## SUPPLEMENTARY DATA

## Table S1

Primer name	Sequence 5' to 3'	Description
Rv8cF-BamHI	GCGGATCCATGAGTGAGCAGGTGGAAAC	Forward for <i>gfp- cwsA</i> <sub>tb</sub> fusion
Rv8cR-XbaI	TTGCTCTAGATCAGGAGCGCGGTTGCACCT	Reverse for sense <i>cwsA</i> <sub>tb</sub> overproduction, and <i>gfp</i> fusions
Rv8c-solR- XbaI	TTGC <b>TCTAGA</b> TCATTTGCTGCGACGTTGGGTCCGG	Reverse for <i>gfp- cwsA</i> <sub>tb</sub> - <i>soluble part</i> fusion
MVM469	GCGACCAGTACTAAAGGAGAAGAACTTTTCACT	Forward for <i>gfp</i>
Rv8cF- BamHI-B2H	TCTAGA' <b>GGATCC'</b> CATGAGTGAGCAGGTGGAAAC	Forward for <i>cwsA</i> <sub>tb</sub> for bacterial two hybrid assay
Rv8cR-KpnI- B2H	TTACTTA' <b>GGTACC'</b> CGGGAGCGCGGTTGCACCTCGA	Reverse for <i>cwsA</i> <sub>tb</sub> for bacterial two hybrid assay
Rv8c-solR- KpnI-B2H	TTACTTA' <b>GGTACC'</b> CGTTTGCTGCGACGTTGGGTCC	Reverse for <i>cwsA<sub>tb</sub></i> -soluble part for bacterial two hybrid assay
Wag31F- BamHI-B2H	TCTAGA' <b>GGATCC'</b> CATGCCGCTTACACCTGCCGA	Forward for <i>wag31</i> for bacterial two hybrid
Wag31R- KpnI-B2H	TTACTTA' <b>GGTACC'</b> CGGTTTTTGCCCCGGTTGAATTGAT CGA	Reverse for <i>wag31</i> for bacterial two hybrid
Rv8cF-KO	AAAA'CTGCAG'CTCGGGGTGGAACTC	Forward upstream <i>cwsA</i> <sub>smeg</sub> for gene knockout
Rv8cR-KO	GGAATTC'CATATG'TTAATTAA'GACGACAGCCCGTTC GCCAAC	Reverse downstream <i>cwsA</i> <sub>smeg</sub> for gene knockout
Rv8cF-PacI	AGAACC' <b>TTAATTAA</b> 'GAGCCCCACCAGGGAGGAAGCC GAACGATGAGTGAGCAGGTGGAAAC	Forward for <i>cwsA</i> <sub>tb</sub> -soluble <i>part</i> in pLR52
Rv8c-solR- SwaI	ATCGG' <b>ATTTAAAT'</b> TCATTTGCTGCGACGTTGGGTCCG G	Reverse for <i>cwsA</i> <sub>tb</sub> -soluble part in pLR52
Msmeg0023F- ClaI	CC'ATCGAT'TGGTTGCCGAACAGTGCGAT	Forward for <i>cwsA</i> <sub>smeg</sub> under native promoter
Msmeg0023F- XbaI	TTGC' <b>TCTAGA'</b> TTCAGGGGCTTGGGGGGCGACCTC	Reverse for <i>cwsA</i> <sub>smeg</sub> under native promoter
MR326	AGAG <b>GATCCC</b> ATGGCCGCGGGGGGGGGGGGGGGGGGGGGGG	Forward for <i>wag31</i> C- terminal domain in BACTH vectors
MVM831	TTAC <b>TTAGGT</b> ACCCGGTTTTTGCCCCGGTTGAATTGAT CGA	Reverse for <i>wag31</i> C- terminal domain in BACTH vectors
MVM920	GGGAATTCCATATGCCGCTTACACCTGCCGA	Forward for wag31 in wag31-mCherry fusion
MVM921	GCAGTACTAACAACAACCTGCAGATGGTGAGCAAGG GCGAGGA	Forward for <i>mCherry</i>
MVM922	GTAGTCTAGATTACTTGTACAGCTCGTCCATGC	Reverse for <i>mCherry</i>
MVM923	CACAGTACTGTTTTTGCCCCGGTTGAATT	Reverse for <i>wag31</i> in <i>wag31-mCherry</i> fusion
msmeg23 F	CCAGAGCAGATGTCCGATTG	Forward for qRT-PCR of

		cwsA
msemg23_R	GCTTCCTGGATCGTCTCG	Reverse for qRT-PCR of
		cwsA
sigAsmeg_F	CCCACCGGGAATTCGTAAG	Forward for qRT-PCR of
		sigA
sigAsmeg_R	TTGCCGGGCTTGCCTT	Reverse for qRT-PCR of
		sigA

