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EccA1, a Component of the *Mycobacterium marinum* ESX-1 Protein Virulence Factor Secretion Pathway, Regulates Mycolic Acid Lipid Synthesis

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SUMMARY

Pathogenic mycobacteria, which cause multiple diseases including tuberculosis, secrete factors essential for disease via the ESX-1 protein export system and are partially protected from host defenses by their lipid-rich cell envelopes. These pathogenic features of mycobacterial biology are believed to act independently of each other. Key ESX-1 components include three ATPases, and EccA1 (*Mycobacterium marinum* MMAR_5443; *M. tuberculosis* Rv3868) is the least characterized. Here we show that *M. marinum* EccA1's ATPase activity is required for ESX-1-mediated protein secretion, and surprisingly for the optimal synthesis of mycolic acids, integral cell-envelope lipids. Increased mycolic acid synthesis defects, observed when an EccA1-ATPase mutant is expressed in an *eccA1*-null strain, correlate with decreased in vivo virulence and intracellular growth. These data suggest that two mycobacterial virulence hallmarks, ESX-1-dependent protein secretion and mycolic acid synthesis, are critically linked via EccA1.

INTRODUCTION

Mycobacterium tuberculosis has infected one-third of all people and kills two million people every year (Koul et al., 2011; Lönnroth et al., 2010). The disease burden is worsened because mycobacteria are becoming increasingly resistant to antibiotics. Despite this, no new antimycobacterial antibiotic has been brought to clinical use for almost 50 years, and many mycobacterial pathogenic mechanisms, which might provide novel drug targets, are only beginning to be understood (Koul et al., 2011).

Mycobacteria are resistant to many of the bactericidal mechanisms of host immunity, at least in part because of their cell envelope composed primarily of mycolic acids (Niederweis et al., 2010). These mycolic acids are covalently attached to underlying arabinogalactan-peptidoglycan and likely form the inner leaflet of an asymmetric outer membrane, whose outer leaflet is composed of diverse lipids (Hoffmann et al., 2008; Minnikin et al., 2002; Niederweis et al., 2010). Many of these surface-exposed lipids, such as trehalose dimycolate (TDM), phthiocerol dimycocerosate (PDIM), and phenolic glycolipid (PGL), are implicated in mycobacterial virulence (Chopra and Gokhale, 2009; Minnikin et al., 2002). Moreover, even minor mycolate structural changes have major effects on mycobacterial virulence (Dubnau et al., 2000; Glickman et al., 2000; Yuan et al., 1998). Thus, cell-wall mycolates and their associated lipids are major factors in the ability of mycobacteria to cause disease in metazoan hosts.

Little is known about how mycobacteria move proteins and other macromolecules across this unusual cell envelope. An export system, ESX-1 (Abdallah et al., 2007; Bitter et al., 2009), is required for protein secretion critical to the virulence of pathogenic mycobacteria including *M. tuberculosis* and its close genetic relative, *M. marinum* (Gao et al., 2004), a natural pathogen of fish and frogs that causes a systemic tuberculosis-like infection in these hosts (Cosma et al., 2003). ESX-1 is composed largely of proteins encoded by a genetic locus that is altered or partially deleted in closely related nonpathogenic species such as the vaccine strain of *Mycobacterium bovis*, Bacillus Calmette-Guerin, and *Mycobacterium microti* (Abdallah et al., 2007; Lewis et al., 2003; Pym et al., 2002). Key components of the ESX-1 protein secretion system include three ATPases: EccCa1 (*M. marinum* MMAR_5445; *M. tuberculosis* Rv3870) (ESX core component [Ecc] Ca1) and EccCb1 (*M. marinum* MMAR_5446;

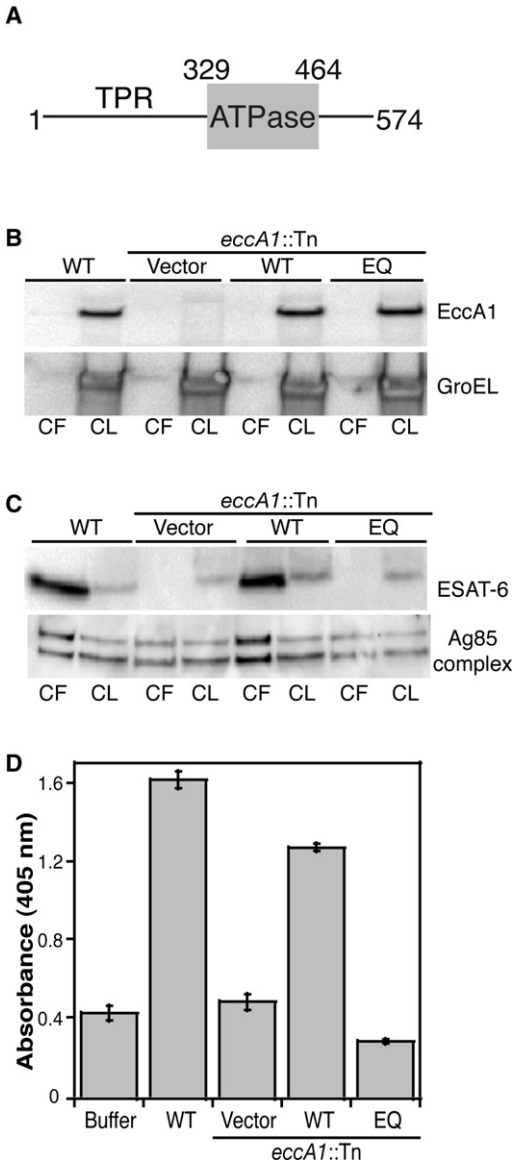

Figure 1. EccA1's ATPase Activity Is Needed for ESAT-6 Secretion and Hemolytic Activity

(A) EccA1 contains an N-terminal tetratricopeptide repeat (TPR) domain and a C-terminal ATPase domain.

(B) Wild-type and plasmid-encoded wild-type or EQ EccA1 are expressed at similar levels. CF, culture filtrate; CL, whole-cell lysate; GroEL, a loading control.

(C) EQ-EccA1 expression cannot restore ESAT-6 secretion. Ag85 complex is a loading control, and 8-fold more normalized CF than CL was analyzed in the Ag85 blot, whereas a 1:1 CF:CL ratio was analyzed in all other blots.

(D) Wild-type EccA1 complements *eccA1*::Tn's hemolytic defect, but EQ-EccA1 expression is similar to the buffer control. Averaged data are from three independent experiments and are mean values ± SEM.

See also Figure S1.

