTGACCCGATCGAGGCGCAAGCTCTGCTGGCTACCTACGGCAAGGAC
CGCGAGTCGCCGCTGTGGCTCGGCTCGCTGAAGTCGAACATCGGCCACGCGCAGGCCGCC
GCGGGGGTCGCCGGCGTCATCAAGATGGTCCAGGCGCTCCGGCACGAAGTCCTGCCGCCG
ACGCTGCACGTCGACCGGCCTACCCCCGAGGTCGACTGGTCGGCCGGTGCCGTCGAACTG
CTGACGGAAGCCCGCGAGTGGCCGCGCAACGGGCGCCCGCGCCGGGCCGGGGTCTCCGCG
TTCGGCGTCAGCGGCACGAACGCGCACCTGATCCTGGAGGAGGCGCCCGCCGAAGAGCCG
GTGCCCACACCCGAGGTTCCCCTGGTGCCGGTCGTGGTCTCCGCGCGGAGCAGGGCGTCC
CTGGCCGGTCAGGCCGGTCGCCTCGCCGGATTCGTGGCGGGTGACGCGTCCTTGGCCGGT
GTGGCCCGGGCGCTGGTGACGAACCGGGCCGCGCTGACCGAGCGCGCGGTCATGGTCGTG
GGCTCTCGCGAAGAAGCCGTGACGAACCTGGAAGCGCTGGCCCGCGGCGAAGACCCGGCC
GCGGTGGTCACCGGCCGGGCGGGTTCGCCGGGCAAGCTCGTCTGGGTCTTCCC

PCR I (41862-43533 bp)

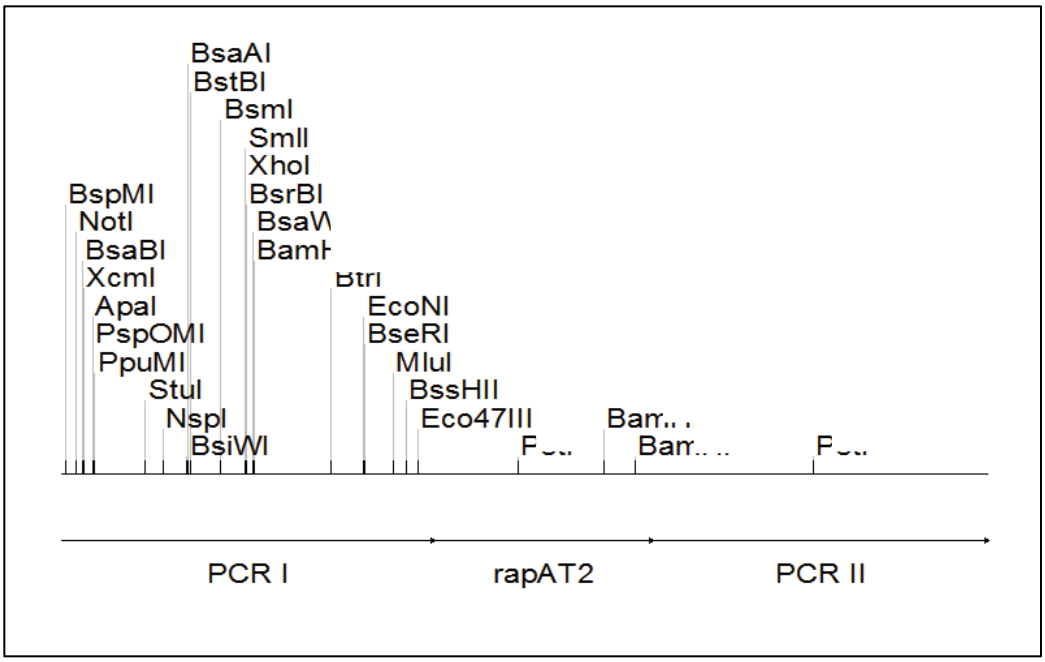
ACCACTCGACACTCACGCCGTTCACGTACAGGTGAGCCAGGGCACCGACAGCGGCCTGCG
CCTCATGGTCACCGTGCAGCATCGCGATGCCATCGACCAGGCGGGCCAGTGACCGGTCGG
CACCCAGCTCGACGAACACCGCATCCTCGAACGAGGCCACCTGCTCGCCGAACCGGACCG
TGTCCCGGACCTGCCGCACCCAGTACTCAGCGGTCATCACCTGATCACCAGCGGCCATGG
CGACCTGCGGCGTCCGGTAGGTCAGGCCTTCAGCGACCGCCCGGAACTCCTCCAGCATCG
GTTCCATACGGGCGGAATGGAACGCGTGACTGGTCGCCAGCCGCGTCCACTTCCCCAGCC
CCTCCGCGGCCTGCAGCACGGCGGCCTCATCACCGGAGAGAACCACCGACGACGGCCCGT
TGACCGCGGCGATCTCCACACCCTCACCCAGCACGGCCCGAGCCTCATCCTCCGAGACCG
GGACAGCGACCATCACCCCACCCGCAGGCAGAGCCTGCATCAGACGAGCCCGCGCCGACA
CCAAAGTGCAGGCATCCTCCAACGACCACAACCCGGAGACGTATCCGGCGGCGAGCTCAC
CGACAGAGTGACCGACCACCGCATCCGGCCGTACACCCCACGATTCCAGCAACCCGAACA
GAGCCACCTGCAAAGCGAACAGGGCCGGCTGGGCATACCCGGTCTCATTCACATCGAGAT
CGGGCACATCCAGCAGATCCCACACCTGCTGATGGATCCGCGCGAAGACGGGGAACGCGG
CGGCCAGTTCCTCACCCATACCAGCACGCTGCGACCCTGGCCAGCCGGCTGGGCATACCC
GGTCTCATTCACATCGAGATCGGGCACATCCAGCAGATCCCACACCTGCTGATGGATCCG
CGCGAAGACGGGGAACGCGGCGGCAAAGTTCCTCACCCATACCAGCACGCTGCGGACCCC
TGGCCAGGGAATACCGAG