**'BOLD LETTERS'** indicate restriction sites.

**Supplementary Figures and legends** 



FIG. S1. Localization of *gfp-cwsA* in *M. tuberculosis*. Actively growing cultures of *M. tuberculosis Pami::gfp-cwsA* were induced with 0.2% acetaminde for 48 h, harvested and fixed in 4% paraformaldehyde for 24 h. The cells were examined by bright-field (i) and fluorescence (ii) microscopy as described in the text. Arrow – midcell (new pole) localization; arrowhead – polar localization.

M.tuberculosis	1MSEQVEIKLIPKEKLIKGLAYSAVGPVDVIKGLLELGVGLGLQSARSIAAGLKKKYKEGKLA	62
M.canettii	1 MSEQVETRLTPRERLTRGLAYSAVGPVDVTRGLLELGVGLGLQSARSTAAGLRRRYREG RLA	62
M ofricanum	1	62
m.an canun		02
M.bovis	1MSEQVEIRLIPKERLIKGLAYSAVGPVDVIKGLLELGVGLGLQSARSIAAGL <u>RKKYKEG</u> KLA	62
M.vanbaalenii	1	62
Maihum	1	62
m.giivuin		02
M.kansasii	1 MSEKVETRLTPRERLTRGLTYSAVGPVDVTRGVVGLGV QSAQSTASEVRRRYREG RLA	58
M.smeamatis	1 MPARADVRLAPRORLTRGLKYTAVGPVDITRGVLGIGA DTAQATAAELRRRYASG KLQ	58
M marinum	1	58
		50
M.avium	1 MRAKAEDKLIPKEKLAKGLIYSILGPLDLIKGVAGLSG QSARSIAEQLKKKYKEG KLA	58
M.intracellulare	1OSAQSTAAQLRRRYREGRLA	58
M avium naratubaraulasia	1 MPAKA EDDI TODEDI A DOL TYSTI ODI DI TOOVACI SV	50
m.avium-paracubercuosis		50
M.colombiense	1 MSAKTUSKLIPRORLARGLSYSAQGPVDVIRGVVGLSV QSAQSIA SQLRRRYQEG RLA	58
M.parascrofulaceum	1	58
M rhodesiae		62
m.moucsec		02
M.abscessus	1MSLLIKSEEPAVKLISGURLAKGLKEAALAPIDVSKGIAGLSFGLAKSAISAAGKLLKKGK	61
M.massiliense	1 MSLLTKSEEPAVKLTSGORLARGLKEAALAPIDVSRGTAGLSFG LAKSATSAAGRLLRRG K	61
M thermore is tibile		54
		54
m.tusciae	1 DGAQSSAAWIGDLYBRSRLKVSAVGPVDVIRGALGLGV DGAQSSAAWIGDLYBRSRLKDQLG	54
M.ulcerans	1HSAQSTASELERRYPOGRLA	50
T naurometahola	1 MATELLDDVOPADETSOAPSNAORVATGEGOSETGPLNLARGITGIGIS LASHVI HTEENI TEKT	67
1.paulonicanola		07
M.tuberculosis	63 REVAAAQETLAQELTAAQDVVANLPQALQDARTQRRSKHHLWIFAGIAAAILAGGAVAFS	122
M canettii		122
M C .		400
M.atricanum	63 KEVAAAQEI LAQEL TAAQU VVANLPQALQUAK I QKK SKHHLWI FAG TAAA I LA	122
M.bovis	63 REVAAAQETLAQELTAAQDVVANLPQALQDARTQRRSKHHLWIFAGIAAAILAGGAVAFS	122
M vanhaalenii	63 A FLAAA O FA LA VEVAAA O FVVAGL POAL FK.	121
		140
M.gilvum	63 TELAAAQQVVANLPEVVQNARKPKKKVKPLLLAGVAVAVLAGGAVTFS	110
M.kansasii	59 REIAAAQETLAQELAAAQEVVANLPQVLQEARRSQRRGSKKRVWVIAGAATVVVVAGGAVAFT	121