M. tuberculosis Rv3871), which are thought to function in transport of proteins across the cytosolic membrane (Abdallah et al., 2007; Bitter et al., 2009), and EccA1 (*M. marinum* MMAR_5443; *M. tuberculosis* Rv3868), a protein whose role in the ESX-1 system, although essential (Gao et al., 2004), is less well characterized. EccA1 contains two predicted domains, an N-terminal tetratricopeptide repeat domain and a C-terminal AAA ATPase domain (Figure 1A). *M. tuberculosis* EccA1 forms homohexamers like other AAA ATPase proteins and has measurable ATPase activity (Luthra et al., 2008). However, it is unclear whether EccA1's ATPase activity is important for its cellular function, which also is poorly defined. Here we show that an ATPase-defective mutant of EccA1 unexpectedly binds several enzymes involved in cell-envelope biogenesis and impedes mycolic acid synthesis. Moreover, an *eccA1*-null *M. marinum* strain expressing this mutant is attenuated in vivo and more defective for intracellular growth in macrophages, even though the *eccA1*-null strain already is defective in ESX-1-mediated secretion. Thus, EccA1 is involved in two previously unlinked virulence-associated pathways.

RESULTS

EccA1's ATPase Activity Is Needed to Secrete ESAT-6 and Lyse Cells

To gain insight into EccA1's function and to determine whether EccA1's ATPase activity is needed, we constructed an *M. marinum* EccA1 point mutant, E398Q (EQ), that lacks the catalytic glutamic acid in the conserved nucleotide-binding site and is likely an ATP-locked form of EccA1 because it cannot hydrolyze ATP (Hanson and Whiteheart, 2005; Zhang and Wigley, 2008) (see Figure S1 available online). Wild-type or mutant *eccA1* was expressed in a previously characterized *eccA1* transposon-disrupted mutant strain, *eccA1*::Tn (Gao et al., 2004). Immunoblotting with an anti-EccA1 antibody detected no EccA1 in *eccA1*::Tn cells, confirming the antibody specificity, whereas similar levels of wild-type or mutant EccA1 were detected in complemented strains (Figure 1B). EccA1 deficiency inhibited secretion of ESAT-6, an archetypal ESX-1 substrate (Abdallah et al., 2007; Gao et al., 2004), whereas expression of wild-type EccA1 restored secretion (Figure 1C). Reconstitution of *eccA1*::Tn with the EQ mutant did not, demonstrating that EccA1's ATPase activity is required to secrete ESAT-6. In addition, *eccA1*::Tn lacked the ability to lyse red blood cells, a property previously demonstrated to require ESX-1 (Gao et al., 2004). This defect was complemented by wild-type EccA1, but not by EQ EccA1 (Figure 1D). Thus, two assays of ESX-1 function demonstrate a requirement for EccA1 ATPase activity.

EQ EccA1 Interacts with Lipid Synthases

Studies using similar ATPase-defective mutants have identified both functional roles where nucleotide hydrolysis is needed as well as partner proteins preferentially binding to the ATP-bound form of the protein of interest (Hanson and Whiteheart, 2005; Zhang and Wigley, 2008). We therefore tested whether EQ EccA1 interacted more strongly with some partner proteins in order to gain insight into EccA1's cellular role. SDS-PAGE analyses of cytosolic complexes containing wild-type or EQ EccA1 showed that many more proteins larger than 100 kDa copurified with EQ EccA1 than did with wild-type EccA1 (Figure 2A). These coprecipitated proteins were identified by mass spectrometry (Tables S1 and S2). Whereas the numbers of

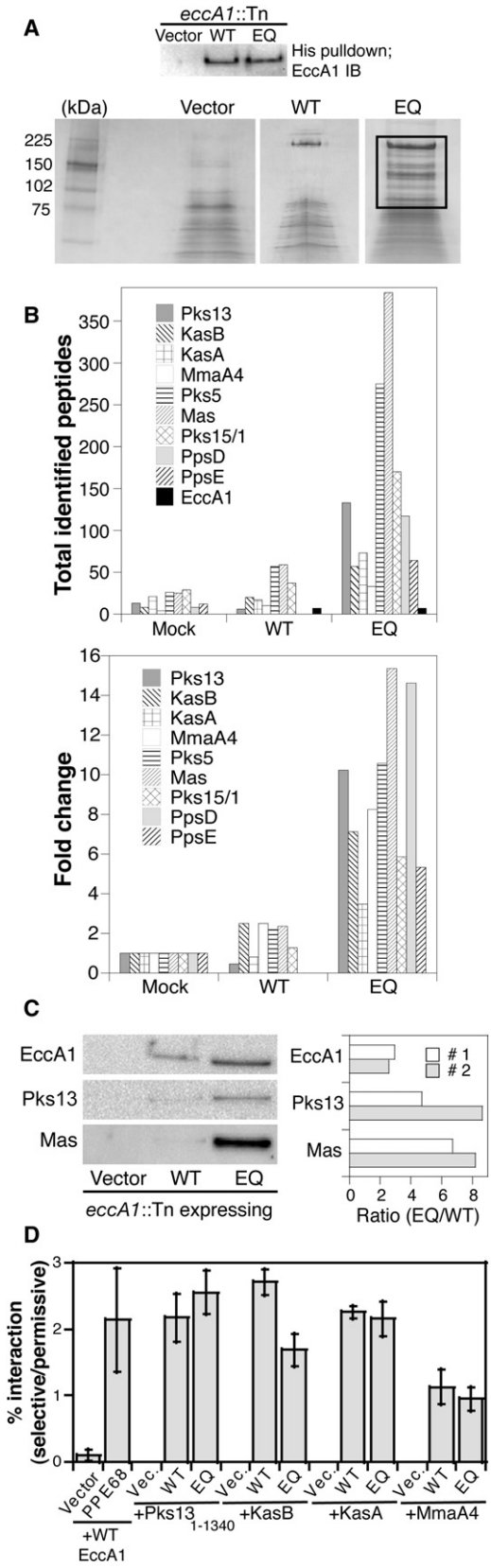

Figure 2. EQ EccA1 Interacts More Stably with Lipid Synthases

(A) Cytosolic complexes containing EccA1 were isolated and visualized by SDS-PAGE and silver staining. EQ EccA1 coprecipitates more proteins larger than 100 kDa compared to wild-type.

(B) Mass spectrometric analyses of coprecipitating proteins identified multiple polyketide synthases as interacting better with EQ EccA1.

(C) Immunoblotting of coprecipitation reactions with specific antibodies shows more Pks13 and Mas interacting with EQ EccA1 (left). Quantitated values from two independent experiments are shown (right).

(D) All EccA1 variants interact with $Pks13_{1–1340}$, KasB, and KasA as strongly as a control in a two-hybrid experiment, and less well with MmaA4. Percent interaction is calculated from the ratio of colonies on selective plates to those on nonselective plates. Averaged data are from two or more independent experiments, and mean values ± SEM are shown.

See also Figure S2 and Tables S1 and S2.

