Discovery of an essential mycobacterial polar growth factor involved in cell envelope integrity and lipomannan and lipoarabinomannan transport

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ABSTRACT

Mycobacteria grow and divide differently compared to well-studied model bacteria. They insert new cell envelope at their poles instead of along their side walls; and, they lack obvious homologs of many well-conserved cell growth and division proteins. Furthermore, while mycobacteria share several similarities to Gram-positive bacteria, unlike these organisms they possess an outer membrane, which is abundant in long-chain fatty acids and several glycolipids and lipoglycans. A better understanding of the unique factors that make this unusual structure could lead to therapeutic targets for pathogenic mycobacteria. Here, we study a gene of previously unknown function – *msmeg_0317* - predicted to be essential and associated with mycobacterial cell growth and division proteins. We find that transcriptional depletion of *msmeg_0317* leads to loss of polar

growth, disruption of the mycobacterial outer membrane, and cell death. Surprisingly, we also observe that depletion results in the accumulation of cell-associated lipoglycans lipomannan and lipoarabinomannan (LM/LAM), while overexpression of CwdA leads to increased shedding of LM/LAM. LM/LAM have been extensively studied in relation to infection but their role in bacterial physiology is less clear. Altogether, our data suggest that MSMEG_0317, renamed CwdA, is involved in the transport of LM/LAM to the mycobacterial outer membrane, and reveal unexpected connections between the correct localization of LM/LAM, polar growth, and the structural integrity of the mycobacterial cell envelope.

IMPORTANCE. Some of the most successful antibiotics target bacterial cell envelope synthesis. However, the cell envelope of mycobacteria is significantly different from that of other, more well-studied, bacteria. Its core structure consists of a covalently-linked network of peptides, carbohydrates, and fatty acids. Additionally, there are several lipids non-covalently interspersed throughout. Many of the enzymes which synthesize these lipids are known, but how they are transported remains largely unclear. Here, we discover that an essential protein, CwdA, is involved in the transport of LM/LAM, abundant lipoglycans in the mycobacterial cell envelope. Depletion of CwdA leads to the loss of polar growth and the disintegration of the mycobacterial outer membrane. These results suggest that the proteins which transport molecules across the mycobacterial cell envelope may represent an abundant source of novel drug targets for the treatment of mycobacterial infections, like tuberculosis.

INTRODUCTION

Mycobacterium tuberculosis (Mtb), the etiological agent of human tuberculosis (TB), is responsible for approximately 1.4 million deaths each year. One of the pathogen's distinguishing features is its unusual cell envelope. Like nearly all other bacterial species, the plasma membrane is surrounded by peptidoglycan, a rigid mesh-like structure made up of carbohydrate chains crosslinked by peptide bridges. However, in contrast to the peptidoglycan of other well-characterized bacteria, mycobacterial peptidoglycan is covalently linked to the highly branched hetero-polysaccharide arabinogalactan, which is, itself, covalently bound to extremely long-chained fatty acids called mycolic acids. Electron microscopy has revealed that the outer cell envelope is a lipid bilayer, and, as such, it is referred to as the outer membrane, or, alternatively, the mycomembrane (1, 2). In addition to this core structure, several lipids are abundantly and non-covalently interspersed across the plasma membrane and outer membrane (3, 4). This complex cell envelope is a double-edged sword: it is a formidable barrier to many antibiotics, yet provides a number of potentially targetable structures. Indeed, two of the four first-line TB antibiotics target cell-envelope biosynthesis.

In addition to their unusual cell envelope, mycobacteria differ from other well-studied rod-shaped bacteria in important ways. Notably, mycobacteria elongate from their poles rather than along their side walls. The details of polar growth are poorly understood (5-7). Many key proteins important for cell elongation in other species appear to be missing in mycobacteria (6, 8). Likewise, it is almost completely unknown how proteins involved in synthesizing the different layers, and free lipids, work together to build this complex cell envelope.