M.s.meamatis		112
M		440
M.marinum	59 REVAAAQEATAQELAAAQEVVANLPQVTQEARRKQGRSKRPWVTAGAVTVVVAGGAVAFT	119
M.avium	59 RDLAAAQETLAQELAAAQEVVTGLPQALQDARR AQRRGKRPWI IAGVAVAVLA GGAAAFS	118
M intracellulare		118
		400
M.avium-paratuberculosis	59 KULTAAQETLAQELAAAQEVVIGLPQALQDAKRAQKRGKPWVIAGVAVAVLAGVAVAVLAGGAAAFS	120
M.colombiense	59 RD LAAAQET LAQE LAAAQEVVT SL PQA LQDAR R AQRRGKR PWVFAG VA VA VLA GGA VA F S	118
M parascrofulaceum		118
		400
M.modesiae	63 QELAAAQDI TAQELAAAQEVVSNLPQALLGAPPTRSVGGKARGKRKKKPLLFAATGVAVLAGGAVAFS	130
M.abscessus	62 A V <mark>S S</mark> E V Q D A V A D V V D T L P E V V S G A R K R K L P R A L T G L A V V G L L G A G A V A F S	111
M massiliense		111
M II		440
M.thermoresistiblie	55 KULAAAUUALALELAAAUEVVSGLPUALUDAKKARKKKKPLIVAA TAAVILVGGATIVI	113
M.tusciae	55 KELAAAQDT IAA ELAAAQEVVANLPQALQ KARTRRKRPLLLAALGVAVLA GGAVAIS	112
M. ulcerans		111
Tanavaratabala		400
i .paurometabola	08 LALAKKVVHTVKSDUPAAPADDAAPSLPVKGGGVKKPLLTTLVVALVLAVGGTAPK	123
M.tuberculosis		145
Moonottii		145
M.canetti		145
M.atricanum	123 IVKKSSRPEPSP-RPPSVEVQPRP	145
M.bovis	123 IVRRSSRPEPSP-RPPSVEVOPRP	145
Myanhaalanii		144
m.vanbaacim		144
M.gilvum	111 ILKKSAQPDPSP-LPPSVEVIPKP	133
M.kansasii		144
Memoramotic		136
m.ancynaus		130
M.marinum	120 VVKK85SQFEF8F-KFPSVDVQFKF	142
M.avium	119 I VRRSVREK PQEPQS-R PPSVDVQPRP	144
M intracellulare		144
		450
M.avium-paratuberculosis	12/ I VKKSVKEKPQEPQS-KPPSVDVQPKP	152
M.colombiense	119 LVRRSVRAKPQEPQS-RPSVDVQPRP	144
M parascrofulaceum		144
M -h - d - i		450
m.modesiae	131 IIRREEFNEFFFILGFSVFM-FKF	153
M.abscessus	112 VLRRSGQP EPSP - LAPSVDPQPQP	134
M.massiliense		134
M thermomentibile		120
m.ulermoresisublie		130
M.tusciae	113 I I KKPPPQ EPPI TLQPSVPVSPKP	136
M.ulcerans	112 VVRRSSQPEPSP-RPSVNVQPRP	134
T naurometahola		179
r.paulometabola		173

FIG. S2. Amino acid sequence alignment of *cwsA* homologues. CwsA homologs (DUF2562 domain containing) from mycobacterial species and closely related *Tsukamurella paurometabola* were aligned with ClustalX program. The conservation of amino acids with the same properties is indicated with colors, according to ClustalX coloring scheme (http://www.jalview.org/help/html/colourSchemes/clustal.html).