AT2

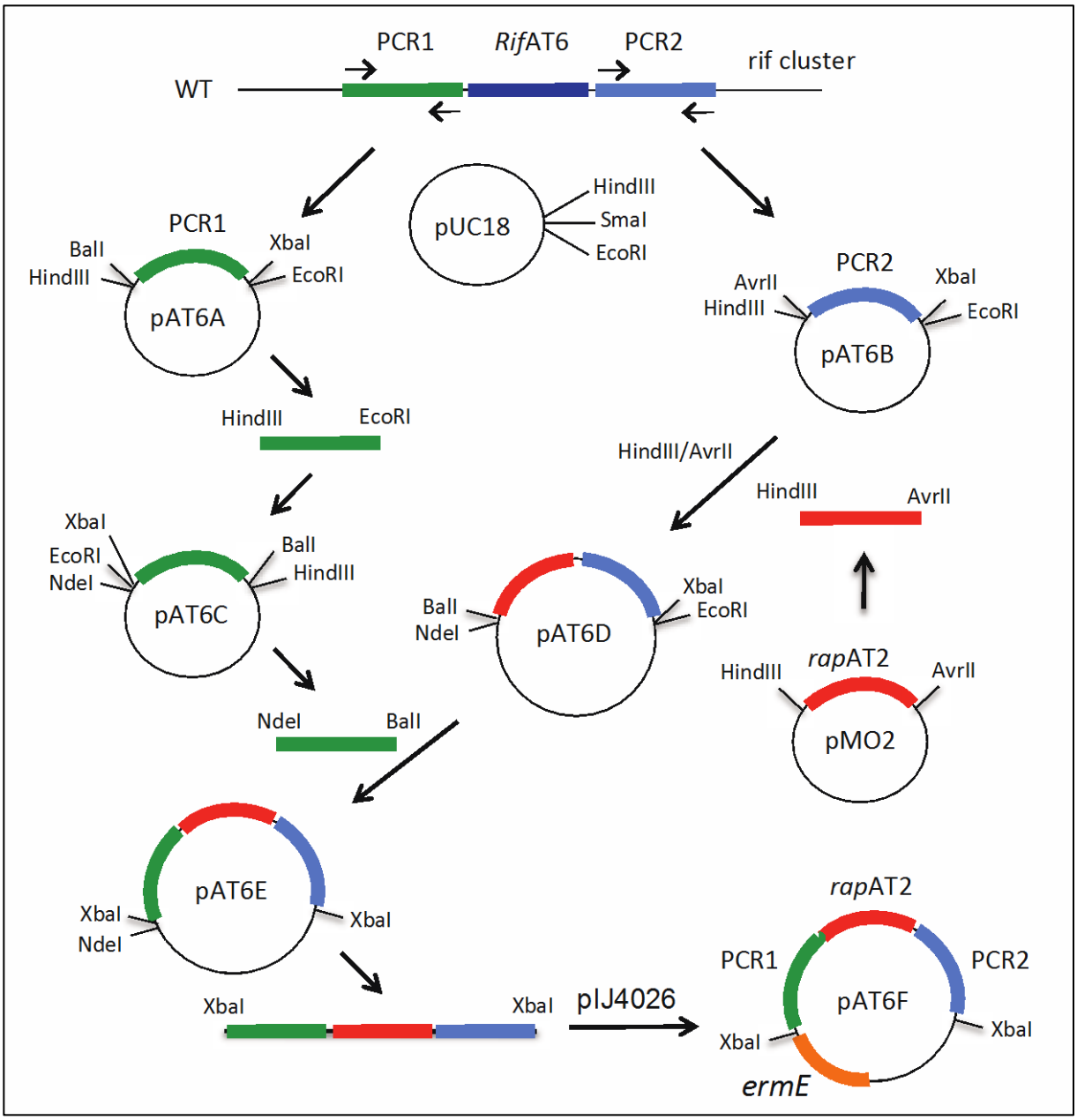
GCGGACCGGCCGGGTCGACCTGCCGAAGTACGCCTTCGACCACCGGCACTACTGGCTGCG
GCCCGCCGAGTCCGCGACCGACGCGGCTTCGCTGGGCCAGGGGGCGGCCGACCACCCGTT
GCTGGGCGCGGTCGTCGAGCTGCCGCAGTCCGACGGCCTGGTGTTCACCTCGCGGCTGTC
CGTGCGGACGCACCCGTGGCTGGCCGACCACGCGGTCGGTGGCGTGGTCATCCTCCCCGG
CTCCGGGCTGGCCGAACTGGCCGTCCGGGCCGGCGACGAAGCCGGGTGCACCGCCCTCGA
CGAGCTGATTATCGAAGCTCCGCTGGTCGTGCCCGCCCAAGGCGCGGTCCGCGTCCAGGT
CGCGTTGAGCGGCCCGGACGAGACCGGCTCGCGCACGGTGGACCTCTACTCCCAGCGCGA
CGGCGGCGCGGGGACGTGGACGCGGCACGCCACCGGCGTGCCGTCGACGGCCCCCGCTCA
GGAACCCGAGTTCGACTTCCACGCCTGGCCGCCCGCGGATGCCGAGCGGATCGACGTCGA
GACCTTCTACACCGACCTGGCCGAGCGTGGTTACGGCTACGGGCCGGCGTTCCAGGGGCT
GCAAGCGGTGTGGCGGCGCGACGGCGATGTCTTCGCCGAGGTCGCCCTGCCCGAGGACCT
GCGCAAGGACGCGGGCCGGTTCGGCGTCCACCCGGCGCTGCTCGACGCGGCGCTGCAGGC
CGCCACGGCCGTGGGCGGCGACGAGCCCGGTCAGCCGGTGCTGGCGTTCGCGTGGAACGG
CCTGGTCCTGCACGCCGCGGGCGCGTCGGCCCTGCGGGTCCGGCTCGCGCCGAGCGGCCC
GGACACGCTGTCCGTGGCAGCCGCCGACGAAACCGGCGGCTTGGTCCTGACCATGGAATC
GCTGGTCTCCCGGCCGGTTTCGGCCGAGCAGCTCGGCGCCGCGGCCGACGCGGGCCACGA
CGCGATGTTCCGCGTCGACTGGACCGAGCTGCCTGCCGTGCCCCGCGCGGAACTGCCGCC
GTGGGTGCGGATCGACACCGCCGACGACGTCGCGGCCTTGGCGGAGAAGGCGGACGCACC
ACCGGTGGTGGTCTGGGAAGCCGCCGGGGGAGACCCGGCCCTGGCCGTGAGTTCCCGGGT
GCTCGAGATCATGCAGGCCTGGCTGGCCGCGCCCGCGTTCGAGGAGGCCCGGCTGGTCGT
GACGACCCGCGGCGCGGTACCCGCCGGCGGTGACCACACACTGACCGACCCGGCCGCGGC
CGCGGTGTGGGGCCTGGTCCGGTCCGCGCAGGCGGAACACCCGGACCGGGTCGTCCTGCT
GGACACCGACGGCGAAGTTCCGCTGGGCGCGGTGCTGGCCTCCGGTGAGCCGCAGCTCGC
GGTGCGCGGAACGACGTTCTTCGTGCCCCGGCTGGCCCGCGCCACCCGGCTCTCGGACGC
GCCTCCTGCGTTCGACCCGGACGGGACCGTGCTGGTCTCGGGCGCCGGATCGCTGGGCAC
CT

PCR II (44488-45989 bp)

