Unraveling the pleiotropic role of the MceG ATPase in *Mycobacterium smegmatis*

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Abstract

The Mce systems are complex ABC transporters that are encoded by different numbers of homologous operons in Actinobacteria. While the four Mce systems of *Mycobacterium tuberculosis* are all energized by a single ATPase, MceG, each system appears to import different fatty acids or sterols. To explore if this behavior can be extended to saprophytic mycobacteria, whose more complex genomes encode more Mce systems, we have identified and characterized the MceG ortholog of *Mycobacterium smegmatis*. This bacterium relies on MceG to energize its 6 Mce systems that contribute to a variety of cellular functions including sterol uptake and cell envelope maintenance. In the absence of MceG, *M. smegmatis* was not able to utilize cholesterol or phytosterols as carbon sources implying that this ATPase is necessary to energize the Mce4-sterol transport system. Other phenotypic alterations observed in the Δ MceG mutant, such as cell envelope modifications, suggest a pleiotropic functionality of the Mce systems that are particularly important for stress responses. Several Δ MceG phenotypes were recapitulated in a strain lacking only the unique C-terminal region of MceG, suggesting an important functional or regulatory function for this domain.

1 Introduction

2 Mycobacteria are gram-positive members of the genus Actinobacteria with a high G+C genome
3 and a characteristic hydrophobic cell envelope that is rich in mycolic acids and other long-chain lipids and
4 glycolipids. The complex cell envelopes together with the ability to metabolically adapt to environmental
5 changes have allowed mycobacteria to adopt different lifestyles as animal pathogens (e.g.,
6 *Mycobacterium tuberculosis*, *Mycobacterium leprae* and *Mycobacterium bovis*) or soil inhabitants (e.g.,
7 *Mycobacterium smegmatis* and *Mycobacterium vanbaalenii*).

8 The understanding of the genetics, physiology, and evolutionary development of these bacteria
9 was facilitated by genome sequence analysis which revealed the presence of encoded *mce* operons,
10 complex transporters that share homology to ABC importers and are found exclusively in Actinobacteria
11 (Casali and Riley, 2007). The biochemical and physiological roles of Mce systems and their components
12 remain still unraveled. The Mce4 system has been characterized in mycobacteria and *Rhodococcus* as a
13 relatively specific cholesterol uptake system (Pandey and Sassetti, 2008; Mohn *et al.*, 2008; Klepp *et al.*,
14 2012). The Mce1 and Mce2 systems have been involved in the uptake of different cell wall lipid
15 components as mycolic acids or sulfolipids (Dunphy *et al.*, 2010; Marjanovic *et al.*, 2011; Santangelo *et al.*,
16 2009). Thus, while existing data are consistent with each Mce system importing a different class of lipids,
17 their specific roles in *M. tuberculosis* infection and survival remain to be defined. Saprophytic
18 mycobacteria, such as *M. smegmatis*, have much larger genomes than the pathogenic ones, likely due to
19 the more diverse environmental conditions to which they are exposed. In this sense, the *M. smegmatis*
20 genome encodes six distinct Mce systems, showing more complexity than the four Mce systems encoded
21 by *M. tuberculosis*. However, the Mce4 system of *M. smegmatis* was found to contribute to cholesterol
22 uptake as in *M. tuberculosis* and a strain lacking all six Mce systems had pleiotropic defects (Klepp *et al.*,
23 2012). Thus, while there is some functional similarity between Mce systems of these two species, their
24 roles in *M. smegmatis* remain relatively ill-defined.

25 Each Mce system contains two transmembrane proteins with homology to the permease subunits
26 of ABC transporters, along with several putative secreted or cell surface proteins (Casali and Riley, 2007).
27 Remarkably, in mycobacteria, an ATPase is not encoded in any of the *mce* operons. However, other
28 actinobacterial *mce* operons encode a single ATPase, and a homolog of this gene, *mceG*, is present in the

1 *M. tuberculosis* chromosome. These proteins contain the conserved Walker A and B motifs required for
2 ATP binding, as well as the ABC transporter family signature (LSGGQ) with no more than one mismatch
3 (Casali and Riley, 2007). Taking into account that only one *mceG*-like gene is present in *M. tuberculosis*, it
4 has been proposed that this unique ATPase generates the energy for substrate translocation by all of the
5 Mce systems of this bacterium. Genetic interaction mapping techniques support this hypothesis and
6 phenotypic studies verified that the *mceG* gene of *M. tuberculosis* is necessary for a proper cholesterol
7 uptake (Joshi *et al.*, 2006; Pandey and Sassetti, 2008) consistent with a fundamental role in Mce4
8 function.