Given the unusual physiology of mycobacteria, perhaps it is unsurprising that greater than 25% of the genes encoded by the mycobacterial genome are annotated as having unknown function (9). In our previous studies on the mycobacterial division complex, ‘the divisome’, we precipitated known divisome and elongasome components from *M. smegmatis* (Msm), and identified multiple candidate mycobacterial-specific divisome proteins in the precipitate, (8, 10). One of these was MSMEG_0317, a protein of unknown function that is highly conserved in actinobacteria. The Mtb homolog of *msmeg_0317*, *rv0227c*, is predicted to be essential (11, 12). Here, we sought to understand the role of MSMEG_0317 during mycobacterial growth and division.

MSMEG_0317 belongs to the DUF3068-domain super-family of proteins, which are exclusively found in actinobacteria. One member of this family in corynebacteria has channel activity, so this domain has been renamed PorA (13, 14). In addition, by structure-based homology prediction, MSMEG_0317 shares limited homology to mycobacterial lipoproteins, LppX and LrpG, which transport lipids, phthiocerol dimycocerosates (PDIMs) and triacylglycerol (TAG), respectively, to the outer mycobacterial membrane (15, 16).

We find that MSMEG_0317 localizes to sites of new cell wall synthesis and is indeed essential for cell viability in Msm. Transcriptional depletion leads to loss of cellular elongation, reductive division, and eventually cell lysis. Surprisingly, we find that lipomannan (LM), and lipoarabinomannan (LAM) – two large lipoglycans abundant in the mycobacterial cell envelope - accumulate as MSMEG_0317 is depleted and are shed into the culture media more abundantly upon MSMEG_0317 overexpression. By cryo-electron microscopy, we find that the cell envelopes of depleted cells are devoid of outer membrane and are highly disrupted. Together, these results show that MSMEG_0317

plays an important role in LM/LAM transport to the outer membrane, polar elongation, and the structural organization of the mycobacterial cell wall. As such, we propose to rename *msmeg_0317*, *cwdA*, for cell wall disruption, mutant A.

RESULTS

***cwdA* is essential for polar growth**

To determine the essentiality of *cwdA*, we used an allele swapping strategy (17). Briefly, in a strain of Msm whose only copy of *cwdA* was at the L5 phage integration site (18), we exchanged *cwdA* for either an empty vector or for another copy of itself (Fig. 1A). Consistent with *cwdA* being essential for cell growth, we observed approximately 1000-fold fewer colonies when we exchanged *cwdA* with an empty vector compared to exchanging it for another copy of itself (Fig. 1A).

To further confirm the essentiality of *cwdA*, we constructed a strain in which the only copy of the gene was tetracycline inducible (Fig. 1B). Removal of anhydrotetracycline (ATC) from the culture media prevented cell growth (Fig. 1B). By time-lapse microscopy we observed that, while cells expressing CwdA became longer, on average, as cell density increased within the microfluidic device (Fig. 2A,C & Movie S1), cells depleted for CwdA stopped elongating but continued to divide (reductive division), became wider, and, in many instances, eventually lysed (Fig. 2B,C & Movie S2). Together these data show that *cwdA* is essential for polar elongation in Msm.

CwdA localizes to the sites of cell wall synthesis

As our data suggested CwdA is important for polar growth, we hypothesized that it would localize to the poles, which are the sites of new cell wall synthesis in mycobacteria. To test this hypothesis, we made a translational fusion to CwdA with mRFP expressed by its native promoter. By allele swapping, we find that *cwdA-mrfp* restores bacterial growth (Fig. S1) and thus encodes a functional *cwdA*. Fluorescence microscopy at a single time-point showed that CwdA-mRFP localizes to mid-cell and to the poles (Fig. 3A). In polar growing bacteria, the site of division eventually becomes the site of elongation. Thus, in addition to localizing to the poles, elongation-complex proteins will also localize to the site of division early during the next cell cycle, making it difficult to distinguish true septal localization from static images. To disentangle whether CwdA also localizes to the septum, we visualized CwdA-mRFP by time-lapse microscopy. By constructing kymographs averaged over many cells, we compared the fluorescence probability distribution over the course of the cell cycle to the earliest known markers for the division complex (FtsZ-mCherry2B) and the elongation complex (eGFP-Wag31). CwdA-mRFP localizes to the site of division after FtsZ, but before Wag31 (Fig. 3B). These data are consistent with CwdA being a member of both the mycobacterial division and elongation complexes, but its essential function is as part of the elongation complex.