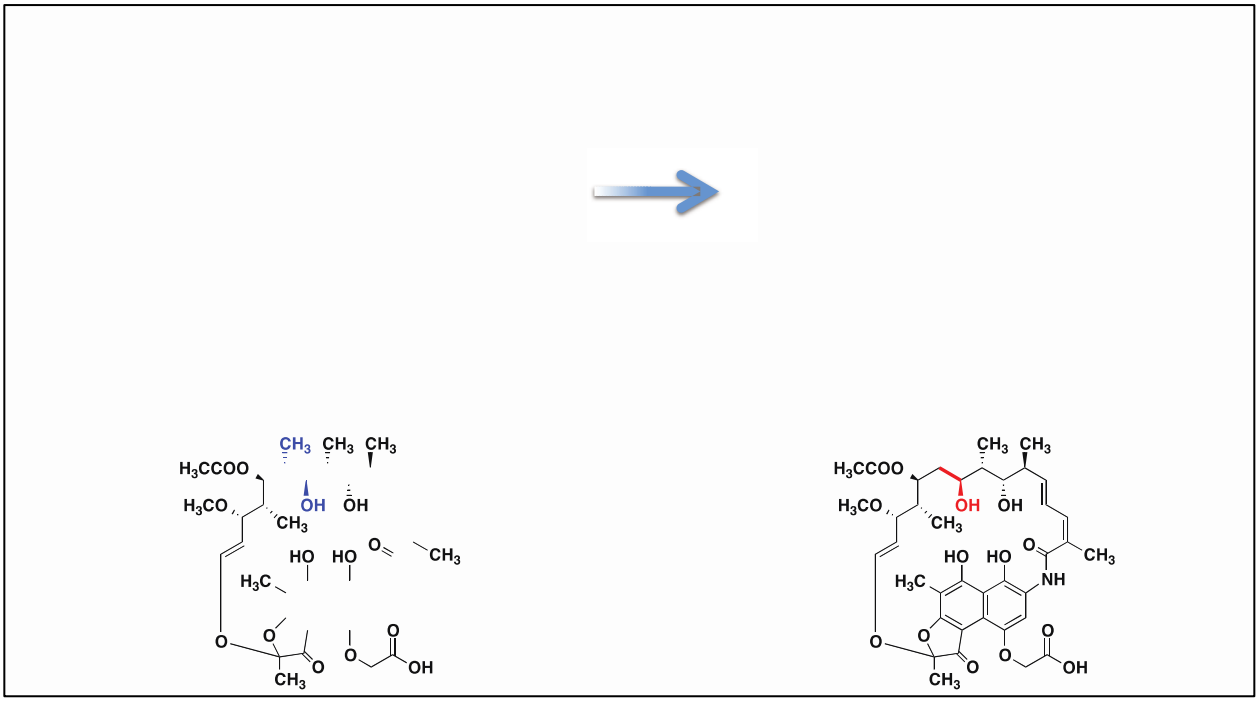
Supplemental Figure 1. Nucleotide sequence of PCR1/*rap*AT2/PCR2 of pAT6F



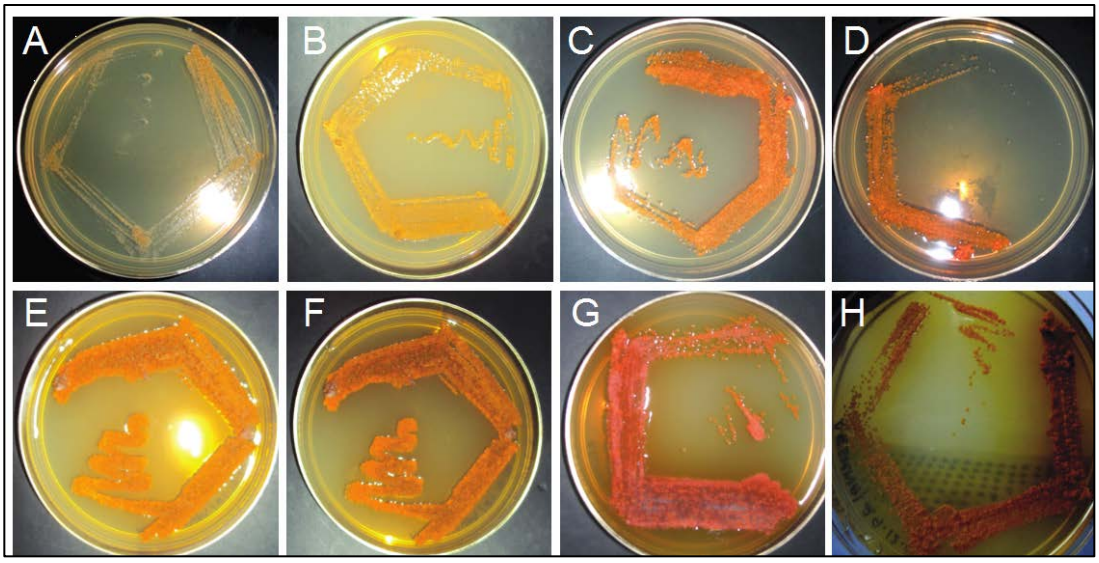
Supplemental Figure 2. Restriction map of hybrid DNA obtained from nucleotide sequences (Figure S1) formed in the double cross over clones. Red arrows indicate the restriction sites for BamHI and PstI that were used.



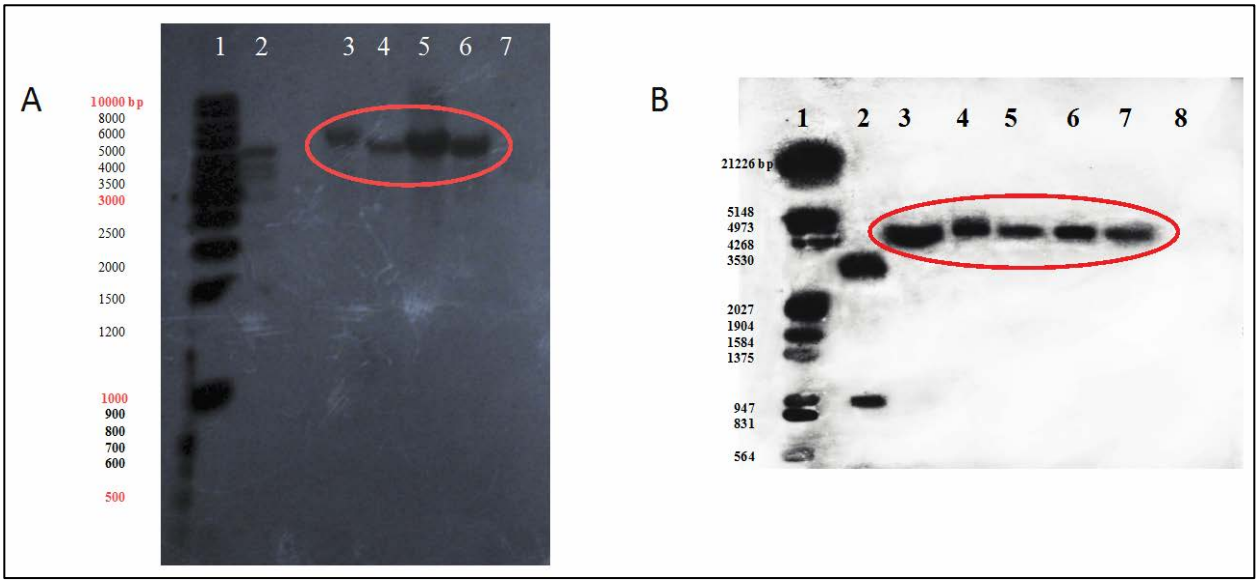
Supplemental Figure 3. Schematic overviews of plasmid pAT6F construction