9 In this work, we investigated the function of the MceG ortholog in *M. smegmatis*. In contrast to *M.*
10 *tuberculosis*, we found that this protein was essential for cholesterol uptake. In addition to this Mce4-
11 associated function, we found that the lack of the MceG protein produced subtle alterations in colony
12 morphology and significant aggregation when cultured in liquid media devoid of detergents. These
13 phenotypes suggest that the identified MceG ATPase in *M. smegmatis* performs a pleiotropic function.
14 The similarity between these phenotypes and those reported for a mutant lacking all six *mce* operons
15 supports the singular role of MceG through the Mce systems. Finally, we found that the conserved C-
16 terminal extension of MceG is necessary for its function, implying that unique enzymatic or structural
17 features are critical for this class of ABC-like transporters.

18 19 **Results**

20 ***Identification and in silico analysis of the mceG ortholog of M. smegmatis***

21 An *in silico* analysis of the six *mce* operons of *M. smegmatis* revealed that none of them
22 contained a gene coding for an ATPase required to energize the substrate translocation performed by
23 ABC transport systems. Instead, in *M. smegmatis* and related mycobacteria these *mceG* genes appear to
24 be encoded in at least partially syntenic regions, as each locus encodes the same ribosomal proteins
25 (Table S3). This genomic organization is unique to mycobacteria as MceG orthologues are encoded
26 immediately upstream of the *mce* operons in species of *Nocardia*, *Rhodococcus* and *Streptomyces* (Table
27 S4). Reciprocal BLAST searches verified that these genes represented the only orthologs of MceG in
28 each genome. This means that MceG appears to be encoded by a single gene in all these organisms

1 suggesting that its plays a critical and pleiotropic role to sustain the activity of all the Mce systems present
2 in the same cell.

3 In *M. smegmatis*, *MSMEG_1366* gene was identified as the putative MceG ortholog (88% amino
4 acid identity with MceG of *M. tuberculosis*). Other candidates, like *MSMEG_5807* and *MSMEG_3056*
5 genes that are also annotated as putative ATPases of ABC transporters showed lower identities, 34% and
6 36%, respectively. These two proteins are located in typical ABC transporter operons and shared greater
7 similarity with *M. tuberculosis* proteins other than MceG. The analysis of the *MSMEG_1366* and related
8 orthologues genomic regions showed that this gene appears to constitute a single transcription unit and no
9 putative specific regulatory genes or recognizable upstream palindromic sequence that might indicate the
10 existence of an operator region were localized in its vicinity (Fig. 1).

12 ***Expression of mceG is induced during growth in cholesterol.***

13 To investigate the function of the *MSMEG_1366* (*mceG*) gene in *M. smegmatis*, we determined
14 its expression levels in different *in vitro* culture conditions. Although the activity of MceG has been related
15 to cholesterol uptake, none of the consensus operator sequences recognized by the two regulatory
16 proteins that control cholesterol catabolism in *M. smegmatis* (i.e., KstR and KstR2 regulators) (Kendall *et al.*,
17 2007; Kendall *et al.*, 2010; Uhía *et al.*, 2011; García-Fernández *et al.*, 2014; García-Fernández *et al.*,
18 2015), were observed in the upstream region of *mceG* gene, strongly suggesting that this gene is not
19 directly controlled by these regulators. Nevertheless, we investigated the expression of this gene by
20 comparing its transcript abundance in cells growing in glycerol or cholesterol as the only carbon and
21 energy source. The results confirmed that *mceG* is upregulated in cells growing in cholesterol compared
22 with the cells growing in glycerol, showing 3-fold induction in the exponential phase (Fig. 2). These
23 observations are consistent with global transcriptomic analyses performed on *M. smegmatis* in which
24 *mceG* was induced 3.5-fold in cholesterol containing media (Uhía *et al.*, 2012) and suggests that the
25 expression of *mceG* must be regulated under demand by other specific or pleiotropic systems still
26 unknown. Concomitantly with the upregulation of *mceG* gene, the expression of the *mce4* operon
27 (*MSMEG_5902-MSMEG_5893*) was also upregulated in the presence of cholesterol as demonstrated by
28 the microarray analyses (Uhía *et al.*, 2012). Nevertheless, in contrast with *mceG* gene, the *mce4* operon

