Supporting Information

Antibacterial Activity of and Resistance to Small Molecule Inhibitors of the ClpP Peptidase

Short Title: ClpP Inhibition as an Anti-Tuberculosis Strategy

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Synthesis

General. All reagents were purchased from Sigma-Aldrich or VWR. All synthesis reactions were monitored by thin layer chromatography using precoated silica gel 60 plates (particle size 0.040–0.063 mm). Column chromatography was performed using 60 Å (230–400 mesh ASTM) silica gel. NMR analyses were performed on a Bruker Advance Ultrashield Spectrometer (400 or 300 MHz). All spectra were referenced to residual solvent signals in $CDCl_3$ (7.24 ppm for ^1H, 77.0 ppm for ^{13}C.) Chemical shifts are reported on the δ scale and coupling constants J are in Hz. Multiplicities are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), or m (multiplet).

General Procedure for the Preparation of Thiol Esters. A 100-mL round-bottomed flask equipped with a rubber septum was evacuated and put under a positive pressure of nitrogen, then, charged with 50 mL of methylene chloride, thiophenol (1 eq, 50 mmol) and pyridine (1 eq, 50 mmol) and cooled in an ice bath while an acyl chloride (1 eq, 50 mmol) was added by syringe over 5 min. The resulting suspension of white solid was stirred for an additional 5 min at 0 °C and at room temperature for 30 min and then poured into 100 mL of distilled H_20. The aqueous phase was separated and extracted with 25 mL of methylene chloride, and the combined organic phases were dried over Na_2SO_4, filtered, and concentrated. Flash chromatography on silica with a mobile phase of 15:1 Hex:EtOAc afforded the desired thiol ester.

S-Phenyl heptanethioate
^1H NMR (400 Mhz, $CDCl_3$) 7.44 (s, 5H), 2.69 (t, 2H) 1.74 (quint, 2H), 1.3 (m, 6H), 0.93 (d, 3H) ^{13}C NMR (300 Mhz, $CDCl_3$) 197.60, 134.48, 129.28, 129.14, 127.95, 43.74, 31.44, 28.64, 25.57, 22.46, 14.05 LRMS (FAB) 223 [M+H]+, 245 $[M+Na]^+$ HRMS (FAB) calculated [M+H]+ 223.1157, found $[M+H]^+$ 223.1150; 92% yield

S-Phenyl propanthioate
^1H NMR (300 Mhz, $CDCl_3$) 7.39 (s, 5H), 2.67-2.74 (q, 2H), 1.22-1.27 (t, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 198.32, 134.56, 129.34, 129.19, 127.85, 37.12, 9.65 LRMS (FAB) 167 [M+H]+, 189 $[M+Na]^+$ HRMS (FAB) calculated [M+Na]+ 189.0350, found $[M+Na]^+$ 189.0352; 94% yield

S-Phenyl nonanethioate
^1H NMR (300 Mhz, $CDCl_3$) 7.42-7.52 (s, 5H), 2.75-2.84 (t, 2H), 1.71-1.78 (m, 2H), 1.26-1.48 (m, 10H), 0.76 (t, 3H) ^{13}C NMR (400 Mhz, $CDCl_3$) 197.61, 134.49, 129.29, 129.16, 127.96, 43.75, 31.82, 29.24, 29.11, 28.98, 25.62, 22.67, 14.13 LRMS (FAB) 251 [M+H]+, HRMS (FAB) calculated [M+H]+ 251.1470, found $[M+Na]^+$ 251.1475; 97% yield

S-phenyl undec-10-enethioate
^1H NMR (400 Mhz, $CDCl_3$) 7.44 (s, 5H), 5.83 (m, 1H) 4.95 (dd, 2H), 2.67 (t, 2H), 2.06 (q, 2H), 1.73 (quint, 2H), 1.32 (m, 10 H) ^{13}C NMR (400 Mhz, $CDCl_3$) 197.57, 139.16,

