

Table S1: RT-qPCR primers.

Gene	Primers ^a	Sequence (5' → 3')	Product location ^b	Product length (bp)	Annealing temperature (°C)	PCR efficiency ^c	
						Liquid media	Solid media
<i>rpfA</i>	rpfAF	GAGTCCGGCGGCAACTGGTC	+160 to	133	61	1.014 ± 0.022	1.020 ± 0.023
	rpfAR	GCTGGGACTTGCTCGCCTGG	+292				
<i>rpfB</i>	SCO3150F	AGTACGGCGGTCTGGACTA	+1220 to	111	60	N/A	N/A
	SCO3150R	CTTATCTGCTGGGAGCGACT	+1330				
<i>rpfC</i>	SCO3098F	GGCTCTACCAGTTCGACTCC	+236 to	61	57	1.045 ± 0.027	1.057 ± 0.029
	SCO3098R	TGCGCACGTAGAGCTTCTG	+296				
<i>rpfD</i>	SCO0974F	GTTTCGTACGGCTACCAGGTG	+1079 to	90	60	1.041 ± 0.034	1.054 ± 0.038
	SCO0974R	GTCCGCTCTTCACGGAGAT	+1168				
<i>rpfE</i>	SCO7458F	ACCGGCAACGGCTACTAC	+199 to	147	66	N/A	N/A
	SCO7458R	CATCCCCTGCAGACGGG	+345				

^a Forward and reverse primers are denoted with letters F and R, respectively.

^b Nucleotide sites are numbered relative to the start codon.

^c Values are presented as mean ± standard deviation.