EccA1-derived peptides were similar in bacterial samples expressing either wild-type or EQ EccA1, EQ-containing complexes were enriched for multiple proteins involved in mycobacterial lipid synthesis (Bhatt et al., 2007; Chopra and Gokhale, 2009; Minnikin et al., 2002) including Pks13, KasB, KasA, MmaA4, Pks5, Mas, Pks15/1, PpsD, and PpsE (Figure 2B), as detected either by total peptides isolated (top) or when normalized to peptides found in the *eccA1*::Tn sample (bottom). Pks13, KasB, KasA, and MmaA4 are important enzymes in the fatty acid synthase II (FAS-II) system that makes mycolic acid and mycolate-containing lipids such as TDM (Bhatt et al., 2007; Dubnau et al., 2000; Takayama et al., 2005; Veyron-Churlet et al., 2005), whereas four of the other enzymes are polyketide synthases that produce PDIM and/or PGL, two other surface-exposed lipids (Chopra and Gokhale, 2009; Minnikin et al., 2002). We did not detect evidence of interaction under these conditions with a previously reported binding partner, EspC (DiGiuseppe Champion et al., 2009). To verify enzyme association with EQ EccA1, antibodies were raised against an enzyme involved in mycolate synthesis, Pks13, and one involved in polyketide synthesis, Mas (Figure S2A). Whereas all strains produced similar amounts of Pks13 and Mas (Figure S2B), immunoblotting showed more coisolation with EQ EccA1 than with wild-type (Figure 2C, left). Normalized for the amount of isolated EccA1, quantities of coprecipitated Pks13 and Mas were approximately 2-fold greater with EQ than with wild-type (Figure 2C, right).

EccA1 Interacts with $Pks13_{1–1340}$, KasB, KasA, and MmaA4

To determine whether interaction between EccA1 and these enzymes involved in mycolic acid synthesis depends on other mycobacterial proteins, their interaction was assessed in an *Escherichia coli* bacterial two-hybrid system (McLaughlin et al., 2007). Wild-type and mutant EccA1 interact with $Pks13_{1–1340}$, KasB, and KasA in this assay (Figure 2D) to the same extent as wild-type EccA1 and PPE68 (*M. marinum* MMAR_5448; *M. tuberculosis* Rv3873), whose interaction has been documented previously (Teutschbein et al., 2009). Interaction with MmaA4 also occurs, but to a lesser extent (Figure 2D). All mycobacterial proteins were expressed to a similar extent in *E. coli* (Figure S2C). There was no difference in interactions with wild-type or EQ EccA1 in *E. coli*, suggesting that additional mycobacterial proteins regulate these interactions in

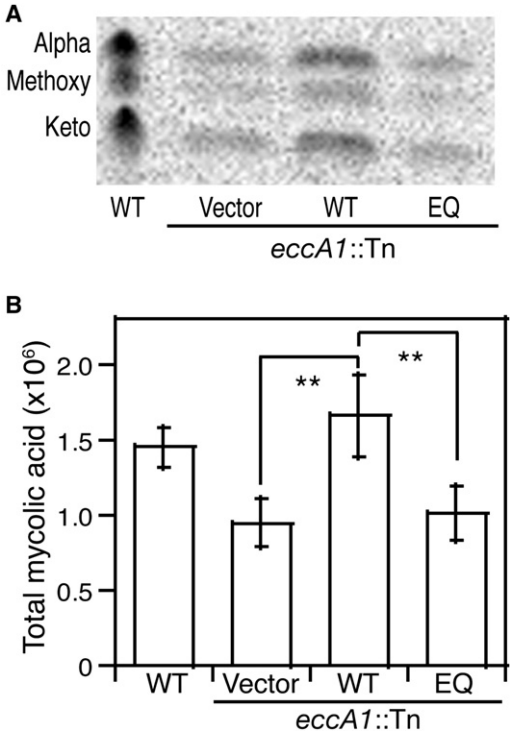

Figure 3. EccA1 and the FAS-II Mycolic Acid Synthesis System Interact Functionally

(A) Radiolabeled and isolated mycolic acid methyl esters were resolved.

(B) Quantitation revealed mycolic acid synthesis defects in *eccA1*::Tn and (*eccA1*::Tn)/EQ. Averaged data are from three or more independent experiments, and mean values ± SEM are shown (**$p \leq 0.03$).

mycobacteria or that the known interactions among Pks13, KasB, KasA, and MmaA4 (Veyron-Churlet et al., 2005) regulate binding to EccA1.

EccA1 Enhances Mycolic Acid Synthesis

Pks13's main function is to condense together the two fatty acid chains that comprise mycolic acids, the longer meromycolate chain produced by FAS-II and the shorter α branch produced by the fatty acid synthase I (FAS-I) complex (Bhatt et al., 2007; Portevin et al., 2004; Takayama et al., 2005). KasB and KasA are integral components of FAS-II (Veyron-Churlet et al., 2004, 2005) and help make the meromycolate chain that Pks13 condenses with the α branch (Bhatt et al., 2007; Takayama et al., 2005), whereas MmaA4 is a methyltransferase that modifies the meromycolate chain (Dinadayala et al., 2003; Yuan and Barry, 1996) as it is elongated by FAS-II.

We therefore tested whether mycobacterial strains lacking functional EccA1 have defects in mycolic acid synthesis. Three distinct types of mycolic acids (alpha, methoxy, and keto) exist in *M. marinum* (Daffé et al., 1991), like in *M. tuberculosis* (Bhatt et al., 2007; Minnikin et al., 2002; Takayama et al., 2005). Regardless of EccA1 expression, all three classes of mycolic acids incorporated radiolabeled [1-^{14}C]-tetracosanoic acid (Figure 3A). However, *eccA1*::Tn produced 30%–40% less total mycolic acid than wild-type (Figure 3B). Wild-type EccA1 complemented this defect, but EQ EccA1 did not (Figure 3B), demonstrating that EccA1's ATPase activity is necessary for optimal mycolic acid production in *M. marinum*.

EQ EccA1 Sensitizes Cells to Mycolic Acid Stress

To determine the functional significance of the decreased mycolate synthesis, we examined the response of *eccA1*::Tn and (*eccA1*::Tn)/EQ to ethionamide, an antibiotic that inhibits mycolic acid synthesis, by monitoring their mycolic acid production in the presence of 0.5 μg/ml ethionamide (Figure 4A, left), an antibiotic concentration likely below the minimum inhibitory concentration (Heifets, 1988). Mycolic acid production was equivalently inhibited by ethionamide in wild-type and *eccA1*::Tn (Figure 4A, left). Interestingly, mycolate production was 2-fold more inhibited in the strain expressing EQ EccA1 than in wild-type, *eccA1*::Tn, or (*eccA1*::Tn)/WT (Figure 4A, left), suggesting that EQ-EccA1 expression further sensitized mycobacteria to stress affecting mycolic acid production, possibly because EQ EccA1 sequestered some of the enzymes involved in the mycolate synthesis pathway. Increased sensitivity to ethionamide was demonstrated as well by ethionamide's lower half-maximal growth-inhibitory concentration (IC_{50}) for the EQ-EccA1 expressing strain (Figure 4A, right). This suggests that stable EQ-EccA1 interaction with FAS-II enzymes amplifies the defect in mycolate synthesis induced by ethionamide, since expression of wild-type EccA1 did not increase sensitivity to the antibiotic. Moreover, sensitization by EQ EccA1 to ethionamide appeared specific because neither *eccA1*::Tn nor (*eccA1*::Tn)/EQ was more sensitive to two hydrophobic antibiotics, rifampin and erythromycin (Figure 4B). The increased resistance of *eccA1*::Tn and (*eccA1*::Tn)/EQ to rifampin and erythromycin compared to wild-type suggests that their decreased mycolic acid production did not result in a simple permeability defect, as has been reported for a *kasB*-null strain (Gao et al., 2003).