CwdA is involved in LM/LAM transport

The corynebacterial homolog of *cwdA* was recently implicated in LM/LAM biosynthesis (19), as a deletion mutant of the *Corynebacterium glutamicum* homolog resulted in disappearance of LAM and accumulation of truncated LM, which was clearly detectable by faster migration on SDS-PAGE. To test if this is also the case in

mycobacteria, we used the same electrophoretic approach to analyze LM/LAM, and thin layer chromatography (TLC) to examine potential changes in other lipid species upon depletion of CwdA, before and after the onset of cell death (Fig. S2B). In contrast to the results obtained in *C. glutamicum*, we observe a dramatic and transient increase in the total amount of cell-associated LM/LAM during depletion (Figs. 4A & S2A), up until the onset of cell death. There were no obvious changes in the migration of LM/LAM on SDS-PAGE, implying that the impact of CwdA depletion on LM/LAM biosynthesis was minimal. Additionally, other lipids, including the precursor of LM/LAM biosynthesis such as Ac₁PIM₂, showed no reproducible change (Figs. 4B,C & Fig. S2A).

These data are not consistent with the proposed biosynthetic function of the corynebacterial CwdA homolog. Instead, the transient increase of LM/LAM prior to cell death suggests a potential role for CwdA in cell envelope trafficking of these lipoglycans. LM/LAM are thought to be embedded in the plasma membrane, and also transported to the outer membrane (20, 21). Furthermore, LM/LAM in the outer membrane are likely to be shed into the extracellular milieu (22-24). Interestingly, sequence-based homology modeling indicated that CwdA shares some homology to LppX and LprG, proteins known to transport lipids from the inner membrane to the outer membrane in mycobacteria. Therefore, we hypothesized that depletion of CwdA results in the transient increase of cell-associated LM/LAM due to the disruption of LM/LAM trafficking. Based on this hypothesis, we reasoned that over-expression of CwdA may facilitate more trafficking, which could deplete cell-associated LM/LAM and increase shedding. To test this, we constructed strains which inducibly expressed CwdA from a multi-copy plasmid. Consistent with our hypothesis, upon induction with ATC we observed less LM/LAM

associated with Msm cells (Figs. S3A & S4) and more LAM secreted in the culture supernatant (Fig. S3B). These data suggest a model whereby CwdA is involved, directly or indirectly, in the transport of LM/LAM to the outer membrane of mycobacteria.

CwdA is important for cell envelope integrity

Our model predicts that CwdA-depleted cells should have greatly altered cell envelopes. Thus, we visualized the cell envelope of wild type and mutant cells by high resolution cryo-electron microscopy (cryo-EM). As has been previously observed (1, 2), wild type Msm cells exhibit a distinct plasma membrane and outer membrane that is approximately 50-nm thick (Fig. 5A-C). Consistent with our model, we find that cells depleted for CwdA have largely unstructured cell walls, and appear devoid of electron density in the outer membrane (Fig. 5D-F). We also observe dramatic fraying of the outer membrane, and large invaginations of plasma membrane (Fig. 6). Thus, CwdA is important for maintaining the structural organization of the mycobacterial cell envelope.

Collectively, our data show that CwdA is an essential polar growth factor involved in transporting, either directly or indirectly, LM/LAM to the outer membrane. Further, our data suggest that the correct localization of LM/LAM is important for the structural organization and integrity of the mycobacterial cell envelope.

DISCUSSION

The extraordinary diversity of the bacterial kingdom has led to a plethora of bacterial cell shapes and geometries of cell growth and division. Unlike well-studied model bacteria, several organisms, including the major human pathogen *Mycobacterium*

tuberculosis, grow from their poles rather than their side-walls. Our understanding of polar growth is at a nascent stage compared to our knowledge of side-wall growth. Mycobacteria, and related organisms, have an additional challenge of orchestrating the biosynthesis and transport of several additional covalently and non-covalently attached lipids and glycans to their thick layer of peptidoglycan. How these organisms coordinate the biogenesis of this complex structure to elongate and divide is almost completely unknown.