134.49, 129.29, 129.16, 127.96, 114.20, 43.73, 33.81, 29.27, 29.21, 29.06, 28.95, 28.90, 25.60, LRMS (FAB) 278 [M+H]+, 300 $[M+Na]^+$, HRMS (FAB) calculated [M+H]+ 277.1626, found $[M+H]^+$ 277.1615; 86% yield from carboxylic acid

S-phenyl 3-phenylpropanethioate
^1H NMR (400 Mhz, $CDCl_3$) 7.23-7.44 (m, 10H), 3.00-3.05 (m, 4H) ^{13}C NMR (400 Mhz, $CDCl_3$) 196.75, 139.94, 134.52, 129.46, 129.24, 128.60, 128.42, 127.63, 126.45, 45.17, 31.41 LRMS (FAB) 278 [M+H]+, 300 $[M+Na]^+$, HRMS (FAB) calculated [M+H]+ 277.1626, found $[M+H]^+$ 277.1615; 98% yield

General Procedure for the Preparation of β-Lactones from Thiol Esters and Aldehydes. A 100-mL round-bottomed flask equipped with a rubber septum was evacuated and put under a positive pressure of nitrogen. The flask was charged with 50 mL of dry THF and diisopropylamine (1.1 eq, 11 mmol), and then cooled in an dry ice and acetone bath (-78 °C) while *n*-butyllithium solution (2.25 M in hexanes, 1.035 eq, 10.35 mmol) was added via syringe over 2 min. After 30 min, thioester (1eq, 10.0 mmol) was added drop wise via syringe over 5 min. After 30 min, a solution of the aldehyde (1 eq, 10.0 mmol) in 12.5 mL of THF was added dropwise over 20 min via a syringe cooled externally with dry ice. The reaction mixture was stirred at -78 °C for 60 min and then allowed to warm to 0 °C over 120 min. Saturated NH_4Cl solution (50 mL) was then added, and the resulting mixture was partitioned between 50 mL of water and 50 mL of diethyl ether. The organic phase was extracted with three 50-mL portions of 1N HCL, three 50-mL portions of sat. Na_2CO_3 solution and two 50-mL portions of saturated NaCl solution, dried over $NaSO_4$, filtered, and concentrated to afford a pale yellow oil. Flash column chromatography on silica gel (gradient elution with ethyl acetate: hexanes) gave single diastereomeric β-Lactones.

Figure 1, β-lactone 2 - Trans 4-butyl 3-pentyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 4.2 (m, 1H), 3.1-3.2 (m, 1H), 1.8 (m, 2H), 1.6 (m, 2H), 1.4 (m, 2H), 1.34-1.49 (m, 6H), 1.02 (t, 3H), 0.94 (t, 3H); ^{13}C NMR (400 Mhz, $CDCl_3$) 165.16, 82.91, 54.09, 32.31, 31.47, 28.17, 26.68, 22.34, 18.05, 16.99, 13.92 LRMS (FAB) 185 [M+H]+, 207 $[M+Na]^+$ HRMS (FAB) calculated 207.1361 $[M+Na]^+$, found 207.1366 $[M+Na]^+$; 28% yield

Figure 1, β-lactone 3 - Trans-4-butyl 3-heptyloxetan-2-one.
^1H NMR (400 Mhz, $CDCl_3$) 4.25 (m, 1H), 3.15 (m, 1H), 1.8-1.9 (m, 2H), 1.7-1.8 (m, 2H), 1.55-1.7 (m, 2H), 1.4-1.55 (m, 4H), 1.2-1.4 (m, 6H), 0.95-1.05 (t, 3H), 0.9-0.95 (t, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 171.70, 77.98, 56.16, 36.51,36.44, 31.71, 29.25, 27.88, 27.01, 22.61, 18.45, 16.93, 13.89; LRMS (FAB) 212 [M+H]+, 235 $[M+Na]^+$ HRMS (FAB) calculated 235.1674 $[M+Na]^+$, found 235.1680 $[M+Na]^+$; 21% yield