EQ EccA1 Decreases Virulence in Zebrafish and Intracellular Growth in Macrophages

Protein secretion through ESX-1 is important for *M. marinum* virulence (Gao et al., 2004), and both *eccA1*::Tn and (*eccA1*::Tn)/EQ do not secrete ESAT-6 (Figure 1C). Because (*eccA1*::Tn)/EQ made significantly less mycolic acid when mycolate synthesis was stressed (Figure 4A, left), we tested whether EQ EccA1 would distinctly affect *M. marinum* virulence in zebrafish, a situation likely to require maximal mycolic acid synthesis both for bacterial growth and to repair cell-envelope damage from host defenses (Bhatt et al., 2007; Lacave et al., 1989; Niederweis et al., 2010; Rachman et al., 2006; Takayama et al., 1972). To perform these experiments, wild-type or EQ *eccA1* was integrated into the chromosome at the *attB* site (Stover et al., 1991) to maintain stable expression during the relatively long time frame of the zebrafish experiments. Like plasmid-expressed EccA1 (Figure 1B), wild-type and mutant EccA1 were expressed at equivalent levels (data not shown). Zebrafish infected with wild-type *M. marinum* all died by 20 days postinfection (Figure 5A). *eccA1*::Tn showed only a small loss of virulence in this experiment, but a higher-infecting inoculum was used here compared to a previous study (Gao et al., 2004). Expression of wild-type EccA1 in *eccA1*::Tn restored the small loss of virulence, but ATPase-defective EccA1 markedly attenuated the *eccA1*::Tn strain. By the time (*eccA1*::Tn)/EQ-infected zebrafish

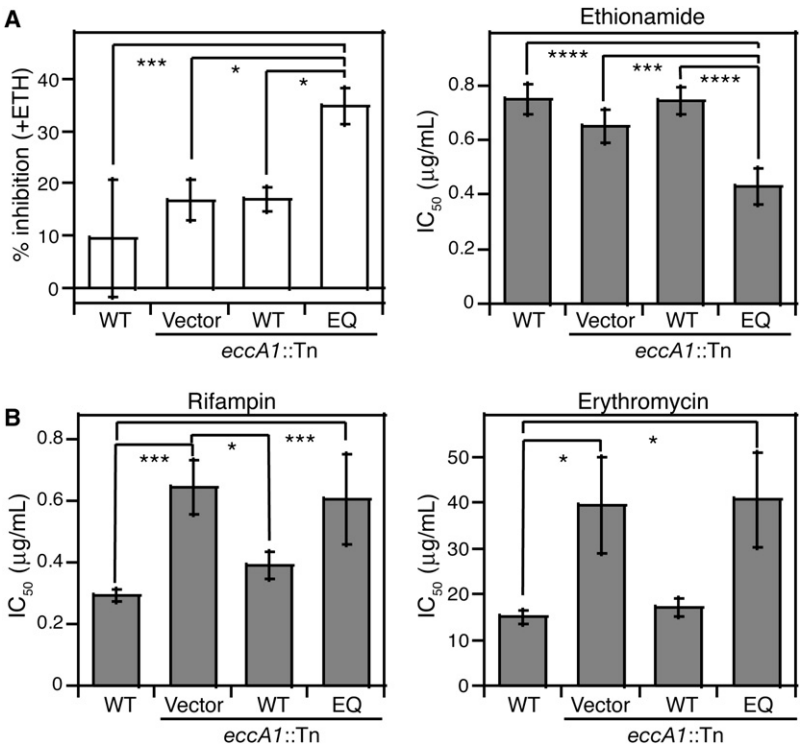

Figure 4. EQ-EccA1 Expression Also Sensitizes Cells to Ethionamide Stress

(A) Left: ethionamide inhibition of mycolic acid synthesis is plotted. (*eccA1*::Tn)/EQ is most inhibited. Averaged data are from at least four independent experiments, and mean values ± SEM are shown (*$p < 0.05$; ***$p \leq 0.01$). Right: IC_{50} values for growth-inhibition sensitivity to ethionamide are shown. (*eccA1*::Tn)/EQ is uniquely sensitized to ethionamide. Mean values ± SEM are averaged data from five independent experiments (***$p \leq 0.01$; ****$p \leq 0.002$).

(B) IC_{50} values for growth-inhibition sensitivity to two hydrophobic antibiotics, rifampin and erythromycin, are shown. (*eccA1*::Tn)/EQ is similar to *eccA1*::Tn in being more resistant to these antibiotics. Mean values ± SEM are averaged data from four or more independent experiments (*$p < 0.05$; ***$p \leq 0.01$).

began to die 24 days postinfection, all the zebrafish infected with wild-type, *eccA1*::Tn, or (*eccA1*::Tn)/WT had died (Figure 5A).

To explore this in vivo result, we determined the effects of EQ EccA1 on *M. marinum* growth. All strains grew similarly in 7H9 broth (Figure 5B). *eccA1*::Tn showed mildly decreased growth in primary bone marrow-derived macrophages, a defect fully complemented by expression of wild-type protein. In contrast, expression of EQ EccA1 markedly diminished growth of *eccA1*::Tn in macrophages (Figure 5C). Statistical analyses revealed significant differences between both *eccA1*::Tn and (*eccA1*::Tn)/EQ with wild-type or (*eccA1*::Tn)/WT after 48 hr. These data demonstrate that EQ EccA1 diminishes bacterial virulence in the absence of wild-type EccA1, a circumstance in which ESX-1-dependent secretion is already lost.