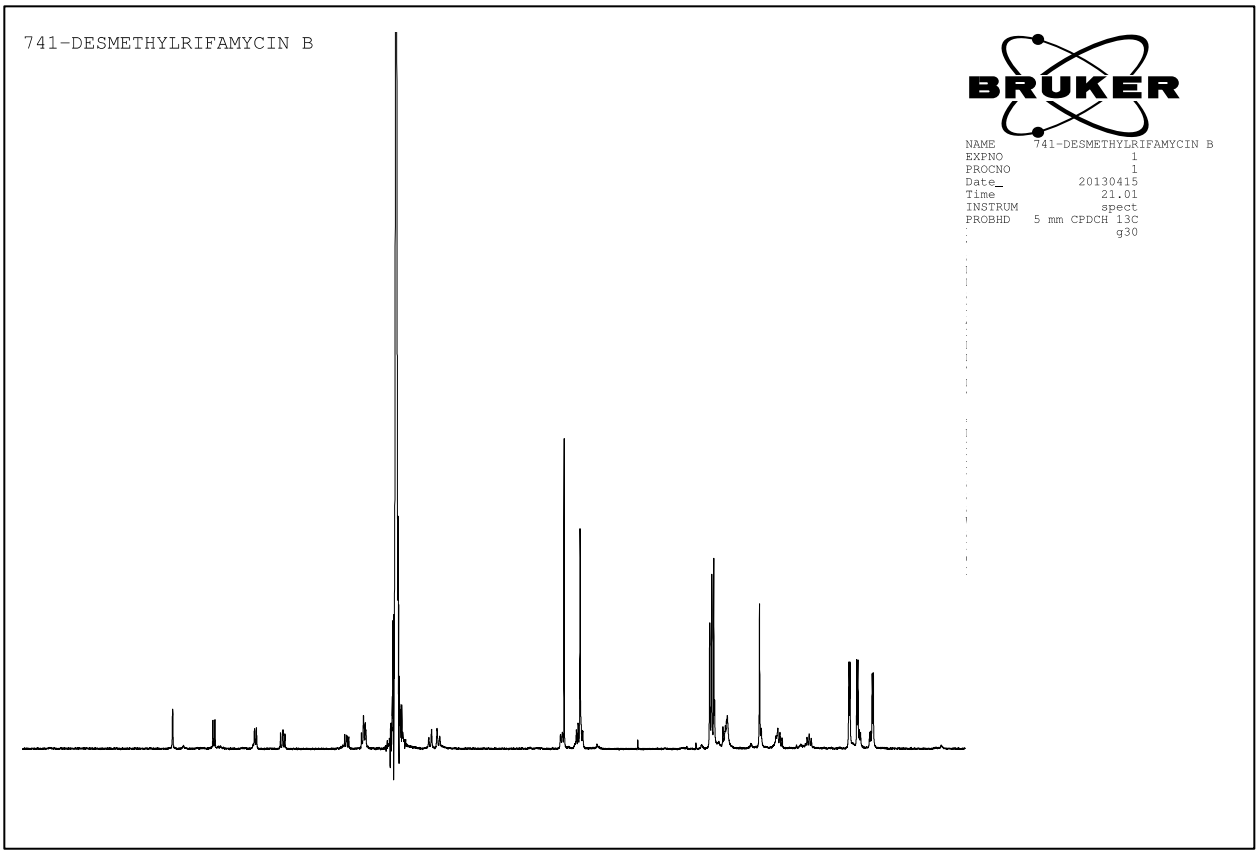
Supplemental Figure 4. Domain replacement strategy for the formation of 24-desmethylrifamycin B.



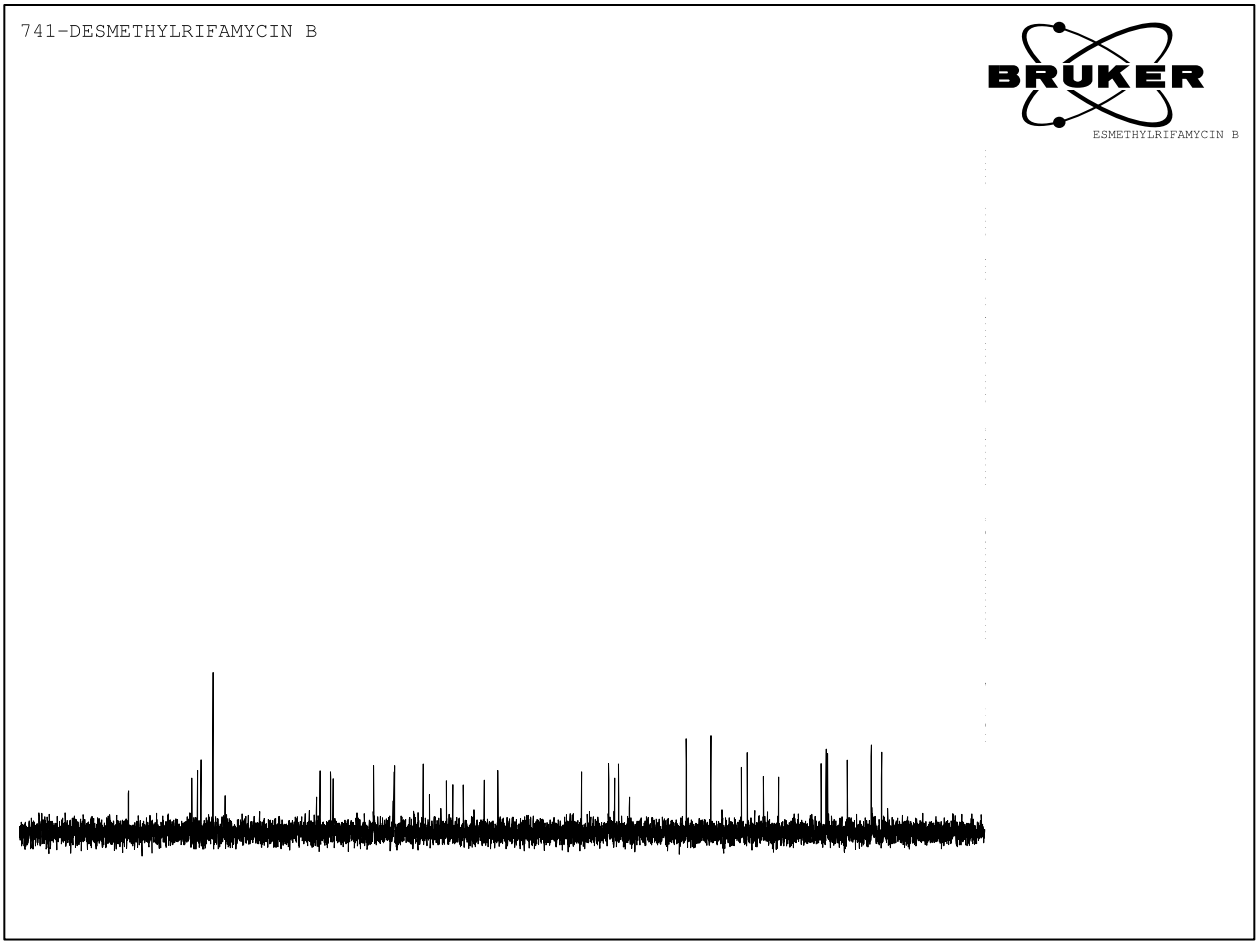
Supplemental Figure 5. Phenotypic appearances of single- and double-crossover mutants of *A. mediterranei* S699 on solid media. **A-D**, single cross-over clones. No pigmentation is seen around the culture of the single cross over clones, which indicates the absence of rifamycin production. **E-G**, double cross-over clones. Pigmentation is seen around the culture of double cross over clones, which indicates rifamycin production is resumed. **H**, wild-type of *A. mediterranei* S699.