Figure 1, β-lactone 4 - Trans-4-butyl-3-(non-8-en-1-yl)oxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 5.7-5.8 (m, 1H), 4.9-5.0 (dd, 2H), 4.2 (m, 1H), 3.1 (m, 1H), 2.0 (m, 2H), 1.5-1.7 (m, 8H), 1.4-1.52 (m, 8H), 0.99-1.03 (t, 3H), ^{13}C NMR (400 Mhz,

$CDCl_3$) 171.69, 139.08, 114.24, 77.98, 56.15, 36.51, 33.76, 29.24, 29.16, 28.96, 28.83, 27.87, 26.98, 18.46, 13.81; LRMS (FAB) 239 [M+H]+, 627 $[M+Na]^+$ HRMS (FAB) calculated 239.2011 $[M+H]^+$, found 239.2018 $[M+H]^+$; 18% yield

Figure 1, β-lactone 5 - Trans-4-isopropyl-3-methyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 3.84-3.86 (dd, 1H), 3.22-3.31 (m, 1H), 1.45-1.48 (d, 3H), 1.10-1.13 (d, 3H), 0.93-1.01 (d, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.02, 84.25, 48.80, 32.40, 18.05, 16.78, 12.85 LRMS (FAB) 129 [M+H]+, 151 $[M+Na]^+$ HRMS (FAB) calculated 129.0916 $[M+H]^+$, found 129.0919 $[M+H]^+$23% yield

Figure 1, β-lactone 6 - Trans-4-isopropyl-3-pentyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 3.94-4.16 (dd, 1H), 3.2-3.6 (m, 1H), 1.9 (m, 1H), 1.8 (m, 1H), 1.7 (m, 1H), 1.34 (m, 6H), 0.9-1.3 (m, 9H), ^{13}C NMR (400 Mhz, $CDCl_3$) 171.69, 82.85, 54.10, 32.34, 31.49, 28.18, 26.71, 22.37, 18.10, 17.01, 13.97 LRMS (FAB) 185 [M+H]+, 207 $[M+Na]^+$ HRMS (FAB) calculated 207.1361 $[M+Na]^+$, found 207.1366 $[M+Na]^+$26% yield

Figure 1, β-lactone 7 - Trans-3-benzyl-4-propyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 7.1-7.5 (m, 5H), 4.3-4.4 (m, 1H), 3.4-3.5 (m, 1H), 1.7-1.9 (m, 1H), 1.6-1.7 (m, 1H), 1.4-1.6 (m, 2H), 1.1-1.3 (m, 2H), 0.9 (t, 3H); ^{13}C NMR (400 Mhz, $CDCl_3$) 170.87, 137.13, 128.89, 127.08, 126.33, 90.39, 57.27, 36.26, 33.75, 18.10, 13.70 LRMS (FAB) 204 [M+H]+, 227 $[M+Na]^+$ HRMS (FAB) calculated 227.1048 $[M+Na]^+$, found 227.1053 $[M+Na]^+$; 47% yield

Figure 1, β-lactone 8 - 4,4-Diethyl-4-methyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 3.31-3.36 (q, 1H), 1.96-2.02 (m, 1H), 1.75-1.87 (m, 3H), 1.29-1.31 (d, 3H), 0.98-1.02 (t, 3H), 0.94-0.98 (t, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.64, 84.97, 51.38, 29.25, 24.53, 8.77, 8.03, 7.62 LRMS (FAB) 129 $[M+H]^+$ 151 $[M+Na]^+$ HRMS (FAB) calculated 129.0916 $[M+H]^+$, found 129.0919 $[M+H]^+$42% yield

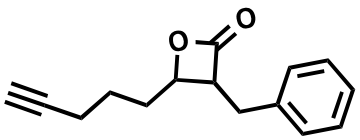
Figure 1, β-lactone 9 - 4,4-Diethyl-4-pentyloxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 3.15-3.20 (t, 1H), 2.00-2.04 (m, 1H), 1.73-1.83 (m, 7H), 1.32 (broad, 4H), 0.91-1.00 (t, 9H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.25, 85.00, 56.98, 31.60, 29.26, 27.61, 24.48, 24.31, 22.36, 13.98, 8.20, 7.42 LRMS (FAB) 199 $[M+H]^+$ 221 $[M+Na]^+$ HRMS (FAB) calculated 221.1518 $[M+Na]^+$, found 221.1520 $[M+Na]^+$39% yield