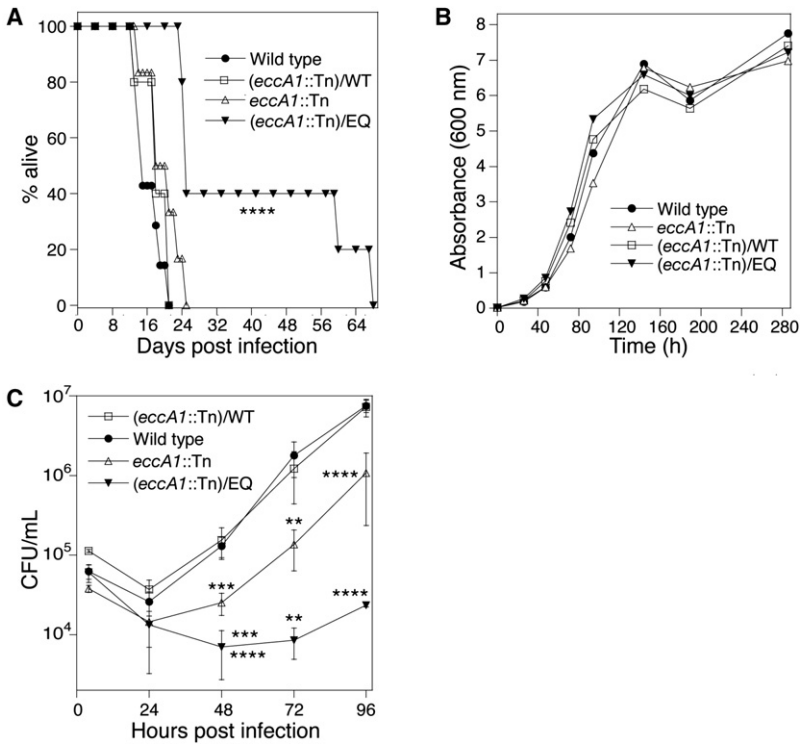

Figure 5. EQ EccA1 Further Decreases Virulence and Intracellular Growth

(A) An EQ-expressing integrant strain is attenuated for zebrafish killing (****$p \leq 0.002$ compared to wild-type, log-rank test; $n \geq 5$ for each strain).

(B) All strains grew similarly in 7H9 media.

(C) *eccA1*::Tn is defective for intracellular growth in bone marrow-derived macrophages. Wild-type EccA1 complements this defect, but EQ EccA1 worsens growth. Data are averaged from three independent experiments (**$p \leq 0.03$; ***$p \leq 0.01$; ****$p \leq 0.002$).

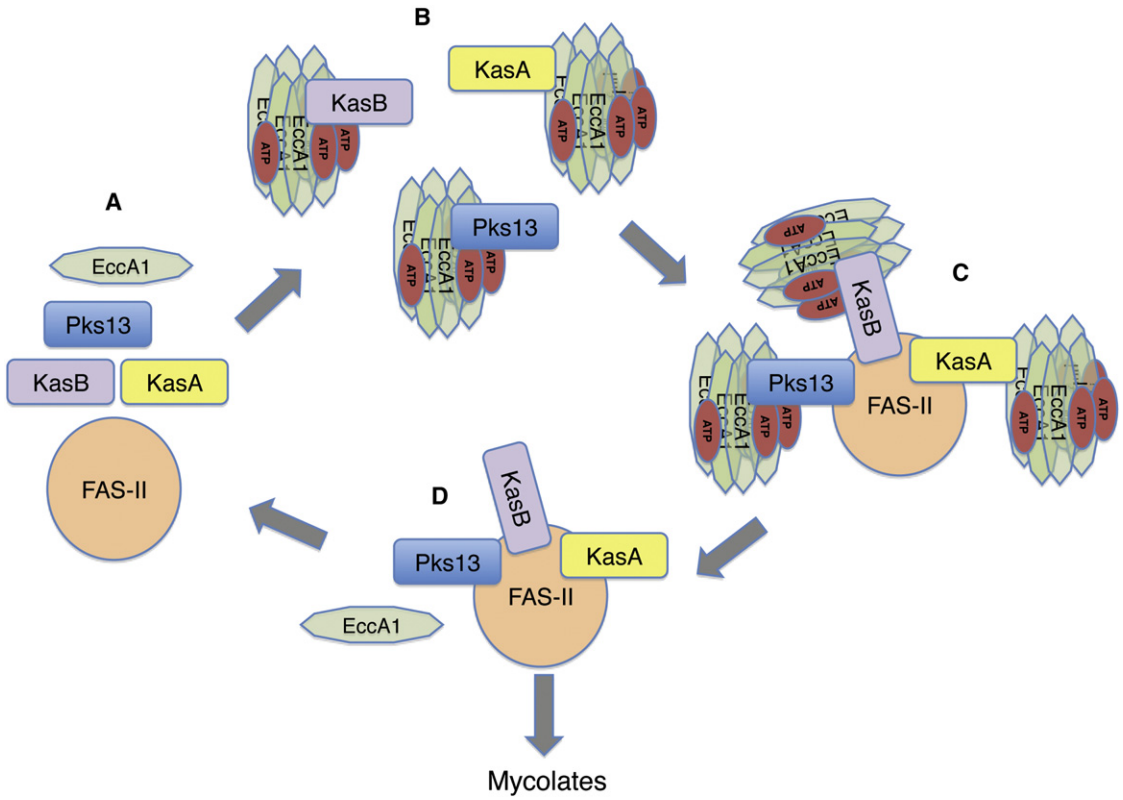

Figure 6. A Model of EccA1's Interaction with FAS-II Proteins

For efficient mycolic acid production, EccA1 must interact with FAS-II components such as Pks13, KasB, and KasA (A–C), and release them in an ATP hydrolysis-linked manner to the rest of FAS-II (D). (*eccA1*::Tn)/EQ is further sensitized to mycolic acid stress, relative to *eccA1*::Tn, because it has a lower amount of fully functioning Pks13, KasB, and KasA, important in (D). EccA1's interactions with these partner proteins are shown to be direct and exclusive here, but other mycobacterial proteins may aid these interactions or be part of these complexes. Three ATPs (shown in red) are bound to hexameric EccA1 here, but the real number is unknown.

DISCUSSION

Current models suggest that the mycobacterial ESX-1 system affects virulence through secretion of protein effectors with host-modulatory effects. Mycobacterial cell-wall lipids also are known to be important for virulence, but there has been little evidence for crosstalk between these key mechanisms of pathogenesis. Our studies suggest that EccA1, a core component of the ESX-1 secretion system (Abdallah et al., 2007; Bitter et al., 2009), physically associates with multiple lipid synthases, preferentially in the ATP-bound state. Disruption of EccA1 ATPase activity creates more stable interactions with these critical cellular enzymes. The functional importance of these interactions is supported by the observation that *eccA1*::Tn mycobacteria make less mycolic acid compared to strains expressing wild-type EccA1. Furthermore, we suggest that expression of ATPase-deficient EccA1 worsens mycolate synthesis when the synthesis system is under stress, perhaps because it sequesters enzymes required for efficient mycolate production.

Based on this, we propose that a normal role for EccA1 is to make mycolate synthesis more efficient, perhaps by shuttling relevant enzymes to sites of mycolate production (Figure 6). This is an unexpected role for an ATPase that also has a more expected role in ESX-1-mediated substrate transport out of the mycobacterial cell. In this way, EccA1 ties together two of the most important virulence mechanisms of pathogenic mycobacteria, ESX-1-mediated secretion and synthesis of its hydrophobic cell envelope. We speculate that the decreased pathogenicity of an *M. marinum* strain expressing the EQ mutant may be due to its interference with mycolic acid synthesis, beyond the defects found in *eccA1*::Tn. Whereas absence of EccA1 appears to be sufficient to abolish ESX-1-mediated secretion, at least of ESAT-6, CFP-10 (Gao et al., 2004), and EspE (*M. marinum* MMAR_5439; *M. tuberculosis* Rv3864) (Carlsson et al., 2009), the ATPase-defective EccA1 further inhibits mycolate production under conditions of bacterial stress. This correlates with decreased pathogenesis, even when ESX-1-mediated secretion is already absent due to loss of EccA1. It is well known that different mutations in the *esx-1* locus exhibit varying severities in virulence loss or intracellular growth, despite the fact that all block secretion (Abdallah et al., 2007; Gao et al., 2004; McLaughlin et al., 2007). We speculate that other genes in the ESX-1 locus besides EccA1 also may contribute to cell-envelope synthesis, and thus have more than one mechanism by which they mediate virulence.