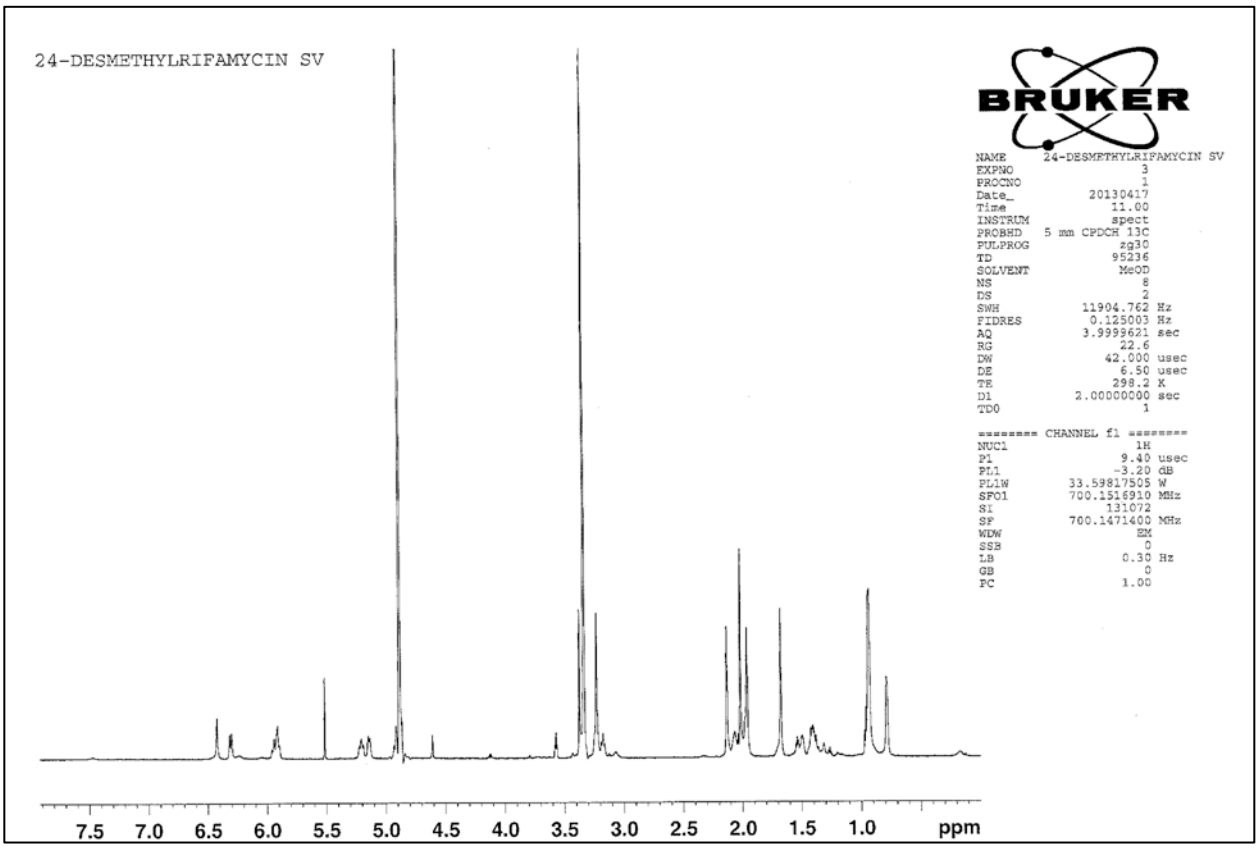
Supplemental Figure 6. Southern blot hybridization of single and double crossover clones. **A**. Blot of four single crossover clones' DNA digested with BamHI and probed with [α^{32}P]-labeled pIJ4026. Lane 1, DNA ladder; Lane 2, BamHI-digested pIJ4026 (positive control); Lane 3-6, BamHI-digested DNA of four S.C.O. clones; Lane 7, BamHI-digested chromosomal DNA of *A. mediterranei* S699. Signals were obtained in all four S.C.O. clones but no signal was seen in the BamHI-digested chromosomal DNA of *A. mediterranei* S699 (negative control); **B**. Double crossover clones digested with BamHI and probed with DIG-labeled *rap*AT2. Lane 1, Marker lambda DNA digested with EcoRI/HindIII, Lane 2, pMO2 digested with EcoRI and HindIII (positive control), Lane 3, S.C.O., Lane 4-7, D.C.O., Lane 8, chromosomal DNA of *A. mediterranei* S699. Positive signals are obtained in S.C.O. and all the D.C.O. clones (encircled in red). No signal is seen for *A. mediterranei* S699 (negative control).



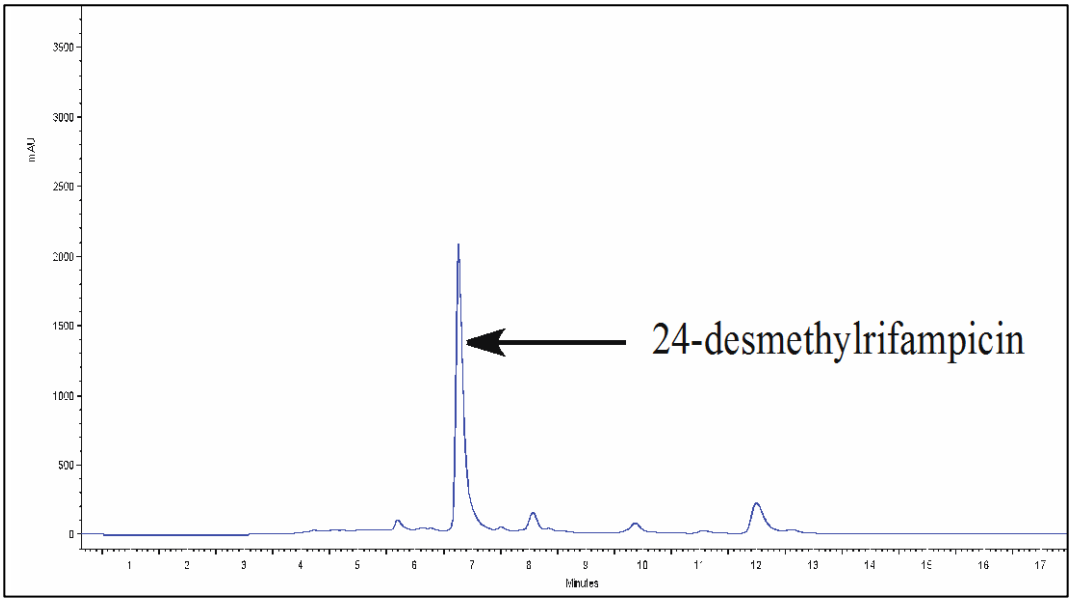
Supplemental Figure 7. ^{1}H NMR spectrum of 24-desmethylrifamycin B



