SUPPLEMENTAL MATERIAL



Supplemental Figure S1: ClpC1-FLAG is functional. (A) C-terminally FLAG-tagged ClpC1 has a ~30% lower ATPase rate than untagged ClpC1. (**B**) Degradation of 10 μ M GFP^{ssrA}, ^{MSMEI_3879}GFP, or GFP^{MSMEI_3879} by 1 μ M ClpXP1P2, ClpC1P1P2, or ClpC1^{FLAG}P1P2 demonstrates that ClpX does not recognize MSMEI_3879 constructs, while ClpC1^{FLAG} retains the ability to recognize these substrates. (**C** – **H**) *M. smegmatis* was transformed with an integrative CRISPRi plasmid carrying either (**C**, **E**) a non-targeting control sgRNA or (**D**, **F**, **G**, **H**) a ClpC1-targeting sgRNA. Additionally, some plasmids carried a supplemental copy of (**E**, **F**) wild-type *clpC1*, (**G**) *clpC1* with a C-terminal FLAG-tag, or (**H**) *clpC1*with both a FLAG-tag and inactivating mutations to ATPase active sites ("EQ"). All supplemental *clpC1* loci incorporate codon substitutions that escape sgRNA targeting, indicated by asterisks.



Supplemental Figure S2: Candidate interaction partners bind to ClpC1 *in vitro*. Binding of 0.1 μ M^{7xHis-SUMO}ClpC1^{FLAG} to purified (A) MSMEI_3879, (B) DnaJ1, (C) DnaJ2, (D) GntR, (E) Mg chelatase, or (F) XRE was assayed by microscale thermophoresis in the presence of 1 mM ATP γ S. Data were fit to a Hill binding equation, and fit parameters are shown.



Supplemental Figure S3: Phylogenetic analysis of MSMEI_3879 homologs. Homologs of MSMEI_3879 in Mycobacteriaceae were compiled, aligned, and used to generate a phylogentic tree. The average per-residue BLOSUM62 score of each sequence to MSMEI_3879 is shown as a heatmap strip. A representation of the sequence alignment, colored according to residue type, is shown alongside the tree. *M. smegmatis* MSMEI_3879 (blue) and *M. tuberculosis* OpIA (red) are indicated.

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Mycolicibacterium smegmatis MC2 155									М	SMEI_3879(4	446 aa)		
Mycolicibacterium fortuitum strain CT6			WP_076182160.1 (687 aa)										
Mycolicibacterium bo	enickei strain	PDNC014	WP 090426366.1 (687 aa)										-
В			ī	660 67	70 61	80 6	90	700	710	720	730	740	750
Mycolicibacterium sm	negmatis MC2 15	5								1	M L H	SGG	GSMT.
(NC_008596.1)	rtuitum etrain	CTTE	CTG	GTGAGCGGGTAC	U N P	CTCGAGGGAT	CCTG <mark>G</mark> CGC	GACGACGGCI	ACGACGGC	GACCTGCTGT	IGTTGCACI	CCGGAGGCG	GCTCGATGACG.
(NZ CP011269.1)	icuicum sciain	010	CTG	GTGAGTGGTTAT	IGTGAATCGA	CTGGAAGGGT	CCCTG-CGC	GCCGACGGCI	ACGACGGG	GACCTGTTGC	IGTTGCACI	CAGGCGGTG	GATCGATGACG.
Mycolicibacterium bo	enickei strain	PDNC014	L	V S G Y	VNR	LEG	SLR	A D G	Y D G	DLLI	LLH	SGG	З Ѕ М Т .
(NZ_CP070348.1)			CTG	GTGAGTGGTTAI	rgtgaaccgg	CTGGAAGGGT	CTCTG-CGC	GCAGACGGC1	ACGACGGT	JACCTGTTGC.	IGTIGCACI	CCGGCGGTG	SATCGATGACG.
C Organism	Sequence ID	S	tart L	50 100	150	200 250	300	350 400	450	500 55	0 600	650	742 End
M. smeamatis	query	(+)	1	this rec	ion missin	a1							446
M. houstonense	WP 06689989	3.1(+)	1	-	. .	5							687
M. fortuitum	WP 06502087	2.1(+)	1	4	4 4								687
M. nivoides	WP 12471306	6.1(+)	1	÷.	÷.								687
M. boenickei	WP 09042636	6.1(+)	1	÷.	44								687
M. septicum	WP 20586732	7.1(+)	1	÷.	44 - C								687
M. perearinum	WP 06493660	8.1(+)	1		83								687
M. hassiacum	WP 02621355	5.1(+)	1		11								685
M. stellerae	WP 12302874	8.1(+)	1										702
M. tokaiense	WP 16390796	5.1(+)	1	1 1								- I.	687
M. murale	WP 19348876	9.1(+)	1	÷	44							· · · ·	669
M. baixiangningiae	WP 19304564	1.1(+)	1 •										· 706
M. agri	WP 13311904	1.1(+)	1	1								- I.	677
M. litorale	WP 18529362	5.1(+)	1 •										. 704
M. chitae	WP 12633336	8.1(+)	1 🕴		140 C							-	· 705
M. confluentis	WP 08515130	1.1(+)	1 •									4 • • • • •	· 706
M. parafortunitum	WP 08314666	1.1(+)	1		1								• 710
M. elephantis	WP 05276161	2.1(+)	1	1	1					I - 18-21			684

Supplemental Figure S4: Comparison of MSMEI_3879 to full-length homologs. (A) Genetic context of *M. smegmatis* MSMEI_3879 compared to the equivalent genomic region in the indicated *Mycolicibacterium* species. **(B)** Sections of genomic sequence show the beginning of the disrupted *M. smegmatis* hydantoinase/oxoprolinase gene and the analogous regions in the intact genes of *M. fortuitum* and *M. boenickei*. Numbering is given from the beginning of the normal intact open reading frame. Amino acid translations of MSMEI_3879 (purple) and hydantoinase/oxoprolinase orthologs (black) are shown. A single nucleotide insertion in the *M. smegmatis* genome (red) disrupts the gene. **(C)** NCBI protein BLAST was used to align MSMEI_3879 (top) with full-length hydantoinase/oxoprolinase orthologs from select *Mycolicibacterium* species. The upstream region missing in MSMEI_3879 is indicated.



Supplemental Figure S5: Structure prediction of MSMEI_3879. AlphaFold2 structure predictions of (A) *M. fortuitum* axcA, which lacks an N-terminal truncation, and (B) *M. smegmatis* MSMEI_3879 are shown as cartoons. The region missing due to truncation of MSMEI_3879 is shown as orange in acxA. (C) Loss of this region exposes hydrophobic residues (green) on structured elements that project from the folded core of the protein.



Supplemental Figure S6: "Full-length" MSMEI_3879 is not degraded by ClpC1P1P2. Proteolysis of purified "full-length" MSMEI_3879, with restored N-terminus, was assayed by SDS-PAGE as in Figure 7B.