Here, we have studied a highly conserved mycobacterial protein, MSMEG_0317, in Msm and propose to rename it CwdA. As predicted (19), we find that the gene which encodes this protein is essential in Msm. The Mtb homolog, *rv0227c*, is almost certainly essential as well, as no transposon insertions have been found in this gene, even in data sets which combine insertion counts from high density mutagenesis across multiple independent experiments (11).

Our data suggest that CwdA's essential function occurs during polar elongation, as cells depleted for CwdA stop elongating, and CwdA-mRFP localizes to the poles. Interestingly, CwdA also localizes to the site of division, yet depleted cells continue to divide. This is consistent with recent work from several groups, which suggests that the essentiality of certain cell envelope structures is different for elongation and division. For example, *C. glutamicum* cells treated with ethambutol, which inhibits the polymerization of arabinan, a core structure in both arabinogalactan and LAM, stop elongating, but continue to divide (25, 26).

What is the function of CwdA? Our work suggests that CwdA is involved in the transport of LM/LAM to the outer membrane, either directly or indirectly, and is important

for maintaining the structural integrity of the mycobacterial cell envelope. Upon depletion, the levels of several other lipids remain constant or show no reproducible change, suggesting that CwdA may be acting on LM/LAM directly, though our lipid profiling was not exhaustive and we cannot rule out the possibility that another structural lipid is affected by CwdA depletion. However, supporting the notion that CwdA specifically acts upon LM/LAM, we observe a temporal correlation, up until the time of cell lysis, between the accumulation of LM/LAM and the disruption of the envelope. There has been a good deal of speculation as to the localization of LM/LAM in the mycobacterial envelope, but several groups have now shown that at least a fraction of LM/LAM resides in the outer membrane (23, 24). Our data are consistent with a model in which LM/LAM are structural components of the outer membrane. However, mutants of Msm that produce diminished amounts or truncated versions of LM/LAM are viable, suggesting that the presence of LM/LAM in the outer membrane may not be essential for viability (27, 28). Why, then, is *cwdA* essential? We speculate that the accumulation of these large lipoglycans in the periplasm of CwdA-depleted cells causes envelope disruption and cell rupture.

One implication from our work is that the transport or correct localization of lipids to the outer membrane is needed for polar elongation, but not division, suggesting that there could be various feedback loops to ensure complete construction of the elaborate mycobacterial cell envelope for insertion of new material to elongate. These checkpoints may be missing during division. This notion is supported by other mutants defective in transporting important outer membrane components like trehalose monomycolate, which, like the *cwdA* mutant, undergo reductive cell division (29).

The mycobacterial cell envelope remains an enduring target for the development of new TB therapeutics. The standard TB therapy uses a combination of four drugs; two of these drugs target cell wall synthesis. In addition to the core, and covalently linked structure of peptidoglycan, arabinogalactan, and mycolic acids, the mycobacterial cell envelope is also rich with many other lipids. The functional role of these lipids in bacterial cell physiology is relatively unexplored but they are likely important, if not essential, components of the cell envelope. Our data argue that a more complete understanding of the transport of these molecules could lead to new targets for the development of anti-TB therapeutics.

Materials and Methods

Bacterial strains and culture conditions: Msm mc²155 was grown in Middlebrook 7H9 broth supplemented with 0.05% Tween80, 0.2% glycerol, 5 gm/L albumin, 2 gm/L dextrose and 0.003 gm/L catalase or plated on LB agar. *Escherichia coli* DH5 α cells were grown in LB broth or on LB agar plates. Concentrations of antibiotics used for Msm is as follows: 20 μ g/ml zeocin, 25 μ g/ml kanamycin, 50 μ g/ml hygromycin, and 20 μ g/ml nourseothricin. Concentrations of antibiotics used for *E. coli* used for *E. coli* is as follows: 40 μ g/ml zeocin, 50 μ g/ml kanamycin, 100 μ g/ml hygromycin, and 40 μ g/ml nourseothricin.