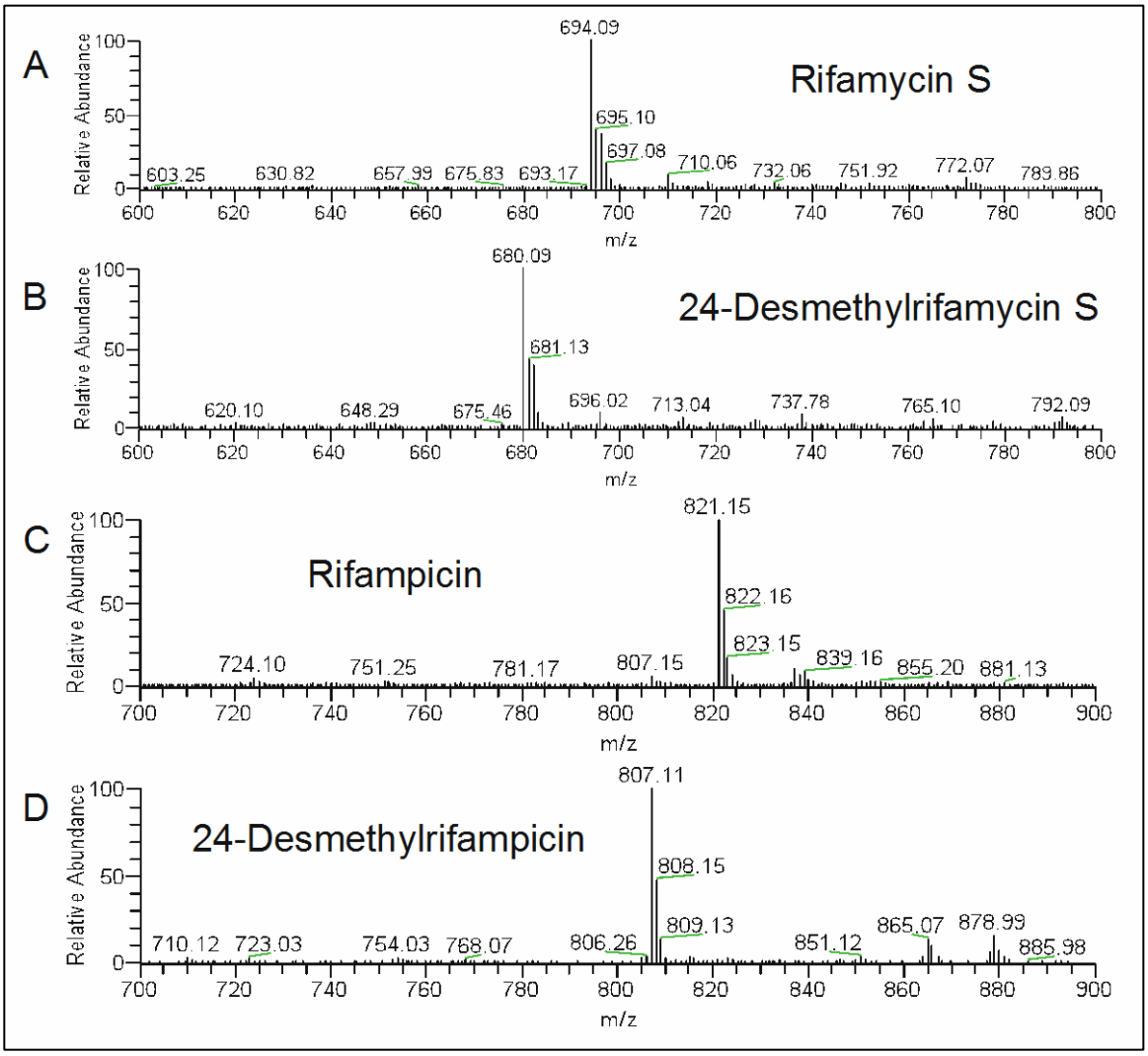
Supplemental Figure 8. ^{13}C NMR spectrum of 24-desmethylrifamycin B



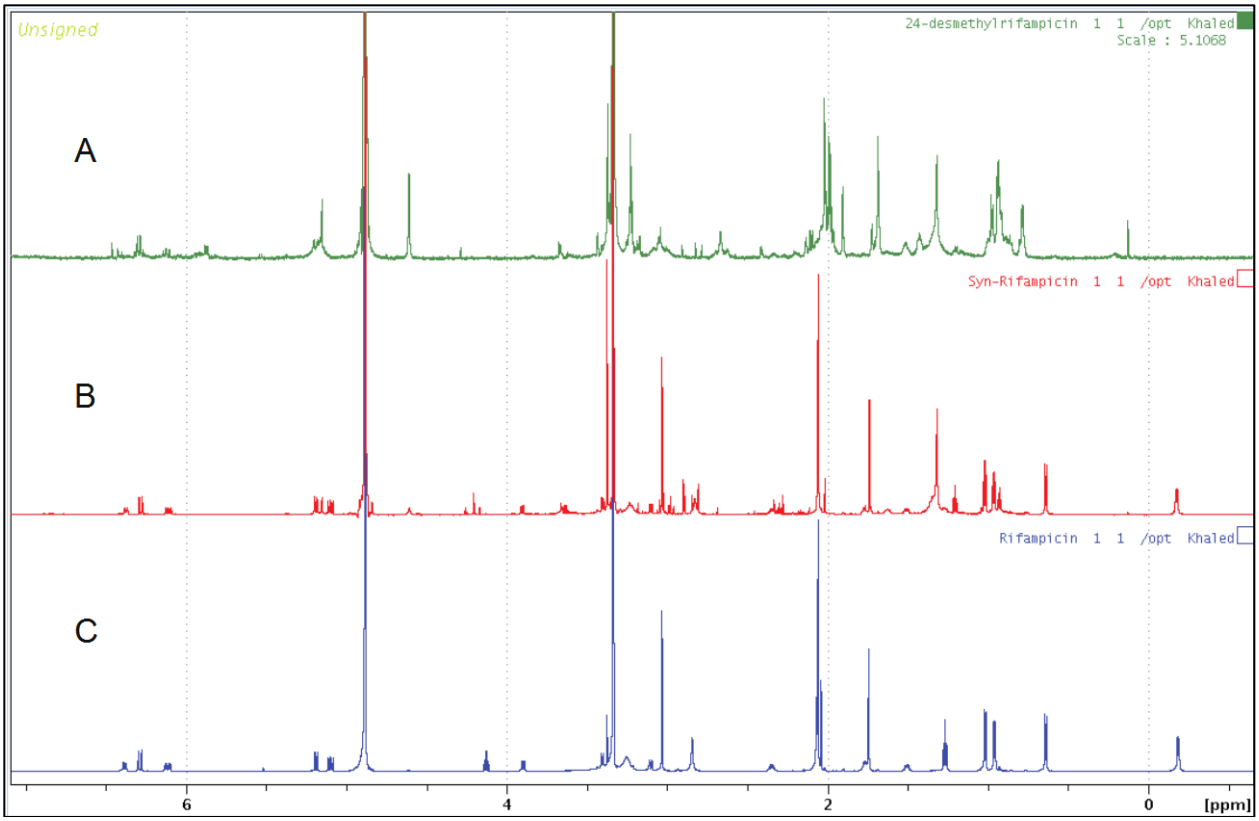
Supplemental Figure 9. ^{1}H NMR spectrum of 24-desmethylrifamycin SV