SIGNIFICANCE

Tuberculosis is an unsolved medical problem with major global health and economic impact that has worsened because of the HIV epidemic. Multidrug resistance is on the rise, making some cases of the disease almost untreatable. Despite this, relatively few disease-causing mechanisms are understood for the causative bacterium, *M. tuberculosis*, and increased understanding is needed before new drug targets are identified and new therapies are developed. The best-characterized mycobacterial disease-related systems are the ESX-1 protein virulence factor export system and the waxy cell envelope composed largely of mycolic acids, which renders the bacteria resistant to host attack. Our studies on EccA1, a protein that is a component of the ESX-1 system, reveal that EccA1 has an unexpected role in regulating mycolic acid lipid synthesis via interaction with mycolic acid biosynthetic enzymes. This interesting link between these two key disease-causing mechanisms indicates that EccA1 represents an "Achilles' heel" affecting two pathways of mycobacterial biology that could be an appealing drug target for future therapeutic development.

EXPERIMENTAL PROCEDURES

DNA

eccA1 (MMAR_5443) was PCR amplified from *M. marinum* genomic DNA, and an N-terminal histidine tag was added. This product was cloned into pLYG206 (Gao et al., 2004) downstream of the *hsp60* promoter to produce pWT68. The E398Q mutation was introduced into pWT68 using overlap extension PCR mutagenesis to produce pEQ68. In order to integrate these *eccA1* variants, we excised the genes from pWT68 and pEQ68 and ligated them into pMV306, which is an integrative vector derived from pMV361 (Stover et al., 1991). For protein expression, we used pET-22b (Novagen) after removing its *pelB* sequence. All construct sequences were verified by DNA sequencing.

Bacterial Strains

M. marinum strain M was used in these studies. The *eccA1*::Tn strain is equivalent to the previously published M3 strain (Gao et al., 2004). Mycobacterial cultures were grown in 7H9 media containing ADC supplement (BD Biosciences), 0.2% glycerol, and 0.05% Tween 80. 7H10 agar plates contained OADC supplement. DNA was introduced into mycobacteria using electroporation. To integrate the *eccA1* variants, the different pMV306 constructs were electroporated into *eccA1*::Tn *M. marinum* and plated on 7H10 agar plates containing 50 μg/ml hygromycin. Hyg^R colonies were grown in liquid culture, and total cell lysates were screened for EccA1 production by immunoblotting using an anti-EccA1 antibody (see below). BL21-CodonPlus (DE3)-RP *E. coli* (Stratagene) was used for protein expression and purification.

Antibodies

Rabbit polyclonal antibodies were generated against EccA1, Pks13 (MMAR_5364), and Mas (MMAR_1767), using either protein or peptide antigens. A His-tagged EccA1 N-terminal fragment containing amino acids 1–298 was purified using Ni-NTA chromatography under denaturing conditions and used to raise the anti-EccA1 antibody. The other two antibodies were raised against unique synthetic peptides conjugated to thyroglobulin. Peptides consisted of amino acids 1653–1670 (Pks13) or 876–889 (Mas), and their specific reactivities were verified (Figure S2A). Immunoblotting experiments additionally used commercial antibodies against GroEL (Enzo Life Sciences), ESAT-6 (Thermo Fisher Scientific), the antigen 85 complex (BEI Resources; NR-13800), lambda repressor (Agilent), and RNA polymerase subunit α (Neoclone).

Solutions

Solution A is 100 mM HEPES-KOH (pH 7.5), 10% glycerol, 0.01% Tween 80, and 50 mM imidazole. Solution B is 100 mM HEPES-KOH (pH 7.5), 10% glycerol, 0.01% Tween 80, 25 mM imidazole, 150 mM KCl, and 5 mM $MgCl_2$. Solution C is identical to solution B except that it also contains 400 mM imidazole.

Functional Tests

Contact-dependent hemolysis assays were done as previously described (Gao et al., 2004) with a few changes. Mid-log-phase cultures were washed in Dulbecco's modified Eagle's medium without phenol red and incubated in a 2.5:1 ratio with sheep red blood cells in a 50 μl reaction for 2 hr. Zebrafish experiments were done with adult zebrafish (Zebrafish International Resource Center, University of Oregon) and at least five zebrafish were infected per tested mycobacterial strain. Mycobacteria (2×10^5) were injected intraperitoneally into each zebrafish and time to death was noted. Macrophage infections were done as previously described (Gao et al., 2004) with a multiplicity of infection of 3 using 129/SvJ bone marrow-derived macrophages, except that infected macrophages were lysed with 0.1% Triton X-100 before plating. Zebrafish studies followed the ethical guidelines of the *M. marinum*-zebrafish infection protocol (protocol 08-124), and macrophage infection studies followed a mouse bone marrow-derived macrophage protocol (protocol TH07-0474). Both protocols received ethical approval from the Institutional Animal Care and Use Committee at Genentech, and all work was done in an Association for Assessment and Accreditation of Laboratory Animal Care International-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 1996).

Coprecipitation of EccA1-Containing Cytosolic Complexes and Identification Using Mass Spectrometry

M. marinum cultures grown in 7H9 media were washed in Sauton's minimal media (Teknova), inoculated into 100 ml Sauton's cultures, and grown to mid-log phase. Cytosolic fractions were collected from harvested, lysed, and fractionated mycobacteria as previously described (Carlsson et al., 2009). Cytosols were diluted 2-fold with solution A to end up in solution B. Talon affinity resin (Clontech) equilibrated in solution B was added to the cytosols in an amount normalized to the cell pellet mass used to generate the cytosolic fraction. Cytosolic complexes containing His-tagged EccA1 variants were batch bound to Talon resin for 90 min at 4°C. Complexes were isolated after passing the cytosols through an empty column, washing the captured resin with ten column volumes of solution B, and eluting with five column volumes of solution C. Eluted complexes were mixed with SDS loading buffer, boiled, and run on 4%–20% Criterion gradient gels (Bio-Rad). After silver staining, visible bands of interest were excised and in-gel digested with trypsin. Extracted peptides were sequenced by liquid-chromatography-coupled ESI tandem MS (LC-MS/MS) on an LTQ-Orbitrap instrument (Thermo Fisher Scientific). Processed MS/MS spectra were searched against the *M. marinum* nonredundant database by using MASCOT (Matrix Science) as a search engine, and were visualized and validated using the Scaffold program (Proteome Software).