Table S2. Oligonucleotides used in this study

Primer name	Sequence (5' – 3')	Function
SCO0974 F	GGCACCGCCGGTATCAGCCGAACA	Disruption of <i>rpfD</i>
SCO0974 R2	GCTGCTGCTCGTCTGCCGTCTTCC	Disruption of <i>rpfD</i>
SCO0974 up	AGTGCTCATTGGGTGGCTC	<i>rpfD</i> complementation and disruption confirmation
SCO0974 down	CAGGTGATACCGGCGAGTG	<i>rpfD</i> complementation and disruption confirmation
SCO3097FWD	CAGCTCACCTCGCAGGCGTCCGGTGAGGGGATCAACCATGATTCCGGGGATCCGTCGACC	<i>rpfA</i> knockout
SCO3097REV	CACGCACCGGGGCGGGAGTCACGGGACCGCCCGCTTATGTAGGCTGGAGCTGCTTC	<i>rpfA</i> and <i>rpfA/C</i> knockout
SCO3098FWD	CCGCGTGGTCCCGCTCGTCTTGTGAAAGGTCTGTCGCATGATTCCGGGGATCCGTCGACC	<i>rpfC</i> and <i>rpfA/C</i> knockout
SCO3098REV	GGGACATACGGTCTCTAACCGTGACCGGAACCTCCTCTATGTAGGCTGGAGCTGCTTC	<i>rpfC</i> knockout
SCO3098 up	CGTGATCTGCGCGGCATGAAC	<i>rpfC</i> knockout confirmation
SCO3098 int	GCAGGCCGCCGTAGTACC	<i>rpfC</i> knockout confirmation
SCO3150FWD	CTGGGGGCCCGATCGGGACCCCTGGAGCGTGTGGGCGTGATTCCGGGGATCCGTCGACC	<i>rpfB</i> knockout
SCO3150REV	GCTCACCCCGCAAGAGTAGCCGGGGCCGGGCGGCTCATGTAGGCTGGAGCTGCTTC	<i>rpfB</i> knockout
3150 up	TTCAAGAACGCCAGAACCTG	<i>rpfB</i> knockout confirmation
3150 down	ACCGTGTGGCGTCGATGAC	<i>rpfB</i> knockout confirmation
3150 internal	AGCAGTTCACCTCACGTCGTC	<i>rpfB</i> knockout confirmation
SCO7458FWD	GGGCGACCGGTGGTCACTGGTGTAAACAGTCTTCTTATGATTCCGGGGATCCGTCGACC	<i>rpfE</i> knockout
SCO7458REV	GTCAACCCGGCGCGCCGGGGCCGGTTTGGCACCGTCATGTAGGCTGGAGCTGCTTC	<i>rpfE</i> knockout
7458 up	ACAAGGAGGCGGTCCACG	<i>rpfE</i> complementation and knockout confirmation
7458 down	GTTGGTCGGCCTACTCGG	<i>rpfE</i> complementation and knockout confirmation
7458 internal	CGACATCCCCTGCAGACG	<i>rpfE</i> knockout confirmation
SCO0974PP 5'	CAGTACCATATGGCCGACGCGGACCTGGGAC	Overexpression of RpfD
SCO0974PP 3'	CAGTACGGATCCCTCAGATCCTGACGCCCGCCGGC	Overexpression of RpfD
SCO3097PP 5'	CAGTACCATATGGCCACCGCGTCCGAGTGGGAC	Overexpression of RpfA
SCO3097PP 3'	CGAAGTGGATCCCTTACTTTCAGGTGCAGCTGCTG	Overexpression of RpfA
SCO3098PP 5'	CAGTACCATATGGCCGACTCCACGAACCTGGGAC	Overexpression of RpfC
SCO3098PP 3'	CAGTACGGATCCCTACTTTTCGCCGATTCGCC	Overexpression of RpfC
SCO3150ΔDUFPP5'	CAGTACCATATGGCGACCGGCTTCCCGC	Overexpression of RpfBΔDUF348
SCO3150PP 5'	CAGTACCATATGGCCAAGGACAAGCGGTCGAG	Overexpression of RpfB
SCO3150PP 3'	CAGTACGGATCCCTCATTCGCCGACCGGGCGCC	Overexpression of RpfB
SCO7458PP 5'	CGAAGTCCATATGGCGCCCTCGGCGCC	Overexpression of RpfE
SCO7458PP 3'	CGAAGTGGATCCCTCAGGCGCAGCCCC	Overexpression of RpfE
PrpfA-F	GCACTGGATCCAAAAGTAGAGGGAGTTCGCG	Cloning of <i>rpfA</i> promoter
PrpfA-R	GCACTGGTACCGAGCGGTTCATGACCGTAGAC	Cloning of <i>rpfA</i> promoter
SCO3152 pFlux Fwd	TGATGAGGATCCCTCTGAGGGGATCCATAGGGC	Cloning of <i>SCO3152</i> promoter
SCO3152 pFlux Rev	TGATGAGGTACCCACGGCGCTCTCACTCAT	Cloning of <i>SCO3152</i> promoter
SCO3098 pFlux Fwd	TGATGAGGATCCGCATCCACTACTGCCTCGG	Cloning of <i>rpfC</i> promoter
SCO3098 pFlux Rev	AAGAAGGGTACCGACGACCGTTCCCGGAGAG	Cloning of <i>rpfC</i> promoter
<i>rpfD</i> pFlux Fwd	TGATGAGGATCCCTTAGTGACCTTGGTGTCCG	Cloning of <i>rpfD</i> promoter
<i>rpfD</i> pFlux Rev	AAGAAGGGTACCCGGTTGATCCTGTTCGGCTG	Cloning of <i>rpfD</i> promoter
<i>rpfE</i> pFlux Fwd	TGATGAGGATCCCGTGTCCATCGTCTCCAC	Cloning of <i>rpfE</i> promoter
<i>rpfE</i> pFlux Rev	TGATGAGGTACCAAGAAGACTGTTACAGGTGAC	Cloning of <i>rpfE</i> promoter
ermEF-B	GCACTGGATCCAGCCGACCCGAGCACGCGC	Cloning of <i>ermE*</i> promoter
ermER-K	GCACTGGTACCGATCCTACCAACCCGGCACGA	Cloning of <i>ermE*</i> promoter

Table S3. Conditions for Rpf protein over expression and purification.

Protein	Size (kDa)*	[IPTG] (mM)	Induction time (h)	Induction temperature (°C)	[Imidazole] (mM) for elution
RpfA	21.7	1	2.5	30	250
RpfB	36.6	0.25	14	16	250
RpfB Δ DUF348	21.5	0.25	14	16	250
RpfC	40.0	1	14	16	150
RpfD	42.6	1	14	16	70
RpfE	10.2	1	2.5	30	250
sIHF	13.3	1	14	16	250

*Calculated excluding the signal peptide but including the 6 \times His tag from pET15b.