Plasmids and strains construction: Before deleting the native copy of *msmeg_0317*, a merodiploid strain was created by inserting a second copy of *msmeg_0317* gene under pTetO promoter using a Kan^R L5 integrating vector. Subsequently, in the merodiploid strain, an in-frame deletion was made by replacing the

native copy of *msmeg_0317* gene with a zeocin resistance cassette flanked by loxP sites using recombineering (18). The plasmid constructs were made using isothermal Gibson assembly whereby insert and vector backbone shared 20-25 bp of homology. *msmeg_0317* and its fluorescent variant were cloned in a Nat^R L5 integrating vector for allele exchange at the L5 site. The *msmeg_0317* depletion strain was made by transforming an episomal Hyg^R marked vector constitutively expressing TetR repressor into a strain expressing a single copy of *msmeg_0317* controlled by the pTetO promoter. mRFP was fused to the c-terminal of *msmeg_0317* gene cloned with 200 bp upstream sequence to capture the native promoter. *ftsZ-mCherry2B* and *eGFP-wag31* genes were cloned under medium strength promoter ptb21 and integrated at tweety (tw) and L5 phage integration sites, respectively.

MSMEG_0317 Depletion: To transcriptionally deplete *msmeg_0317* in Msm, the ATC-inducible Tet-ON system was used. The only copy of *msmeg_0317* was driven by the pTetO promoter, while the TetR repressor was constitutively expressed in trans from an episomal vector. MSMEG_0317 was depleted by removing ATC from the medium and cells were grown for 18 hours. Subsequently, MSMEG_0317 depleted cells were re-diluted in fresh medium, also without ATC, and samples at different timepoints were taken and processed for cryo-electron microscopy. Alternatively, to avoid changes in LM/LAM abundance that have been found to correlated with cell density and growth phase (21), at 18 hours of depletion, half of the culture was removed for lipid extraction and analysis, and replaced with fresh media. Lipid analysis was performed at timepoints before and after the decrease in OD indicating cessation of growth (Fig. S3).

Time-lapse microscopy: An inverted Nikon Ti-E microscope was used for the time-lapse imaging. An environmental chamber was maintained at 37°C. Cells were cultivated in an B04 microfluidics plate from CellAsic; continuously supplied with fresh medium; and imaged every 15 minutes using a 1.4 N.A. phase contrast objective.

Kymograph analysis. Time-lapse images were analyzed in open source image analysis software Fiji (30) and a custom MATLAB program was used to generate kymographs. Specifically, for a single cell, in Fiji, a 5-pixel wide segmented line was drawn from the new pole to the old pole at each time point during the cell cycle. This was repeated on 20-50 cells. These line profiles were then imported into MATLAB, where a custom script was used to generate an average kymograph by 2D interpolation of the individual kymographs.

Lipid extraction and analysis. Extraction, purification and analysis of lipids were as described previously (31). Briefly, crude lipids were extracted from equal wet cell pellet weights of the MSMEG_0317 depletion strains after 18, 24, 27, 30, and 33 hrs with or without ATC. After lipid extraction using chloroform/methanol, LM and LAM were extracted from the delipidated pellet by incubation with phenol/water (1:1) for 2 hrs at 55°C. Phospholipids and PIMs extracted by chloroform/methanol were further purified by *n*-butanol/water phase partitioning, and separated by high performance thin layer chromatography (HPTLC) (Silica gel 60, EMD Merck) using chloroform/methanol/ 13 M ammonia/1 M ammonium acetate/ water (180:140:9:9:23). Phospholipids were visualized via cupric acetate staining. PIMs were visualized with orcinol staining as described (32). LM/LAM samples were separated by SDS-PAGE (15% gel) and visualized using ProQ Emerald 488 glycan staining kit (Life Technologies). To detect LM/LAM in culture

supernatants, the supernatants were initially treated with a final concentration of 50 µg/ml Proteinase K for 4 hours at 50°C. The treated supernatants were electrophoresed on 15% SDS-PAGE. LM/LAM were blotted onto nitrocellulose at 20 V for 45 minutes using semi-dry transfer method. Post-transfer, the membrane was blocked by 5% milk in Tris-buffered saline supplemented with 0.05% Tween-20 (TBST). The blocked membrane was then probed overnight with CS-35 antibody (BEI Resources, NIH) at 1:250 dilution at 4°C. The membrane was washed with TBST five times for five minutes each. Post-washing, it was probed with anti-mouse secondary for one hour at room temperature. Membrane was then washed five times for five minutes with TBST. Thermo Scientific's west dura chemiluminescent reagent was used to develop the membrane.