Fig. S3. Morphology of *M. smegmatis* WT and  $\Delta cwsA$  stationary phase cultures. Respective strains were grown to A600 ~ 2.0 and imaged by brightfield microscopy. Data were analyzed and scored for number of bulged (swollen) cells in each strain. Averages ± standard error from two independent experiments are shown. WT strain: N (number of cells) = 388;  $\Delta cwsA$ : N = 492.



FIG. S4. Complementation of  $\Delta cwsA$ . Morphology of *M. smegmatis* wild-type (i),  $\Delta cwsA$  (ii),  $\Delta cwsA$  *PcwsA::cwsA* (iii) and  $\Delta cwsA$  *Pami::gfp-cwsA<sub>TB</sub>* (iv, v) was examined by microscopy. Panel v - fluorescent image corresponding to panel iv. Note reversal of  $\Delta cwsA$  phenotype in complemented strains (iii and iv). Black arrowheads – bulged regions; white arrowhead – polar localization; white arrow – punctate localization.



FIG. S5. ECFP-CrgA localization in  $\Delta cwsA$ . Actively growing cultures of *M. smegmatis* WT (i, ii) and  $\Delta cwsA$  expressing *Pami::ecfp-crgA* (iii, iv) were imaged by brightfield (i, iii) and fluorescent (ii, iv) microscopy. Quantitation of CrgA localization from two independent experiments (N= 464) revealed a modest increase in ECFP-CrgA polar localization in  $\Delta cwsA$  strain [24.79 ± 0.29%] as compared to wild type strain [17.49 ± 1.97%] but no significant difference in midcell localization [WT = 13.99 ± 2.15% vs  $\Delta cwsA$  = 14.51 ± 1.97%].



Fig. S6. Wag31-mCherry can complement  $\Delta wag31$ . (A) To establish the functionality of Wag31-mCherry, the integrated copy of *Pami::wag31* (Apramycin<sup>R</sup>) in a *M. smegmatis* conditional mutant (Hyg<sup>r</sup>) (Kang et al. 2008) was swapped with *Pami::wag31-mCherry* (Km<sup>r</sup> resistant) as described (Chauhan et al. 2006) and transformants were selected on agar plates containing Hygromycin, Kanamycin and 0.2% acetamide. Colonies were patched on plates containing Hygromycin and kanamycin, but lacking acetamide. Note no growth was seen in the absence of acetamide (compare plates with and without acetamide). (B) Cells from + acetamide plate were propagated in broth containing appropriate antibiotics and 0.02% acetamide and examined by brightfield (i) and fluorescence (ii) microscopy. Arrowheads – polar localization of Wag31-mCherry. As control, *M. smegmatis* producing mCherry fluorescent protein was visualized by brightfield (iii) and fluorescence (iv) microscopy. Note diffuse localization with mCherry protein in panel iv.



FIG. S7. Wag31 levels do not change in a  $\Delta cwsA$  strain. Cell lysates prepared from *M*. *smegmatis* WT and  $\Delta cwsA$  strain were separated in a SDS-PA gel, transferred to nitrocellulose membrane and analyzed by immunoblotting with  $\alpha$ -Wag31 antibodies and  $\alpha$  –SigA (loading control). Lane 1 – Purified His-SigA protein; lane 2 – purified His-Wag31 protein.



FIG. S8. BACTH assays. Indicated gene fusions to T25 and T18 fragments of adenylate cyclase in the BACTH vectors (Table 1) were used in various combinations to transform *E. coli* BTH101

and recombinants plated on LB agar supplemented with X-Gal and IPTG. Recombinant colonies were subsequently propagated in LB broth and  $\beta$ -galactosidase activity measured as described in the text. Values shown are means  $\pm$  standard deviations from at least 3 independent experiments.



FIG. S9. Confirmation of DKO ( $\Delta cwsA \ \Delta crgA$ ) strain by PCR. Genomic DNA from WT and DKO strains was used for PCR amplification of the *cwsA* region using primers msmeg0023\_F and msmeg0023\_R (Table S1). Amplified products were run on 1% agarose gels and photographed. Expected products are: WT = 411 – bp; single cross-over (SCO) = 411- and 1300 – bp; double cross-over = 1300 – bp and V = vector pPP116 (recombination plasmid for *cwsA* deletion; Table 1) = 1300 – bp.