Bacterial Two-Hybrid Experiments

Interactions between EccA1 variants, PPE68 (MMAR_5448), $Pks13_{1-1340}$, KasB (MMAR_3339), KasA (MMAR_3338), and MmaA4 (MMAR_0977) were tested using the BacterioMatch II system (Agilent). Because Pks13 (MMAR_5364) is a very large molecule that might not fold correctly in *E. coli*, only the first 1,340 amino acids were used (Singh et al., 2006). EccA1 variants were cloned into the pTRG vector, whereas the other five tested proteins were cloned into the pBT vector. Cotransformations followed the manufacturer's protocol, and protein interaction percentages were determined from the ratio of colonies on selective plates to colonies on nonselective plates. Nonselective plates contained 25 μg/ml chloramphenicol, 12.5 μg/ml tetracycline, and 50 μM IPTG, whereas selective plates additionally contained 5 mM 3-amino-1,2,4-triazole. To test expression of these fusion proteins by immunoblotting, we first expressed the proteins using the manufacturer's protocol, except we induced expression in SOC media at 37°C with 50 μM IPTG.

Mycolic Acid Synthesis Tests

Early-log-phase mycobacterial cultures growing in Sauton's minimal media were diluted to an A_{600} of 0.4 in 20 ml final volumes. [1-^{14}C]-tetracosanoic acid (Moravek) was added to 0.5 μCi/ml and cultures were labeled for 3 hr. For testing mycolic acid production in the presence of ethionamide, mycobacteria were grown in 15 ml 7H9 cultures in 50 ml tubes for 6 hr after being diluted to an initial A_{600} of 0.3. Either 0.5 μg/ml ethionamide or an equal volume of ethanol was added to each culture, along with 0.4 μCi/ml [1-^{14}C]-tetracosanoic acid, at the start of the experiment. Harvested cells were washed once in water before being resuspended in 500 μl water. Saponification, methylation, and purification to isolate mycolic acid methyl esters (MAMEs) followed an established protocol (Vilchèze and Jacobs, 2007). Washed MAMEs were dried under nitrogen gas and resuspended in dichloromethane, and equal counts were applied to analytical silica gel 60 HPTLC plates (EMD Chemicals). Plates were developed three sequential times with 95:5 hexanes/ethyl acetate and imaged using a Typhoon PhosphorImager (GE Healthcare), and ImageQuant TL (GE Healthcare) was used to quantitate bands on the TLC plates.

Antibiotic Growth-Inhibition Tests

Mid- to late-log-phase mycobacterial cultures grown in 7H9 rich media were diluted to an A_{600} of 0.1. Two-fold serial dilutions of mycobacteria with ethionamide, rifampin, or erythromycin were performed and a final volume of 100 μl was dispensed into each well of a 96-well plate. The tested concentration ranges were 0.018–18 μg/ml (ethionamide), 0.027–28 μg/ml (rifampin), and 0.625–640 μg/ml (erythromycin). Plates were sealed with Parafilm and grown for 48 hr at 30°C with 105 rpm shaking. A_{600} values were determined using a plate reader, and IC_{50} values (m3) were determined after fitting the data to the four-parameter logistic equation (Baud, 1993): $y = m1 + m2/(1 + (x/m3)^{m4})$. Here, y is the observed growth expressed as a percentage of the maximal growth, x is the antibiotic concentration, m1 is the minimal growth asymptotic value, m2 is the difference between the maximal and minimal growth asymptotes, m3 is the IC_{50} value, and m4 is the Hill coefficient for cooperativity.

Statistical Analyses

Statistical significance of the survival of zebrafish cohorts infected with different mycobacterial strains was determined using the log-rank test. Otherwise, significance was determined using one-way ANOVA testing across all the samples analyzed in a given experiment. When calculated F values were greater than critical F values in these analyses, Fisher's least significant difference post hoc tests were applied to determine pairwise significant differences. Nonsignificant F values were found in a subset of the macrophage infection samples (inocula, 4 hr time point, and 24 hr time point). Significance is denoted in the figures as *$p < 0.05$; **$p \leq 0.03$; ***$p \leq 0.01$; and ****$p \leq 0.002$.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2012.01.008.

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REFERENCES

Abdallah, A.M., Gey van Pittius, N.C., Champion, P.A., Cox, J., Luirink, J., Vandenbroucke-Grauls, C.M., Appelmelk, B.J., and Bitter, W. (2007). Type VII secretion—mycobacteria show the way. Nat. Rev. Microbiol. *5*, 883–891.

Baud, M. (1993). Data analysis, mathematical modeling. In Methods of Immunological Analysis. Volume 1: Fundamentals, R.F. Masseyeff, W.H. Albert, and N.A. Staines, eds. (New York: VCH Publishers), pp. 656–671.

Bhatt, A., Molle, V., Besra, G.S., Jacobs, W.R., Jr., and Kremer, L. (2007). The *Mycobacterium tuberculosis* FAS-II condensing enzymes: their role in mycolic acid biosynthesis, acid-fastness, pathogenesis and in future drug development. Mol. Microbiol. *64*, 1442–1454.

Bitter, W., Houben, E.N., Bottai, D., Brodin, P., Brown, E.J., Cox, J.S., Derbyshire, K., Fortune, S.M., Gao, L.Y., Liu, J., et al. (2009). Systematic genetic nomenclature for type VII secretion systems. PLoS Pathog. *5*, e1000507.

Carlsson, F., Joshi, S.A., Rangell, L., and Brown, E.J. (2009). Polar localization of virulence-related Esx-1 secretion in mycobacteria. PLoS Pathog. *5*, e1000285.

Chopra, T., and Gokhale, R.S. (2009). Polyketide versatility in the biosynthesis of complex mycobacterial cell wall lipids. Methods Enzymol. *459*, 259–294.

Cosma, C.L., Sherman, D.R., and Ramakrishnan, L. (2003). The secret lives of the pathogenic mycobacteria. Annu. Rev. Microbiol. *57*, 641–676.

Daffé, M., Lanéelle, M.A., and Lacave, C. (1991). Structure and stereochemistry of mycolic acids of *Mycobacterium marinum* and *Mycobacterium ulcerans*. Res. Microbiol. *142*, 397–403.

DiGiuseppe Champion, P.A., Champion, M.M., Manzanillo, P., and Cox, J.S. (2009). ESX-1 secreted virulence factors are recognized by multiple cytosolic AAA ATPases in pathogenic mycobacteria. Mol. Microbiol. *73*, 950–962.

Dinadayala, P., Laval, F., Raynaud, C., Lemassu, A., Laneelle, M.A., Laneelle, G., and Daffe, M. (2003). Tracking the putative biosynthetic precursors of oxygenated mycolates of *Mycobacterium tuberculosis*. Structural analysis of fatty acids of a mutant strain devoid of methoxy- and ketomycolates. J. Biol. Chem. *278*, 7310–7319.

Dubnau, E., Chan, J., Raynaud, C., Mohan, V.P., Lanéelle, M.A., Yu, K., Quémard, A., Smith, I., and Daffé, M. (2000). Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. Mol. Microbiol. *36*, 630–637.