Sample preparation and image collection for Cryo-EM: Wild type and MSMEG_0317 depleted Msm were pelleted, washed twice with 1X phosphate buffered saline (PBS), and suspended in a very small volume of PBS to get a very dense suspension of bacterial cells. The dense culture was subsequently mixed with 10-nm colloidal gold and deposited onto fresh glow-discharged holey carbon grids. The grids were then blotted with filter paper manually and rapidly frozen in liquid ethane. The frozen grids were transferred under -170 °C into a 300kV Titan Krios equipped with K2 Summit direct detector, a quantum energy filter (Gatan, Pleasanton, CA) and an FEI volta phase plate (FEI). The data was collected using SerialEM (33) and Digital Micrograph software. The motion correction for each movie was done with MotionCor2 (34). The microscope was operated at 300kV with a magnification of 3,600x and 26,000 x for low-mag map and high-mag map respectively. The grey levels of each micrograph are obtained using MATLAB.

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Fig. 1. *cwdA* is essential in *Msm*. (A) L5-integrating vectors carrying either *cwdA* or a gene encoding TetR were transformed into a strain whose only copy of *cwdA* was at the L5 integration site, and transformants were counted. Bars represent means of biological triplicates and are representative data of three independently performed experiments. (B) Optical density over time of a strain whose only copy of *cwdA* is tetracycline inducible, with and without ATC (+/-ATC). Points are means of three biological replicates. Error bars are too small to be visible. Experiment was performed at least 4 times on separate days, and representative data are shown. ATC=anhydrotetracycline. Time on the x-axis corresponds to time after ATC was removed.

Fig. 2. CwdA-depletion causes reductive cell division and eventual cell lysis. (A,B) The strain shown in Fig. 1B was visualized by time-lapse microscopy in the presence (A) or absence (B) of ATC. Cells were loaded into a microfluidic device 18 hours after the removal of ATC (B) or a mock control (A). Representative frames are 3 hours apart. (C) Birth lengths and cell widths over time of depletion (orange = +ATC; red = -ATC). Open circles represent individual cells (length: N = 261 (length) & 253 (width) for +ATC; N = 355 (length) & 254 (width) -ATC). Closed circles are not fits to the data but represent the mean of cells in 2.25-hr (length) or 2.75-hr (width) bins. The experiment was performed twice on separate days. In +ATC cultures, cell density became too high to measure length and width past ~33 hrs. ATC=anhydrotetracycline

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Fig. 3. CwdA localizes to the sites of division and elongation. (A) Merged phase-contrast and fluorescence images of *Msm* cells expressing CwdA-mRFP, eGFP-Wag31 and FtsZ-mCherry2b. (B) Average fluorescent distributions over time (kymographs) from cells aligned from new to old poles and from birth to division (N=20 CwdA-mRFP; N=48 FtsZ-mCherry2B; N=20 eGFP-Wag31).

Fig. 4. LM/LAM accumulate as CwdA is depleted. In a strain whose only copy of *cwdA* is ATC-inducible, (A) LM/LAM, (B) non-polar and polar PIMs, (C) and plasma membrane lipids were analyzed by (A) SDS-PAGE and (B-C) TLC during CwdA depletion (without ATC). Cell pellets were normalized by wet weight to account for differences in cell density. Two other biological replicates are shown in Fig. S2. CL, cardiolipin; PE, phosphatidylethanolamine; PI, phosphatidylinositol.

Fig. 5. Depletion of CwdA leads to loss of outer membrane. The cell envelope of a representative wild type Msm cell (A-C) and a cell depleted for CwdA for 24 hours were (D-F) visualized (A,B,D,E), and measured (C,F) by cryo-electron microscopy. PM = plasma membrane; OM = outer membrane. Scale bar = 100 nm.

Fig. 6. Cryo-electron microscopy of CwdA-depleted cells (A-H) The cell envelopes of CwdA-depleted cells show plasma membrane invaginations (A&E; C&G; D&H) and outer membrane wall fraying (B-D; F-H) (arrows). (A-C; E-G) 24 hours; (D&H) 33 hours of depletion. Scale bar = 100 nm.

