Supporting Information

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SI Materials and Methods

EspR ChIP. *Mycobacterium smegmatis* (50 mL) cultures were grown at 37 °C to $OD_{600} = 0.5$ in 7H9 (0.05% Tween 80, appropriate antibiotics), pelleted, fixed in fresh 4% (wt/vol) paraformaldeyde at room temperature for 20 min, and quenched in 125 mM glycine for 5 min. Pellets were washed twice in cold Tris buffered saline [20 mM Tris·HCl (pH 7.5), 150 mM NaCl] and suspended in 350 µL lysis buffer [50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate] with protease inhibitors. Cells were lysed by sonication in a Diagenode Bioruptor water bath sonicator and pelleted. Cell extracts were further sonicated to shear DNA fragments to a mean size of 200 bp, at which point a sample was stored at −20°C as input material.

Immunoprecipitations (IPs) were performed by incubation with 3 µL monoclonal anti-FLAG M2 antibody (Sigma) at 4 °C overnight, followed by incubation with 50 µL magnetic Dynabeads (Invitrogen) for 2 h while rocking at 4 °C. Beads were subjected to two washes in lysis buffer, two washes with wash buffer [10 mM Tris·HCl (pH 8.0), 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA), and one wash in TE [10 mM Tris (pH 8.0), 1 mM EDTA]. Next, 110 µL elution buffer [50 mM Tris·HCl (pH 8.0), 10 mM EDTA, 1% SDS] was added to each sample, and the beads were incubated for 15 min at 65 °C and pelleted. A second elution was performed with 150 µL of elution buffer 2 (TE, 0.67% SDS) and eluates from the two elution steps were combined. Formaldehyde crosslinks were reversed by incubating IP and input samples at 65 °C overnight. Samples were treated with 250 µL proteinase K solution (TE, 20 µg/mL glycogen, 400 µg/mL proteinase K) and incubated at 37 °C for 2 h. Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol solution (25:24:1), precipitated with LiCl/ethanol, and resuspended in 25 µL TE containing 100 µg/mL RNaseA.

Quantitative RT-PCR was performed using primer pairs (Table S3) that spanned the entire *espACD* upstream intergenic region, using Taq polymerase in the presence of SYBR Green on an Opticon Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). IP samples were used as a direct template for PCR, and reactions were quantified using standard curves generated for each primer pair with input material for that sample. Data were normalized to signal from a nonenriched region (16S rRNA) for each sample.

EMSA. DNA probes were PCR-amplified from a plasmid template and gel purified. The following primer pairs correspond to each probe: BC_{364} (oCD208/oCD143), B_{240} (oMT26/oCD143), C_{124} (oCD208/oMT25). Labeled probes were generated using 5′-5-FAM prelabeled oligonucleotides (Bioneer, Inc.), with the label on one strand only. The B_{48} probe was generated by annealing oligonucleotides oCD166 (5-FAM) and oCD167. Binding reactions were performed with purified protein and 5 nM probe for 30 min at room temperature in EMSA buffer [10 mM Tris (pH 8.0), 50 mM NaCl, 1 mM DTT, 100 µg/mL BSA, 1 mM EDTA, 5% (vol/vol) glycerol, and 30 µg/mL poly(deoxyinosinic-deoxycytidylic) (Sigma)]. Reactions were resolved at room temperature on a 4–20% Tris-glycine gel (Invitrogen) and visualized using a Typhoon Fluorescence Imager (GE Healthcare).

Fig. S1. Single-site probes are insufficient for high-affinity interaction with EspR. (*A*) EMSA with purified EspR and a ^{32}P-labeled 520-bp probe spanning the A site and translational start site of *espA*. EspR$_{dimer}$ concentrations were 0, 1, 2, and 5 μM; lane 2 contains 20 μM His-maltose-binding protein as a nonspecific protein control. (*B*) EMSAs with purified EspR ([EspR$_{dimer}$] = 0, 1, 5 μM) and 5-FAM-labeled probes containing only the indicated site (subscript indicates length of probe in base pairs).

Fig. S2. A 364-bp region centered on the B and C sites is sufficient for complete EspR binding in vivo. The region corresponding to EMSA probe BC$_{364}$ was cloned into pMV30.Kan, as indicated on the map under the *x* axis (thick black bar represents promoter fragment; thin gray line represents plasmid vector sequence), and integrated into the *M. smegmatis* genome. ChIP experiments were performed as described in Fig. 4*B*, with the exception that different primer pairs (Table S3) were used to detect plasmid vector sequence.

Table S1. Crystallographic and refinement statistics

	Se-Met	Se-Met (peak phasing)	Se-Met (high remote phasing)
Data Collection			
X-ray source	ALS Bl 8.3.1	ALS Bl 8.3.1	ALS Bl 8.3.1
Wavelength	1.115889	0.9797	0.9570
Resolution	50–2.5	50–2.7	50–2.7
Space group (twinning operator)	$P3_2$ $(-k,-h,-l)$	$P3_212$	$P3_212$
Cell parameters, Å, °	$a = 148.49$ $c = 129.67$	$a = 149.03$ $c = 129.36$	$a = 149.03$ $c = 129.33$
Molecules per ASU	18	18	18
Unique reflections (test set)	100,721 (5,413)	88,375	94,197
Redundancy	2.1 (2.0)	3.7 (3.7)	3.7 (3.7)
Mn (I/σ)	3.9 (1.5)	5.1 (1.2)	5.2 (3.1)
Completeness	91.05 (86.9)	100 (99.9)	100 (100)
R_{sym}*	0.140 (0.73)	0.258 (0.839)	0.279 (0.959)
Refinement			
R_{work}/R_{free}, %	19.7/23.2		
No. atoms	17,208		
rmsd			
Bond length, Å	0.010		
Bond angles, °	1.249		
Twinning fraction, α	0.5		

ALS Bl, Advanced Light Source Beamline; ASU, asymmetric unit; Se-Met, selenomethionine.
*R_{sym} $\Sigma|I - \langle I \rangle|/\Sigma I I$ where $\langle I \rangle$ is the average intensity over symmetry equivalents.