Figure 1, β-lactone 10 - 4,4-Diethyl-3-(non-8-en-1-yl)oxetan-2-one
^1H NMR (400 Mhz, $CDCl_3$) 5.80(m, 1H), 4.95-5.04 (dd, 2H), 3.19 (t, 1H), 2.40-2.54 (m, 2H), 2.0-2.12 (m, 4H), 1.77 (m, 8H), 1.08-1.17 (m, 8H), 0.94-1.02 (t, 6H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.24, 139.10, 114.22, 85.00, 56.97, 33.75, 29.39, 29.26, 29.15, 28.99, 28.84, 27.92, 24.48, 24.34, 8.20, 7.42 LRMS (FAB) 253 [M+H]+, 275 $[M+Na]^+$ HRMS (FAB) calculated 275.1987 $[M+Na]^+$, found 275.1995 $[M+Na]^+$34% yield

Figure 1, β-lactone 11 - 3-pentyl-1-oxaspiro[3.5]nonan-2-one
^{1}H NMR (400 Mhz, $CDCl_3$) 3.07 (t, 1H), 1.89-1.93 (m broad, 2H), 1.67 (m broad, 8H), 1.33 (m, 6H), 0.92 (t, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.30, 82.163, 58.22, 37.43, 31.61, 31.25, 27.45, 24.96, 23.97, 22.91, 22.38, 22.17, 14.00 LRMS (FAB) 211 [M+H]+, 233 $[M+Na]^+$ HRMS (FAB) calculated 233.1518 $[M+Na]^+$, found 233.1526 $[M+Na]^+$ 56% yield

Figure 1, β-lactone 12 - 3-heptyl-1-oxaspiro[3.4]octan-2-one
^{1}H NMR (400 Mhz, $CDCl_3$) 3.40-3.48 (t, 1H), 2.55-2.58 (t, 1H), 2.42-2.45 (t, 1H), 2.23-2.26 (t, 1H), 1.86-1.95 (m broad, 4H), 1.60-1.63 (m broad, 2H), 1.29-1.32 (m, 8H) 0.88-0.92 (t, 3H), ^{13}C NMR (400 Mhz, $CDCl_3$) 172.08, 91.15, 54.80, 37.77, 34.45, 32.14, 31.74, 29.35, 27.38, 25.57, 23.49, 22.63, 20.36, 14.10 LRMS (FAB) 211 [M+H]+, 233 $[M+Na]^+$ HRMS (FAB) calculated 247.1674 $[M+Na]^+$, found 247.1668 $[M+Na]^+$ 48% yield

Chemical Proteomic Probe: Trans-3-benzyl-4-(pent-4-yl)oxetan-2-one



^{1}H NMR (400 Mhz, $CDCl_3$) 7.2-7.5 (m, 5H), 4.3-4.4 (m, 1H), 3.5-3.6 (m, 1H), 3.2 (m, 2H), 3.0 (s, 1H) 1.4-2.0 (m, 6H) ^{13}C NMR (400 Mhz, $CDCl_3$) 170.87, 137.13, 128.89, 127.08, 126.33, 90.39, 78.32, 68.67, 57.27, 36.26, 33.75, 20.70, 18.10, LRMS (FAB) 228 [M+H]+, 251 $[M+Na]^+$ HRMS (FAB) calculated 251.1048 $[M+Na]^+$, found 251.1056 $[M+Na]^+$; 28% yield