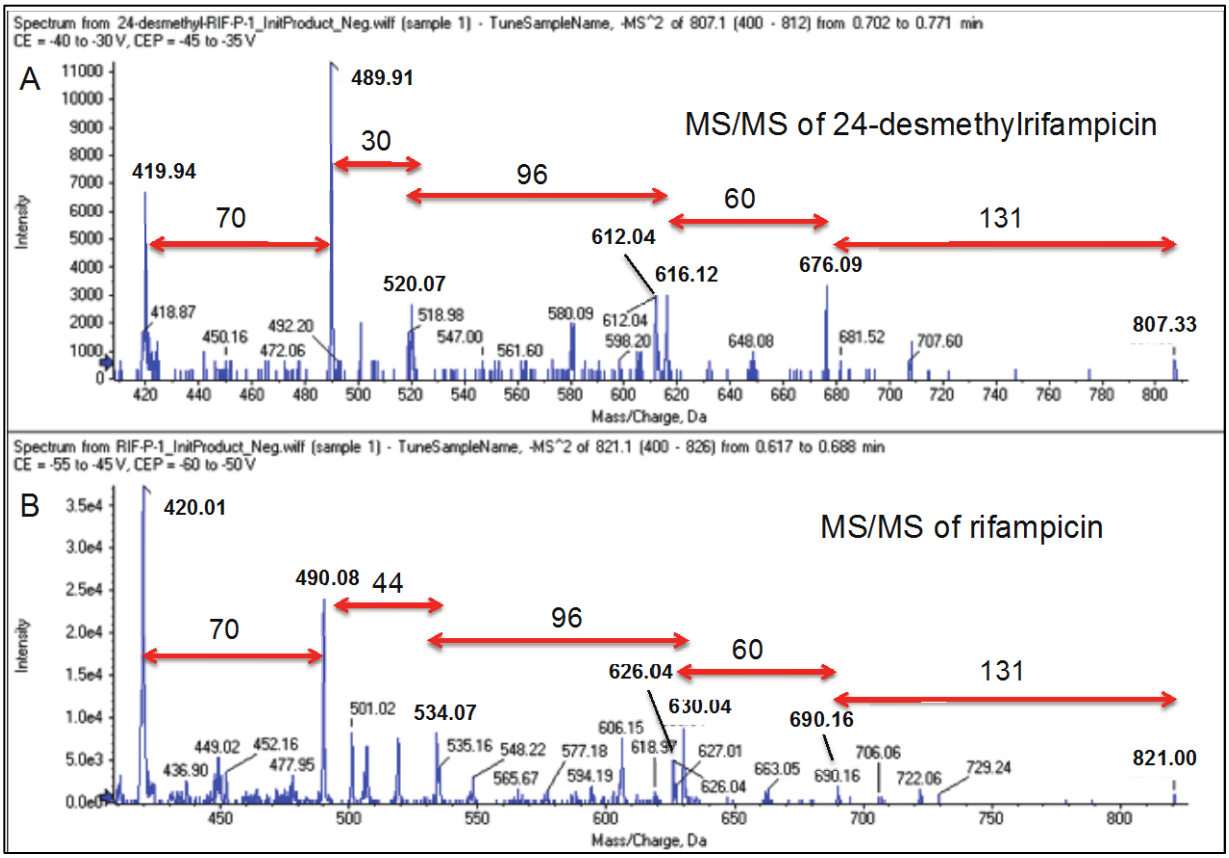
Supplemental Figure 10. Purification of 24-desmenthylrifampicin using HPLC. Peaks were detected at 254 nm.



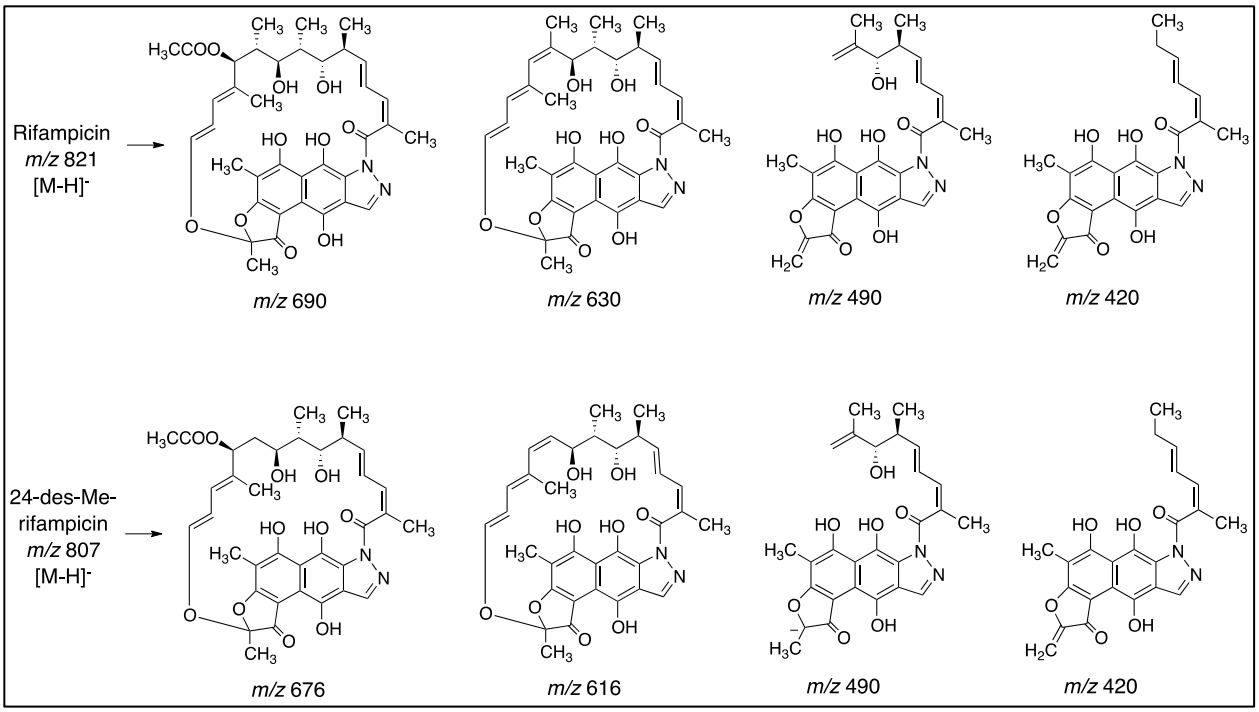
Supplemental Figure 11. (-)-ESI-MS spectra of rifamycin S (**A**), 24-desmethylrifamycin S (**B**), rifampicin (**C**), and 24-desmethylrifampicin (**D**).