Table S2. Strains and plasmids used in this study

Strains/plasmids	Genotype/description	Resistance	Source
M. smegmatis			
mc^{2}155	wild type	—	(1)
CDMS165	mc^{2}155 + pCD165	Zeo	This study
CDMS165/501	CDMS165 + pROW501	Zeo, Kan	This study
CDMS165/182	CDMS165 + pCD182	Zeo, Kan	This study
CDMS165/173	CDMS165 + pCD173	Zeo, Kan	This study
Plasmid			
pLIC-HMK	T7 promoter-6XHis-MBP-TEV	Kan	(2)
pCD99	pLIC-HMK + *espR*$_{Mtb}$	Kan	This study
pH3C	T7 promoter-6XHis-MBP-3C	Kan	(3)
pCD174	pH3C + *espR*$_{Mtb}$	Kan	This study
pCD177	pH3C + *espR*$_{Mtb}$ (amino acids 1–107)	Kan	This study
pMV261.Zeo	*oriM*, *oriE*, groEL promoter	Zeo	This study
pCD165	pMV261.Zeo + *espR*$_{Mtb}$-3X-FLAG	Zeo	This study
pMV306.Kan	*int*, *oriE*	Kan	MedImmune
pROW501	pMV306.Kan + full intergenic region-*espA-luxAB*	Kan	This study
pCD182	pROW501 (aBC)	Kan	This study
pCD173	pROW501 (AbC)	Kan	This study
pCD180	pROW501 (ABc)	Kan	This study
pCD181	pROW501 (Abc)	Kan	This study
pCD189	pMV306.Kan + BC$_{364}$ region	Kan	This study

MBP, maltose-binding protein; TEV, tobacco etch virus.

1 Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR, Jr. (1990) Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. *Mol Microbiol* 4:1911–1919.
2 Kapust RB, Waugh DS (1999) Escherichia coli maltose-binding protein is uncommonly effective at promoting the solubility of polypeptides to which it is fused. *Protein Sci* 8:1668–1674.
3 Hammon J, Palanivelu DV, Chen J, Patel C, Minor DL, Jr. (2009) A green fluorescent protein screen for identification of well-expressed membrane proteins from a cohort of extremophilic organisms. *Protein Sci* 18:121–133.

Table S3. Oligonucleotides used in this study

Purpose	Sequence	
qPCR—*espA* promoter	***Product midpoint position relative to* espA *ORF start (bp)***	
oMT40	agcagccagaggcgattgt	−1,247
oMT41	agtcactgtgagctgtagccat	−1,247
oMT22	gagggtcccaaacgtgagccaat	−1,050
oMT23	tctatagcccgcctgagctattcc	−1,050
oCD212	taaccgctgtcttggcgtgatctt	−914
oCD213	aatgcggtgtctcgccttagtaga	−914
oMT26	aacccatcctcggcaggt	−850
oMT27	tatcccgggataacggcagtgcta	−850
oCD144	aaatcggtccgagcatgtagcact	−784
oCD145	gttatcgggcaacggaaagcgaaa	−784
oMT30	attcaggcttttcttcgcaaggtacc	−650
oMT31	attgcggcggccttatctaaacca	−650
oCD134	gcaacggatttccatcgtcggttt	−491
oCD133	taacccaccatcactcacccgatt	−491
oCD130	aatcgggtgagtgatggtgggtta	−358
oCD131	cgatcggtatgagatctgttgcagga	−358
oCD110	cgtgcaatgcagaaccaaggctat	−17
oCD111	tggatcgatgatgaacgctctgct	−17
qPCR—pMV306.Kan	***Product midpoint position relative to site B (bp)***	
oCD220	acgatagttaccggataaggcgca	−533
oCD221	cgctttctcaatgctcacgctgta	−533
oCD222	aaaggcggacaggtatccggtaa	−399
oCD223	tggcgaaacccgacaggactataa	−399
oCD224	cacctctgacttgagcgtcgattt	−276
oCD225	tatagcccgcctgagctattccacat	−276
oCD228	atcagctagagccgtgaacgaca	329
oCD229	attggagctggtgcagtgaagaga	329
oCD230	tgcaaccttgtcccggtctattct	406
oCD231	cctgatctggctactttcgatgct	406
oCD234	tcggctgcatcctctaagtggaaa	709
oCD235	gttgcaactcctgtgcaactctca	709
qPCR—control		
Msm16SF	gtgcatgtcaaacccaggtaa	control
Msm16SR	gggatccgtgccgtagctaac	control
EMSA probes		
oCD143	atatagcgaacaccggtaccttgc	
oCD166	gcactgccgttatcccgggatagcaaaccacccggaaccagggctatc	
oCD167	gatagccctggttccgggtggtttgctatcccgggataacggcagtgc	
oCD208	ggaatagctcaggcgggctataga	
oMT25	acatggtggtcagctactgagc	
oMT26	aacccatcctcggcaggt	

Quantitative PCR, qPCR.