Preparation of (1*S*,5*R*)-5-methyl-6-oxabicyclo[3.2.0]heptan-7-one (Figure 1: Entry 13). A 25-ml RBF equipped with a rubber septum was evacuated and placed under a positive pressure of nitrogen. To the flask was then added tosyl Chloride (1.1 eq, 1.1 mmol), 4-dimethylaminopyridine (1.5 eq, 1.5 mmol) and dichloromethane (4 mL). After the solid was dissolved, diisopropylamine (4 eq, 4 mmol) was added and allowed to stir for 10 min. Next, a solution of 6-ketoheptanoic acid (1 eq, 1 mmol) in 1 mL DCM was added drop wise. The solution was allowed to stir at RT for 1.5 h and then powdered anhydrous K_2CO_3 (3 eq, 3 mmol) was added in one portion. The reaction slurry was allowed to stir at RT for 4 h and then diluted with hexanes and passed through a silica gel plug to remove excess solids. The solution was then dried with Na_2SO_4 and concentrated *in vacuo*. The product was purified by flash chromatography (SiO_2, eluting with 20% EtOAc) to afford the β-lactone as a colorless liquid.

Table S1. Strains used in this study

Strain	Description	Source/Reference
Streptomyces coelicolor		
M145	Prototroph SCP1-, SCP2-	
B812	M145 *Δ2618-2619::apr*	This Study
B834	M145 *Δ7281-7280::apr*	This Study
B813	M145 *Δ2618-2619::apr* – *pJS372*	This Study
B835	M145 *Δ7281-7280::apr* – *pJS374*	This Study
Escherichia coli		
DH5α	F^- φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (r_K^- m_K^-) phoA supE44 thi-1 gyrA96 relA1 λ	Invitrogen
ET12567	*dam, dcm, hsdS, cat, tet*	50
BW25113	Δ(*araD-araB*)*567* Δ*lacZ4787*(::*rrnB-4*) *lacIp-4000*($lacI^Q$), λ-, *rpoS*369(Am), *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514	50
JK10	clpP::cat, Δlon, slyD::kan, λDE3	13
gKS112	JK10 *pKS215*	This Study
gKS113	JK10 *pKS216*	This Study
ER2566		NEB
gKS037	ER2566 *pKS075*	This Study
Mycobacterium		
M. smegmatis MC2155		ATCC
M. tuberculosis H37Rv		Scott Franzblau

Table S2. Plasmids used in this study.

Plasmid	Description	Source/Reference
pIJ790	*[oriR101], [repA101(ts)], araBp-gam-bet-exo*, Chl^R	50
pUZ8002	RP4 Derivative, OriT-, Kan^R	50
PGem-T-Easy	pUC-derived, *lacZ*, Amp^R	Promega
pMS81	*OriT*, ΦBT1 *attB-int*, Hyg^R	37
pBlueScript KS+	pUC *ori*, MCS, Amp^R	Agilent Technologies (Stratagene)
pJS812	StC49 ΔSCO2618-19::*apr*	This Study
pJS833	St5H1 ΔSCO720-81::apr	This Study

pJS371	1904 bp AleI and SacI fragment from cosmid StC49 containing SCO2618-19 with 146 upstream bp in pBluescript KS+, Amp^R	This Study
pJS372	SCO2618-19 subcloned into PMS81 from pJS372 using EcoRV and SpeI, Hyg^R	This Study
PJS373	1806 bp ScaI and SacI fragment from cosmid StC49 containing SCO7280-81 with 340 upstream bp in pBluescript KS+, Amp^R	This Study
PJS374	SCO7280-81 subcloned into PMS81 from pJS373 using EcoRV and SpeI, Hyg^R	This Study
PJS375	*clpP3* 5'RACE product of GSP2 and AAP cloned into pGEM-T Easy, Amp^R	This Study
pET-21a	pBR322 *ori*, *lacI, Amp^R*	EMD
pKS075	codons 62-425 of *E.* coli ClpX downstream of a hexahistidine tag and TEV protease site, in pET-21a	This Study
pET-22b	pBR322 *ori*, *lacI, Amp^R*	
pKS215	codons 7-200 of *M. tuberculosis* ClpP1 upstream of a hexahistidine tag in pET-22b	This Study
pKS216	codons 13-214 of *M. tuberculosis* ClpP2 upstream of a hexahistidine tag in pET-22b	This Study

Table S3. Primers used in this study.