Supplemental Information

Figure S1: CwdA-mRFP encodes a functional copy of CwdA

Figure S2: Two additional biological replicates of LM/LAM analysis during CwdA depletion

Figure S3: Detection of cell-associated and shed LM/LAM during CwdA over-expression

Figure S4: An additional biological replicate of cell-associated LM/LAM and other lipids during CwdA over expression

Movie S1. Cells whose only copy of *cwdA* is ATC-inducible are imaged over time by phase-contrast microscopy in the presence of ATC. Cells were treated in parallel to those shown in Movie S2, except ATC was included in the culture media at all times.

Movies S2. Cells whose only copy of *cwdA* is ATC-inducible, are imaged over time by phase-contrast microscopy in the absence of ATC. Cells were loaded into the device 18 hours after the removal of ATC.

Table S1: A list of strains used in this study.

Strain	Usage
<i>Mycobacterium smegmatis</i> mc ² 155	Wild type strain, lab stock
mc2 155 L5::pTetO- <i>cwdA</i> -kan ^R	Merodiploid strain used for deleting native copy of <i>cwdA</i>
mc2 155 Δ <i>cwdA</i> L5::pTetO- <i>cwdA</i> -kan ^R	Parental strain used for allele exchange of <i>cwdA</i> at L5 site
mc2 155 Δ <i>cwdA</i> L5::pTetO- <i>cwdA</i> /pTetR	ATC inducible <i>cwdA</i> strain where <i>cwdA</i> is under TetO promoter and the strain carries constitutive TetR repressor; used for transcriptional depletion of <i>cwdA</i>
mc2 155 Δ <i>cwdA</i> L5::pTetO-MS0317-myc/pTetR	ATC inducible C-terminal myc-tagged <i>cwdA</i> strain where <i>cwdA</i> is under TetO promoter and the strain carries

	constitutive TetR repressor; used for transcriptional depletion of <i>cwdA</i>
mc2 155 /pTetOR- <i>cwdA</i> -myc	ATC inducible C-terminal myc-tagged <i>cwdA</i> strain; used for over-expression of CwdA protein
mc2 155 Δ <i>cwdA</i> pL5:pNative- <i>cwdA</i> -mRFP-myc	Strain carrying C-terminal mRFP tagged CwdA protein expressed from native promoter to track the localization of CwdA protein
mc2155 L5::pG-MCK-ptb21-egfp-wag31 tweety::ptb21-ftsZ-mCherry2B	Strain used to track the localization of Wag31 and FtsZ proteins (Rego et al 2017, <i>Nature</i>)

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513 Table S2: A list of primers used in this study

MS_0317_FP	GCTTAATTAAGAAGGAGATATACATAT GAACCGCGCTGTGGCGCTGCGTATCG	Forward primer to amplify <i>cwdA</i> from Msm genome
MS_0317_RP	GGTCCCCAATTAATTAGCTAAAGCTTTC AGATCGGTTCGGTCCGGTGGCAGATCG	Reverse primer to amplify <i>cwdA</i> from Msm genome
MS_0317_1	GGAGCGCAGGTAGTGCCCGGTTCTCG	Forward primer to amplify the upstream region for <i>cwdA</i> knock-out construct
MS_0317_2	GGCGTTACCCAACTTAATCGCCTTGCA GCTCACCCCAGAAGTCCACATGCCGCG	Reverse primer to amplify the upstream region for <i>cwdA</i> knock-out construct
MS_0317_3	CGCGGCATGTGGACTTCTGGGGTGAGC TGCAAGGCGATTAAGTTGGGTAACGCC	forward primer to amplify <i>zeo</i> ^R cassette for