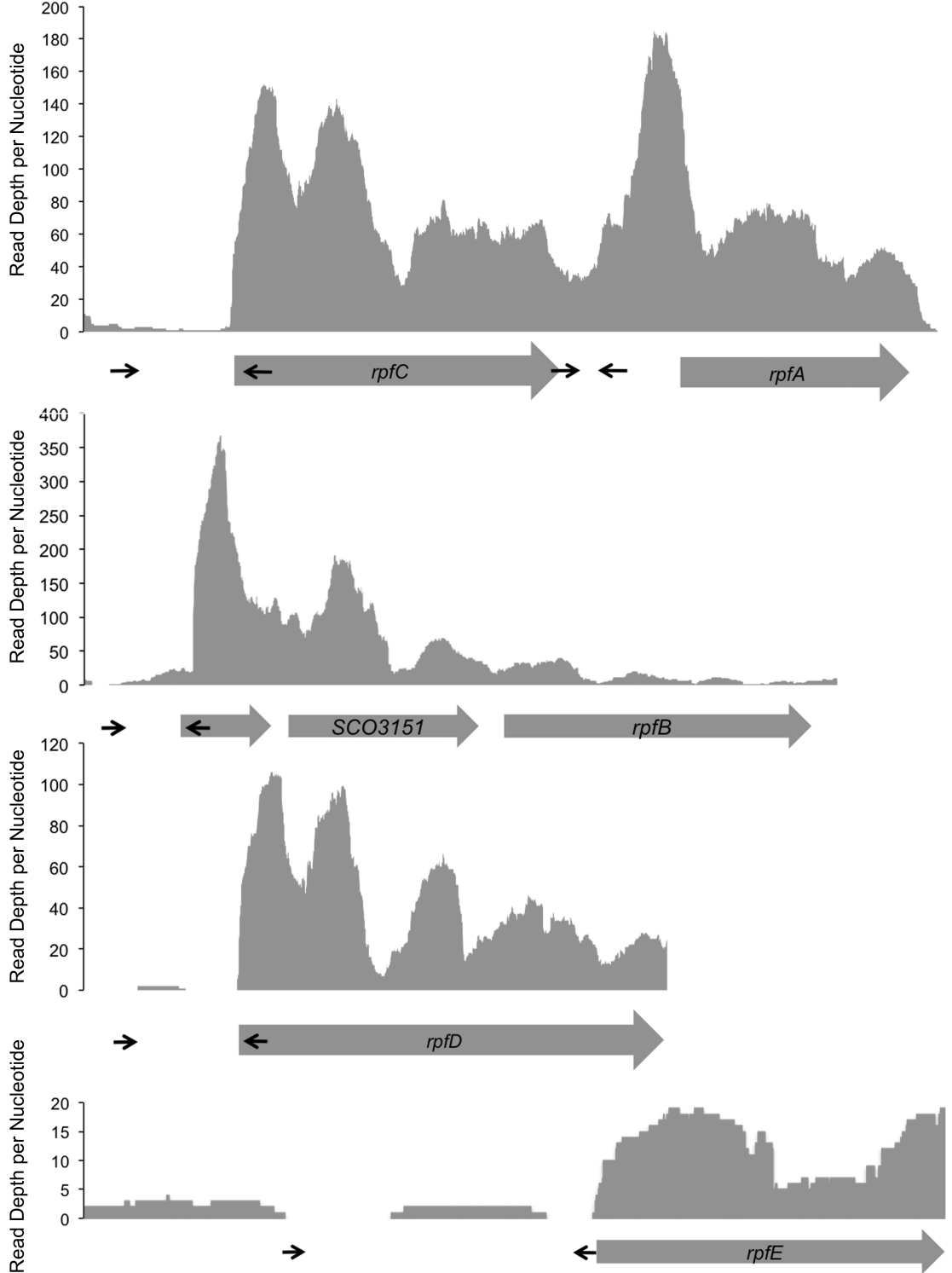


Figure S1. Primer location for luciferase assays. Primers for luciferase assays were positioned to amplify the promoter region for each *rpf* gene, based on previously published RNA sequencing data (Moody *et al.*, 2013). Graphs show the number of reads at each nucleotide for both the protein-coding sequence and region upstream of each *rpf*. Protein-coding sequences are indicated with grey arrows. Primer locations are indicated with black arrows.