Primer Name	Application / Function	Sequence
SCO 2618 KO R	PCR targeting/disruption of *clpP1clpP2*	CCGTGCCAAGGAGCGGCAGACGATAC AGCCTCTTGCCTAACTAGTTGTAGGC TGGAGCTGCTTC
SCO 2619 KO F	PCR targeting/disruption of *clpP1clpP2*	GAGTCCCGGAAAGCGGCTCCAGCCCCG CAGGGAAACGTGACTAGTATTCCGGGG ATCCGTCGACC
SCO 7280 KO R	PCR targeting/disruption of *clpP3clpP4*	GGCGCCCCCGCACCGCGGGGACGCCGGC CCGGCAGCTCAACTAGTTGTAGGCTGGA GCTGCTTC
SCO 7281 KO F	PCR targeting/disruption of *clpP3clpP4*	CGGCCACGACCGGCCACGACTCGACAAGG AGCGCCGATGACTAGTATTCCGGGGATCC GTCGACC
SCO 2618 KO Det R	Verification of *clpP1clpP2* replacement with *apr*	CGCGTTAGGGTCCATGAATA
SCO 2619 KO Det F	Verification of *clpP1clpP2* replacement with *apr*	CTCCTGGTGCTGCCTTATGT
SCO 7281 KO Det F	Verification of *clpP3clpP4* replacement with *apr*	GGCAGAGAAGTCCCCTGTC
SCO 7280 KO Det R	Verification of *clpP3clpP4* replacement with *apr*	CTGGTCCGGCTCACCTC
clpP1clpP2 RT-PCR F	Detection of *clpP1clpP2* replacement with *apr*	CACAGCTGCTGCTCCTTG
clpP1clpP2 RT-PCR R	Detection of *clpP1clpP2* replacement with *apr*	GTCGATGAGGCCGTACTC
clpP3clpP4 RT-PCR F	Detection of *clpP3clpP4* replacement with *apr*	GCCAGGGAGTACGGAATG
clpP3clpP4 RT-PCR R	Detection of *clpP3clpP4* replacement with *apr*	TCTCCTTGGCCTGGAGTG
hrdB RT-PCR F	Detection of *hrdB* transcript	CTCGAGGAAGAGGGTGTGAC
hrdB RT-PCR R	Detection of *hrdB* transcript	TGCCGATCTGCTTGAGGTAG
GSP2	*clpP3* 5' RACE	CGCGGGTCCTGTGCG
GSP1	*clpP3* 5' RACE	CCGCGTGCACGGAGC

MtbClpP1M7_NdeI_F	PCR of ClpP1 from *M. tuberculosis* genomic DNA	GAATGCCATATGCGTTCGAACTCGCAG
MtbClpP1_NotI_R	PCR of ClpP1 from *M. tuberculosis* genomic DNA	GCATTCGCGGCCGCCTGTGCTTCTCCATTGAC
MtbClpP2R13_NdeI_F	PCR of ClpP2 from *M. tuberculosis* genomic DNA	GAATGCCATATGCGCTACATCCTGCCGTCG
MtbClpP2_NotI_R	PCR of ClpP2 from *M. tuberculosis* genomic DNA	GCATTCGCGGCCGCGGCGGTTTGCGCGGAGAG

Table S4. Proteins Identified by Mass Spectrometry from *S. coelicolor* M145

Accession #	Protein name	Protein Score	Unique PSMs
21224335|ref|NP_630114.1|	aconitate hydratase [Streptomyces coelicolor A3(2)]	958.48	18
21221742|ref|NP_627521.1|	delta-aminolevulinic acid dehydratase [Streptomyces coelicolor A3(2)]	145.90	2
21221075|ref|NP_626854.1|	ATP-dependent Clp protease proteolytic subunit 1 [Streptomyces coelicolor A3(2)]	104.61	2
21221076|ref|NP_626855.1|	ATP-dependent Clp protease proteolytic subunit 2 [Streptomyces coelicolor A3(2)]	246.76	4
21220139|ref|NP_625918.1|	20S proteasome alpha-subunit [Streptomyces coelicolor A3(2)]	48.23	1
21220023|ref|NP_625802.1|	pyridoxal biosynthesis lyase, PdxS [Streptomyces coelicolor A3(2)]	391.30	8
21221616|ref|NP_627395.1|	molybdenum cofactor biosynthesis protein, MoaC [Streptomyces coelicolor A3(2)]	68.27	1
21223433|ref|NP_629212.1|	GTP-dependent nucleic acid-binding protein, EngD [Streptomyces coelicolor A3(2)]	288.23	6