		<i>cwdA</i> knock-out construct
MS_0317_4	GCACCTGGATGCCCTGCGTATCGAA GAAACAGCTATGACCATGATTACGCC	reverse primer to amplify <i>zeo</i> ^R cassette for <i>cwdA</i> knock-out construct
MS_0317_5	GGCGTAATCATGGTCATAGCTGTTTC TTCGATACGCAGGGCATCCAGGTGC	Forward primer to amplify the downstream region for <i>cwdA</i> knock-out construct
MS_0317_6	AGACTCCAGTGGCCCTGCAACAACCGC	Reverse primer to amplify the downstream region for <i>cwdA</i> knock-out construct
MS0317_Nat_Fwd	CGATCCGCATGCTTAATTAAG AAGGAGATATACTAATGAACCGCG CTGTGGCGCTGCGT	Forward primer for sub-cloning <i>cwdA</i> into L5 integrating pTetO-Nat ^R vector
MS0317_Nat_Rev	CCTCTAGGGTCCCCAATTAATT AGCTAAAGCTTTCAGATCGGTC GGTCCGGTGGCAGATCG	Reverse primer for sub-cloning <i>cwdA</i> into L5 integrating pTetO-Nat ^R vector
MS0317_KO_Fwd	GGATCACGATCACCGCCACCAC GTAGC	Forward sequencing primer to confirm deletion of <i>cwdA</i> gene
MS0317_KO_Rev	CCAGAACACCAGCGGAGCCACC GGAGTCC	Reverse sequencing primer to confirm

		deletion of <i>cwdA</i> gene
MS0317-Nat_myc-Rev	TCCCCAATTAATTAGCTAAAGCTT TCACAGGTCTTCCTCGCTGATCAGC TTCTGCTCGATCGGTCCGGTCCGGTGGC	Reverse primer for attaching myc tag at C-terminus of <i>cwdA</i>
pNative317-RFP_1	CGATCCGCATGCTTAATTAAGAAGGAG ATATACTAATGAACCGCGCTGTGGCGCTGCGT	Gibson primers used for making <i>pNative_cwdA_mrfp</i> construct
pNative317-RFP_2	CCTTGATGACGTCCTCGGAGGAGGC CGAGCCGCCGATCGGTCCGGTCCGGT GGCAGATCGG	Gibson primers used for making <i>pNative_cwdA_mrfp</i> construct
pNative317-RFP_3	CCGATCTGCCACCGGACCGACCGAT CGGCGGCTCGGCCTCCTCCGAGGACGTCATCAAGG	Gibson primers used for making <i>pNative_cwdA_mrfp</i> construct
pNative317-RFP_4	GGTCCCCAATTAATTAGCTAAAGCTT TCACAGGTCTTCCTCGCTGATC AGCTTCTGCTCGGCCGCGG TGGAGTGGCGGCCCTCGGCGC	Gibson primers used for making <i>pNative_ms_0317_mrfp</i> construct

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Supplemental Figures

Fig. S1. CwdA-mRFP encodes a functional copy. L5-allele swapping with *cwdA* or *cwdA-mRFP* expressed by the native promoter. Bars represent means of 3 biological replicates. Error bars indicate standard deviation.

Fig. S2. Two additional biological replicates of LM/LAM analysis during CwdA depletion. (A) As in Main Text Figure 4, LM/LAM and other lipids were extracted and separated by SDS-PAGE and TLC in cells expressing (+ATC) and depleted for (-ATC) CwdA. Two biological replicates are shown. (B) The optical density of the cultures was measured as CwdA was depleted. To avoid changes in LM/LAM due to cell density, at the indicated timepoints, half of the culture was taken for lipid analysis, and replaced with fresh media. Filled circles indicate means; error bars indicate standard deviation.

Fig. S3. Detection of cell-associated and shed LM/LAM during *CwdA* over-expression. In cells carrying an ATC-inducible copy of *cwdA* on a multicopy plasmid, (A) cell-associated LM/LAM were extracted, separated via SDS-PAGE, and visualized by ProQ Emerald with and without ATC. (B) The culture supernatants of those cells were also probed for LAM by western blot using an anti-LAM antibody (CS-35, BEI resources) specific to the arabinan moiety of LAM. The high molecular weight species marked by * likely corresponds to the recently described LAM-like polysaccharide (35).

Fig. S4. An additional biological replicate of cell-associated LM/LAM and other lipids during CwdA over expression. As in Fig S3, cell-associated were separated and visualized by SDS-PAGE. Other lipids were also separated and visualized by TLC.