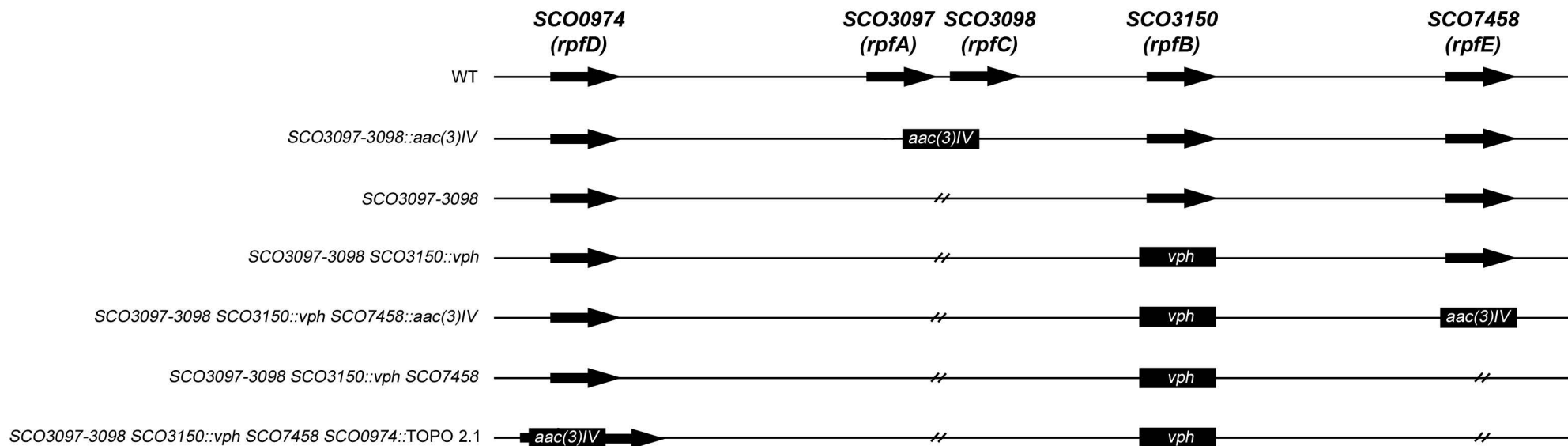


Figure S2 . Construction of multiple *rpf* mutants. *rpf* genes were replaced with antibiotic resistance cassettes in the order shown. Double diagonal lines represent ‘scar’ sequences left after removal of the antibiotic resistance cassettes. Abbreviations: *aac(3)IV*, apramycin resistance cassette; *vph*, viomycin resistance cassette.

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SCO2326      1 GDSAEFQCFSKIVDHESDWNIHATNASSGAYGLV-QAL-----PGSKMASAGDDWKTN
SCO5029      1 -----NHESSWNYQAVNASSGAYGLF-QAL-----PAGKYASAGADWRTN
RpfB/SCO3150 1 ---ADHLNWQGLAACESGGRA-DAVDPSGTYGGLYQFDSATWHGLGGEGR---PEDASA-
RpfC/SCO3098 1 ---ADSTNWDQVAECETGGAW-SQNSGNGYYGGL-QLSQDAWEQYGGLDYAPSADQASR-
RpfE/SCO7458 1 --APLRIDWDAIAACESGNW-QANTGNGYYGGL-QFARSSWIAAGGLKYAPRADLATR-
RpfA/SCO3097 1 ---ATASEWDAVAQCESGGNW-SINTGNGYYGGL-QFSASTWAAAYGGTQYASTADQASK-
RpfD/SCO0974 1 ---ADAATWDKVAACESDQDDW-DINTGNGYYGGL-QFTQSTWEAFGGTRYAPRADLATR-
RpfB-Mtb     1 ---IDGSIWDAIAGCEAGGNW-AINTGNGYYGGV-QFDQGTWEANGGLRYAPRADLATR-