contains an operator signal of KstR and belongs to the *kstR* regulon. Although both *mceG* and the *mce4* operon appear to be regulated by different mechanisms, their coordinated induction for sustaining cholesterol uptake during exponential growth is consistent. However and remarkably, in early stationary phase the *mceG* gene showed higher expression levels in glycerol than in cholesterol (Fig. 2). This result is in agreement with the pleiotropic role played by MceG, suggesting that in glycerol other Mce systems remain active under the stationary phase and demand the ATPase activity to sustain the viability of the cells.

MceG is indispensable for growth on cholesterol

To determine if the identified MceG ortholog is necessary for cholesterol uptake in *M. smegmatis*, we deleted this gene and determined if the resulting mutant (named Δ MceG) was able to grow in media supplied with different sterols as a sole carbon and energy source. While wild-type *M. smegmatis* was able to grow using cholesterol, cholestenone or phytosterols as a sole carbon and energy source, the Δ MceG mutant was unable to grow in these conditions even after more than 120 h of culture (Fig. 3A, 3B and 3C respectively). The inability to utilize cholesterol was also ostensible in mixed carbon source cultures, as the addition of cholesterol (0.70 g L⁻¹) to media containing low concentrations of glycerol (0.18, 0.36, 0.72 and 0.90 g L⁻¹) did not enhance growth (data not shown). This inability to utilize cholesterol was proved to be due to the *mceG* deletion, as complementing the mutant with a wild-type copy of the *mceG* gene expressed from an episomal vector (strain Δ MceG-C) restored its ability to grow in the sterols as a sole carbon and energy source. Interestingly, overproducing MceG in wild-type bacteria by cloning the *mceG* harboring plasmid did not increase the growth rate of wild-type on cholesterol (strain WT-C), suggesting that the ATPase activity, and consequently the uptake of cholesterol, is not the principal bottleneck step for the mineralization of cholesterol (Fig. 3A).

In *M. tuberculosis*, Pandey and Sassetti (2008) showed that the Δ MceG mutant retained some ability to use cholesterol suggesting the presence of a less efficient import system. Therefore, it was possible that the complete inability of the Δ MceG mutant of *M. smegmatis* to grow in the sterol could be due to a defective induction of the cholesterol catabolic pathway.

1 To address this question, we constructed a double mutant (Δ MceG Δ KstR) in which we deleted
2 both the ATPase and the KstR repressor that regulates the upper cholesterol pathway. Therefore, this
3 mutant constitutively expresses cholesterol catabolic genes (Kendall *et al.*, 2007). This double mutant was
4 not able to grow in cholesterol media, indicating that the growth defect of the Δ MceG mutant cannot be
5 attributed to a defective induction of the *kstR* regulon (Fig. 3D). This result also suggests that cholesterol
6 cannot be transported by other MceG-independent mechanisms, since in the double mutant the
7 cholesterol transported by any other mechanism (including passive uptake) should support its growth even
8 at low rates.

9 10 ***MceG is necessary for cholesterol uptake***

11 To determine if MceG was specifically necessary for the cholesterol uptake process performed by
12 the Mce4 system, we quantified the relative ability of wild-type and Δ MceG bacteria to actively import 14 C-
13 labeled cholesterol. Although due to the high hydrophobicity of cholesterol it was impossible to completely
14 eliminate the background of cholesterol adsorbed to the cell envelope, we observed that the wild-type cells
15 imported approximately twice the amount of cholesterol than the mutant (Fig. 4). To determine the
16 background levels of cholesterol bound to the cell envelope, we used DCCD to inhibit the active transport
17 (Monh *et al.*, 2008) and we observed that the Δ MceG was unable to import cholesterol over the
18 background level observed in wild-type DCCD-treated cells. This result reinforced the hypothesis that no
19 other active uptake system is involved in the process and that the MceG is the unique ATPase responsible
20 for the active transport of cholesterol in *M. smegmatis*.