Table S5. Proteins Identified by Mass Spectrometry from *M. smegmatis* MC1255

Accession #	Protein name	Protein Score	Unique PSMs
500047791|ref|WP_011728509.1|	beta-lactamase [Mycobacterium smegmatis]	59.97	1
489989095|ref|WP_003892152.1|	ATP-dependent chaperone protein, ClpB [Mycobacterium smegmatis]	37.38	1
504690048|ref|WP_014877150.1|	D-alanyl-D-alanine carboxypeptidase [Mycobacterium smegmatis]	104.44	1
500051709|ref|WP_011731626.1|	leucyl-tRNA synthetase [Mycobacterium smegmatis]	71.98	1
504691119|ref|WP_014878221.1|	malate dehydrogenase [Mycobacterium smegmatis]	112.26	2
440625868|gb|ELQ87711.1|	ATP-dependent Clp protease proteolytic subunit 1 [Mycobacterium smegmatis]	50.69	1
440625869|gb|ELQ87712.1|	ATP-dependent Clp protease proteolytic subunit 2 [Mycobacterium smegmatis]	47.84	1
500050671|ref|WP_011730588.1|	purine biosynthesis protein, purH [Mycobacterium smegmatis]	55.90	1
489992290|ref|WP_003895347.1|	prokaryotic ubiquitin-like protein, Pup [Mycobacterium smegmatis]	55.76	1
489990142|ref|WP_003893199.1|	thiosulfate sulfurtransferase [Mycobacterium smegmatis]	40.95	1
500048252|ref|WP_011728970.1|	tryptophan synthase subunit beta [Mycobacterium smegmatis]	35.62	1
500047086|ref|WP_011727804.1|	tryptophanyl-tRNA synthetase [Mycobacterium smegmatis]	51.56	1
489992288|ref|WP_003895345.1|	proteasome subunit alpha [Mycobacterium smegmatis]	43.62	1

Construction of *clpP1clpP2* and *clpP3clpP4* null mutants. Strains of *S. coelicolor* in which either the *clpP1clpP2* locus (SCO2618-19) or the *clpP3clpP4* locus (SCO7280-81) was replaced with an apramycin (*apr*) resistance cassette were constructed via polymerase chain reaction (PCR)-targeted mutagenesis (50). The *apr* resistance cassette for replacement of *clpP1clpP2* was prepared using PCR primers SCO2618 KO FOR and SCO2619 KO REV and the Expand High Fidelity DNA polymerase (Roche). The *apr* resistance cassette for replacement of *clpP3clpP4* was prepared using the Expand High Fidelity polymerase (Roche) and PCR primers SCO7280 KO FOR and SCO781 KO REV. (Table S3). The resistance cassettes were introduced individually into *E. coli* BW25113/pIJ790 containing the appropriate WT (StC49 or St5H1) cosmid. Arabinose was added to induce expression of the λ RED recombinase. The resulting recombinant cosmids, StC49 ΔSCO2618-19::*apr* (pJS812) and St5H1 ΔSCO720-81::*apr* (pJS834), were transformed into ET12576/pUZ8002, a non-methylating strain of *E. coli*, and introduced into wild-type *S. coelicolor* M145 through conjugation as previously described (37), yielding *S. coelicolor* B812 and B834, respectively. Double cross-over exconjugants were selected by apramycin resistance and kanamycin sensitivity. The disruption of SCO2618-19 and SCO7280-81 was confirmed via PCR on isolated genomic DNA from *S. coelicolor* using the appropriate KO Det primers (Table S3).