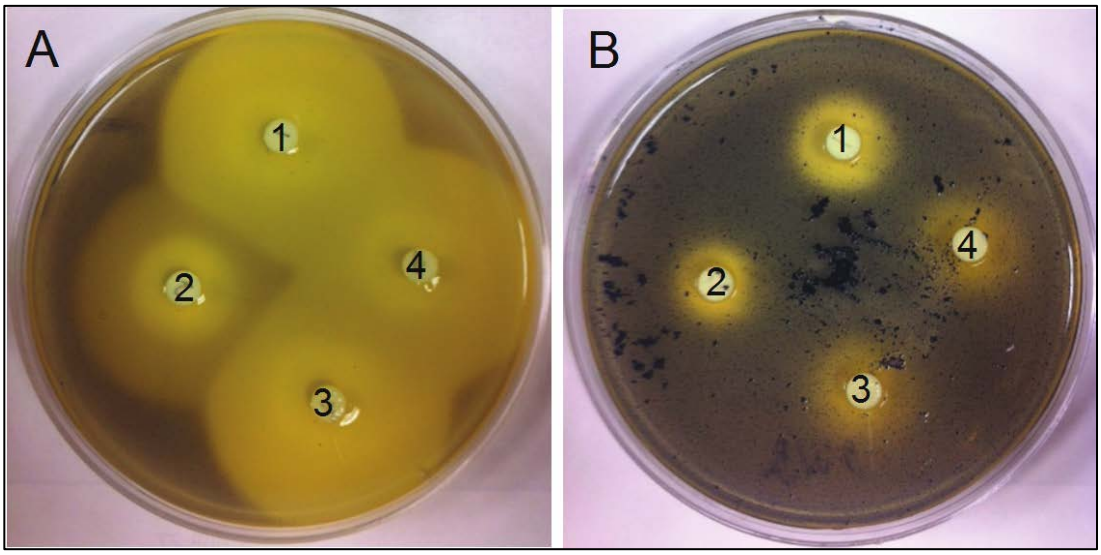
Supplemental Figure 12. ^{1}H NMR spectra of 24-desmethylrifampicin (**A**), chemically synthesized rifampicin (**B**), and commercially available rifampicin (**C**).



Supplemental Figure 13. Comparative MS/MS Analyses of rifampicin and 24-desmethylrifampicin. **A,** 24-desmethylrifampicin; **B,** rifampicin.



Supplemental Figure 14. Characteristic MS fragments of rifampicin and 24-desmethylrifampicin in ESI negative ion mode.



Supplemental Figure 15. Agar diffusion assay of rifamycin S, rifampicin, 24-desmethylrifamycin S, and 24-desmethylrifampicin against *Staphylococcus aureus* (**A**) and *Mycobacterium smegmatis* (**B**). **1,** rifamycin S, **2**, rifampicin, **3**, 24-desmethylrifamycin S, and **4**, 24-desmethylrifampicin.

Supplemental Table 1. Bacterial strains and plasmids used in this study.

Organism/ plasmid	Characteristics	Source or reference
***Amycolatopsis mediterranei* S699**	Rifamycin B producer	Dr. G. Lancini
***Escherichia coli* XL-1 Blue MR**	*mcr*A183 *mcr*CB-hsdSMR-*mrr*173 *end*A1 *sup*E44 *thi*-*rec*A1 *gyr*A96 *rel*A1 lac	Invitrogen
Staphylococcus aureus		ATCC
Escherichia coli		ATCC
M. smegmatis		ATCC
***M. tuberculosis* OSDD 209**	Rifampicin-sensitive *M. tuberculosis*	IGIB, India
***M. tuberculosis* OSDD 321**	Rifampicin-resistant *M. tuberculosis*, which has S531L mutation in the RpoB protein	IGIB, India
***M. tuberculosis* OSDD 55**	Rifampicin-resistant *M. tuberculosis*, which has H526Tmutation in the RpoB protein	IGIB, India
***M. tuberculosis* OSDD 206**	Rifampicin-resistant *M. tuberculosis*, which has S531L mutation in the RpoB protein	IGIB, India
***M. tuberculosis* H37Rv**	Rifampicin-sensitive *M. tuberculosis*	IGIB, India
pUC 18 and pUC 19	~2.7 kb, $Amp^R$for selection in *E. coli*, pBR322 origin of replication58 bp multiple cloning site internal to a-fragment of β-galactosidase gene(*lac*Z)	Lab stock Fermentas
pIJ4026	~4.3 kb, $Amp^R$for selection in *E. coli* and *erm*E for selection in *A. mediterranei* S699	M.J. Bibb, John Innes Institute
pMO2	~ 3.5 kb, $Amp^R$for selection in *E. coli*, 0.85 kb *rap*AT2 inserted at its *Sma*I site	Peter Leadley, University of Cambridge
pAT6A	~ 1.6 kb PCRI fragment cloned in pUC18at its *Sma*I site	This study
pAT6B	~1.5 kb PCRII fragment cloned in pUC18at its *Sma*I site	This study
pAT6C	~ 1.6 kb PCRI fragment cloned in pUC19at its *Sma*I site	This study
pAT6D	0.85 kb *rap*AT2 and ~1.5 kb PCRII fragment in pUC18	This study
pAT6E	~ 1.6 kb PCRI fragment, 0.85 kb *rap*AT2 and ~1.5 kb PCRII fragment in pUC18	This study
pAT6F	~ 1.6 kb PCRI fragment, 0.85 kb *rap*AT2 and ~1.5 kb PCRII fragment in pIJ4026	This study
pWE15	$Amp^R neo^R$ 8.2 kb cosmid vector containing cos sites, ori, SV40 ori and an mvs with T3 and T7 promoters flanking *Bam*HI as cloning site and *Eco*RI and *Not*I restriction sites at the either ends of mcs	Stratagene
pRIF13	$Amp^R neo^R$, *rif*PKS insert from *A. mediterranei* S699 between *Eco*RI in pWE15 cosmid vector	This study
pRIF21	$Amp^R neo^R$, *rif*PKS insert from *A. mediterranei* S699 between *Eco*RI in pWE15 cosmid vector	This study
pRIF24	$Amp^R neo^R$, *rif*PKS insert from *A. mediterranei* S699 between *Eco*RI in pWE15 cosmid vector	This study

Supplemental Table 2. Primers used to amplify the flanking regions of *rif*AT6.

Primer I	XbaI (41862-41892bp of *rif*PKS cluster) 5´-C*TCTAGA*AGGCGCTCGCCCGGCACCTGCGCGACGAACT-3´
Primer II	BalI (43513-43533 bp of *rif*PKS cluster) 5´-C*TGGCCA*GGGAAGACCCAGACGAGCTTG-3´
Primer III	AvrII (45963- 44513bp of *rif*PKS cluster) 5´-C*CCTAGG*GCGGACCGGCCGGGTCGACCTGCCGA-3´
Primer IV	XbaI (45963-45989bp of *rif*PKS cluster) 5´-C*TCTAGA*GGTGCCCAGCGATCCGGCGCCCGAGA-3´
AT6-HindIII-F	HindIII 5´-CCC*AAGCTT*CGGTGCCGTCGAACTGCT-3´
AT6-EcoRI-R	EcoRI 5´-CCG*GAATTC*AGGTGAACACCAGGCCGT-3´