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SCO2326      53 AATQIEWGLDYMKDRYGSTCDAWTFWQSNQWY-
SCO5029      40 PATQIKWGLSYMDNRYGSPCDAWAFWQANHWY-
RpfB/SCO3150 53 -AEQTYRAQ---KLYVRSQADAWPHCGARLRE-
RpfC/SCO3098 55 -SQQIRIAE---KIHASQGIAAWPTCGLLAGLG
RpfE/SCO7458 56 -GEQIAVAE---RLARLQGMSAWGCA-----
RpfA/SCO3097 55 -SQQIQIAE---KVLAQGKGAWPVCGTGLSGA
RpfD/SCO0974 55 -EQQIAGAE---KVLDTQGPGAWPVCSERAGL-
RpfB-Mtb     55 -EEQIAVAE---VTRLRQGWGAWPVCAARAGAR

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* Active site residues

* Key cleft residues

Figure S3. Alignment of distantly related Rpf-like domains from SCO2326 and SCO5029 with RpfB from *Mycobacterium tuberculosis* and the more closely related Rpfs from *S. coelicolor*. Identical amino acid residues are highlighted in black, while similar residues indicated in grey. The red asterisk marks the key catalytic residue, while the blue asterisks mark important substrate-binding residues.

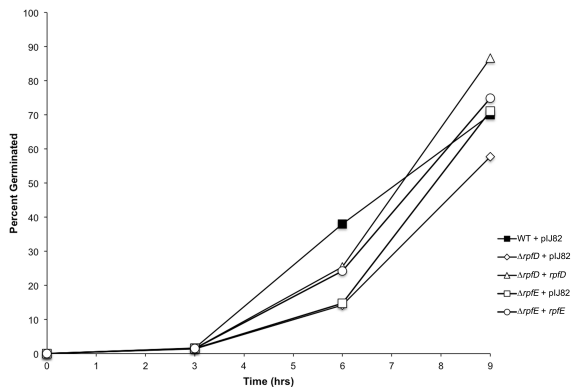


Figure S4. Partial complementation of $\Delta rpfD$ and $\Delta rpfE$ confirmed by examining germination profiles. Spores of complemented and vector alone-containing *rpf* mutants were plated on MS agar overlaid with cellophane discs. Spores were monitored for the presence or absence of germ tubes at the indicated time points. Data are representative of three independent trials (N > 100 per timepoint per strain). WT, wild type.

S.venezuelae	41	ADAA	TWDRVAECESGGQWSANF	GNGMYGGLQFTQDS	WERHGGLAYAPSP	DLASRAQQIAV
S.clavuligerus	41	ADSA	VWDRVAECESGGAWSADI	GNGYYGGLQMSQQT	WEAYGGLEYASGP	DLASRSQQITV
S.sp.S4	41	ADTA	TWDRVAECESGGAWS	TNAGNGYYGGLQVTQEL	WERHGGLSYAP	SADLASRSQQIVV
S.griseus	41	AEAT	TWDRVAECESGGMWSADL	GNGYYGGLQFSQET	WSAYGGTAFAPR	ADLASRSQQISV
S.coelicolor	41	ADST	NWDOVAECETGGAWSQNS	GNGYYGGLQLSQDA	WEQYGGLDYAP	SADQASRSQQIRI
S.vermitilis	41	ASGT	TWDOVAECESGGFSWADI	GNGYYGGLQLSQGN	WEKYGGLDYAP	SADQASRSQQIAV
S.scabies	41	ASGT	TWDOVAECESGGFWSADI	GNGRYGGLQLTQAN	WEKYGGLEYAKT	ADLASRSQQIAV
			*		**	
S.venezuelae	101	AEKALA	-KGSNDWATCAP	IAGLT		
S.clavuligerus	101	AEKVLAA	ECAKAWASCAG	MAGLA		
S.sp.S4	101	AERILDA	EAGTAAWATCAP	IAGLK		
S.griseus	101	AEKVLDD	QGPKAWPSCA	VISGLA		
S.coelicolor	101	AEKTHAS	QGIAAWPTCGL	IAGLG		
S.vermitilis	101	AEKVLAA	KGSSPWSTCG	IAGVLS		
S.scabies	101	AEKVLAD	QGVGVWSTCGL	IHNLG		

Figure S5. Alignment of the Rpf domain of RpfC orthologues from diverse *Streptomyces* species. The key catalytic residue is indicated with a red asterisk, while important substrate-binding residues are marked with blue asterisks.