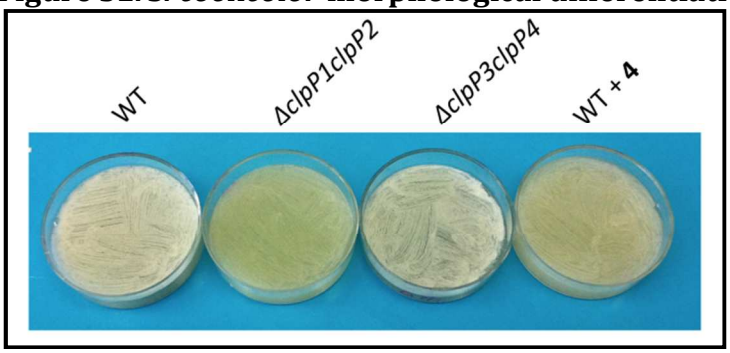
Complementation of *clpP1clpP2* and *clpP3clpP4* null strains. A 1,940-bp fragment was excised from cosmid StC49 containing the SCO2618-19 ORF and 146 bp upstream of the SCO2618 translational start site by restriction digestion with AleI and SacI. The fragment was treated with DNA polymerase I Large (Klenow) Fragment (New England Biolabs). The blunt-ended fragment was then ligated into the SmaI site of pBluescript KS+ to yield pJS371. The fragment containing the SCO2618-19 locus was excised and ligated into pMS81 using EcoRV and SpeI restriction digestion to yield pJS372. pMS81 is a site-specific integrating vector that inserts into the ΦBT1 *attB* site of *S. coelicolor* (50). pJS372 was transformed into ET12576/pUZ8002 and introduced into *S. coelicolor* via conjugation, yielding *S. coelicolor* ΔSCO2618-19::*apr* pJS372 (37). Ex-conjugants were selected based on hygromycin resistance.

A 1,806-bp fragment was excised from cosmid St5H1 containing the SCO7280-81 ORF and 340 bp upstream of the SCO7280 translational start site by restriction digestion with ScaI and SacI. The fragment was treated with DNA polymerase I, Large (Klenow) Fragment (New England Biolabs). The blunt-ended fragment was then ligated into the SmaI site of pBluescript KS+ to yield pJS373. The fragment containing the SCO7280-81 locus was excised and ligated into pMS81 using EcoRV and SpeI restriction digestion to yield pJS374. pJS374 was transformed into ET12576/pUZ8002 and introduced into *S. coelicolor* via conjugation, yielding *S. coelicolor* ΔSCO7280-81::*apr* pJS374 (37). Ex-conjugants were selected based on hygromycin resistance.

Mapping the *clpP3/4* transcription start site via 5' RACE. The transcriptional start site of the *clpP3/4* locus was identified with the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Invitrogen). To map the transcription start

site, total RNA was isolated as previously described from an NMMP culture of wild-type *S. coelicolor* M145 containing 50 μg/mL β-lactone to elevate the levels of the *clpP3clpP4* transcript. First strand cDNA synthesis was performed with 30 pmol of primer *GSP1* and 1.2 μg of RNA template according the manufacturer's protocol for transcripts with high GC content. Following the first strand cDNA synthesis, the reaction was treated with RNase and S.N.A.P. purified. The purified first strand cDNA was treated with terminal deoxynucleotidyl transferase (Tdt) to append a 3´-oligo-dC tail according to the manufacturer's protocol. A 5 μl aliquot of the crude TDT reaction was then used as a template in a Taq polymerase PCR reaction with primer *GSP2* and the Abridged Anchor Primer (AAP, supplied with the kit). The resulting PCR product was ligated into vector pGEmT EASY (Invitrogen) resulting in pJS375, and introduced into *E. coli* strain DH5a. Several pJS375 plasmids containing the transcriptional start site of *clpP3clpP4* operon were isolated and sequenced.

Figure S1. *S. coelicolor* morphological differentiation



Morphological differentiation phenotypes of *S. coelicolor* M145 WT, *ΔclpP1clpP2*, *ΔclpP3clpP4*, and WT treated with β-lactone 4 at 200 μg/mL. Image taken after 48 h on SFM media.