Gao, L.Y., Laval, F., Lawson, E.H., Groger, R.K., Woodruff, A., Morisaki, J.H., Cox, J.S., Daffe, M., and Brown, E.J. (2003). Requirement for kasB in *Mycobacterium* mycolic acid biosynthesis, cell wall impermeability and intracellular survival: implications for therapy. Mol. Microbiol. *49*, 1547–1563.

Gao, L.Y., Guo, S., McLaughlin, B., Morisaki, H., Engel, J.N., and Brown, E.J. (2004). A mycobacterial virulence gene cluster extending RD1 is required for cytolysis, bacterial spreading and ESAT-6 secretion. Mol. Microbiol. *53*, 1677–1693.

Glickman, M.S., Cox, J.S., and Jacobs, W.R., Jr. (2000). A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. Mol. Cell *5*, 717–727.

Hanson, P.I., and Whiteheart, S.W. (2005). AAA+ proteins: have engine, will work. Nat. Rev. Mol. Cell Biol. *6*, 519–529.

Heifets, L. (1988). MIC as a quantitative measurement of the susceptibility of *Mycobacterium avium* strains to seven antituberculosis drugs. Antimicrob. Agents Chemother. *32*, 1131–1136.

Hoffmann, C., Leis, A., Niederweis, M., Plitzko, J.M., and Engelhardt, H. (2008). Disclosure of the mycobacterial outer membrane: cryo-electron tomography and vitreous sections reveal the lipid bilayer structure. Proc. Natl. Acad. Sci. USA *105*, 3963–3967.

Institute for Laboratory Animal Research. (1996). Guide for the Care and Use of Laboratory Animals, Seventh Edition (Washington, DC: National Academies Press).

Koul, A., Arnoult, E., Lounis, N., Guillemont, J., and Andries, K. (2011). The challenge of new drug discovery for tuberculosis. Nature *469*, 483–490.

Lacave, C., Laneelle, M.A., Daffe, M., Montrozier, H., and Laneelle, G. (1989). Mycolic acid metabolic filiation and location in *Mycobacterium aurum* and *Mycobacterium phlei*. Eur. J. Biochem. *181*, 459–466.

Lewis, K.N., Liao, R., Guinn, K.M., Hickey, M.J., Smith, S., Behr, M.A., and Sherman, D.R. (2003). Deletion of RD1 from *Mycobacterium tuberculosis* mimics bacille Calmette-Guérin attenuation. J. Infect. Dis. *187*, 117–123.

Lönnroth, K., Castro, K.G., Chakaya, J.M., Chauhan, L.S., Floyd, K., Glaziou, P., and Raviglione, M.C. (2010). Tuberculosis control and elimination 2010–50: cure, care, and social development. Lancet *375*, 1814–1829.

Luthra, A., Mahmood, A., Arora, A., and Ramachandran, R. (2008). Characterization of Rv3868, an essential hypothetical protein of the ESX-1 secretion system in *Mycobacterium tuberculosis*. J. Biol. Chem. *283*, 36532–36541.

McLaughlin, B., Chon, J.S., MacGurn, J.A., Carlsson, F., Cheng, T.L., Cox, J.S., and Brown, E.J. (2007). A mycobacterium ESX-1-secreted virulence factor with unique requirements for export. PLoS Pathog. *3*, e105.

Minnikin, D.E., Kremer, L., Dover, L.G., and Besra, G.S. (2002). The methyl-branched fortifications of *Mycobacterium tuberculosis*. Chem. Biol. *9*, 545–553.

Niederweis, M., Danilchanka, O., Huff, J., Hoffmann, C., and Engelhardt, H. (2010). Mycobacterial outer membranes: in search of proteins. Trends Microbiol. *18*, 109–116.

Portevin, D., De Sousa-D'Auria, C., Houssin, C., Grimaldi, C., Chami, M., Daffé, M., and Guilhot, C. (2004). A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. Proc. Natl. Acad. Sci. USA *101*, 314–319.

Pym, A.S., Brodin, P., Brosch, R., Huerre, M., and Cole, S.T. (2002). Loss of RD1 contributed to the attenuation of the live tuberculosis vaccines *Mycobacterium bovis* BCG and *Mycobacterium microti*. Mol. Microbiol. *46*, 709–717.

Rachman, H., Strong, M., Ulrichs, T., Grode, L., Schuchhardt, J., Mollenkopf, H., Kosmiadi, G.A., Eisenberg, D., and Kaufmann, S.H. (2006). Unique transcriptome signature of *Mycobacterium tuberculosis* in pulmonary tuberculosis. Infect. Immun. *74*, 1233–1242.

Singh, A., Mai, D., Kumar, A., and Steyn, A.J. (2006). Dissecting virulence pathways of *Mycobacterium tuberculosis* through protein-protein association. Proc. Natl. Acad. Sci. USA *103*, 11346–11351.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., Bansal, G.P., Young, J.F., Lee, M.H., Hatfull, G.F., et al. (1991). New use of BCG for recombinant vaccines. Nature *351*, 456–460.

Takayama, K., Wang, L., and David, H.L. (1972). Effect of isoniazid on the in vivo mycolic acid synthesis, cell growth, and viability of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. *2*, 29–35.

Takayama, K., Wang, C., and Besra, G.S. (2005). Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. Clin. Microbiol. Rev. *18*, 81–101.

Teutschbein, J., Schumann, G., Möllmann, U., Grabley, S., Cole, S.T., and Munder, T. (2009). A protein linkage map of the ESAT-6 secretion system 1 (ESX-1) of *Mycobacterium tuberculosis*. Microbiol. Res. *164*, 253–259.

Veyron-Churlet, R., Guerrini, O., Mourey, L., Daffé, M., and Zerbib, D. (2004). Protein-protein interactions within the fatty acid synthase-II system of *Mycobacterium tuberculosis* are essential for mycobacterial viability. Mol. Microbiol. *54*, 1161–1172.

Veyron-Churlet, R., Bigot, S., Guerrini, O., Verdoux, S., Malaga, W., Daffé, M., and Zerbib, D. (2005). The biosynthesis of mycolic acids in *Mycobacterium tuberculosis* relies on multiple specialized elongation complexes interconnected by specific protein-protein interactions. J. Mol. Biol. *353*, 847–858.

Vilchèze, C., and Jacobs, W.R. (2007). Isolation and analysis of *Mycobacterium tuberculosis* mycolic acids. Curr. Protoc. Microbiol. *5*, 10A.3.1–10A.2.11.

Yuan, Y., and Barry, C.E., III. (1996). A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA *93*, 12828–12833.

Yuan, Y., Zhu, Y., Crane, D.D., and Barry, C.E., III. (1998). The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *Mycobacterium tuberculosis*. Mol. Microbiol. *29*, 1449–1458.

Zhang, X., and Wigley, D.B. (2008). The 'glutamate switch' provides a link between ATPase activity and ligand binding in AAA+ proteins. Nat. Struct. Mol. Biol. *15*, 1223–1227.