21 22 ***Deletion of mceG alters colony morphology***

23 Several studies have shown a relationship between Mce systems, cell envelope composition and
24 colony morphology in mycobacteria (Dunphy *et al.*, 2010; Marjanovic *et al.*, 2011; Santangelo *et al.*, 2009;
25 Klepp *et al.*, 2012). However, the central role of MceG in these processes has not been investigated up to
26 date. In this sense, we observed a clumping phenotype in cultures of the Δ MceG mutant very similar to
27 that described for the *M. smegmatis* mutant deleted in all six *mce*-operons (Klepp *et al.*, 2012). In addition,

1 we observed that the Δ MceG mutant also forms distinctive morphologies in 7H10 media supplemented
2 with Congo Red (Fig. 5A and Fig. 5B), which were similar to the morphologies of the mutant lacking all six
3 *mce*-operons (Klepp *et al.*, 2012). These observations suggested that the loss of MceG contributed to cell
4 envelope alterations in *M. smegmatis*, and is consistent with the hypothesis that the MceG is necessary
5 for the function of all the Mce systems in mycobacteria (Joshi *et al.*, 2006).

6 7 ***Mycolic acid methyl ester (MAME) composition is altered in the absence of MceG***

8 The morphological changes observed in the Δ MceG mutant suggested alterations in the
9 envelope composition. The mycobacterial cell wall possesses unique lipids that are specific to this genus,
10 and, as commented above, the *mce1* operon has been related to the re-importation of mycolic acids in *M.*
11 *tuberculosis* (Forrellad *et al.*, 2014), and the *mce2* operon has been associated with sulfolipids transport in
12 this bacterium (Marjanovic *et al.*, 2011). In contrast to these findings, the total lipid content of a *M.*
13 *smegmatis* mutant lacking all six *mce*-operons described by Klepp *et al.* (2012) showed no differences
14 relative to the wild-type strain. Similarly, we found no major difference in the content of mycolic acid methyl
15 esters (MAMEs) between the Δ MceG mutant and wild-type (Fig. 6A).

16 Taking into account the cell wall remodelling function previously suggested for the Mce systems,
17 we decided to repeat these experiments after exposing the cells to unusual culture conditions that might
18 cause metabolic perturbations. Remarkably, under low aeration conditions the epoxy mycolic acid species
19 present in the wild-type strain disappears and is replaced by a new species with lower R_f. This new lipid
20 species is not detected in the Δ MceG mutant cultured in the same conditions (Fig. 6B). This change in the
21 MAMEs pattern became similar to the observed in the standard conditions (Fig. 6A) after several hours in
22 normal aeration conditions, which can be observed in the early stationary phase (Fig. 6B) and during a
23 second culture (data not shown). In order to confirm the involvement of the Mce systems in cell wall
24 processes as response to other metabolic perturbations, this analysis was repeated adding D,L-norvaline
25 to the culture medium, a valine analogue that is known to induce the starvation-related stringent response
26 in *M. smegmatis* mc²155 (Dahl *et al.*, 2003). This experiment showed a change in the mycolic acid pattern

1 similar to the alteration observed under low aeration conditions (Fig. 6C), reinforcing the idea of the need
2 of the Mce systems in situations of metabolic stress.

4 ***C-terminal domain of MceG***

5 The MceG protein has a high similarity with other well described ATPases and possesses the
6 characteristic features shared by many other ATPases involved in ABC transporters that were first
7 identified in HisP (Hung *et al.*, 1998). However, this analysis also revealed an additional uncommon C-
8 terminal domain that has only been described in a small number of ATPases related to Malk (Böhm *et al.*,
9 2002). Interestingly, the C-terminal domain of MceG is peculiar since it does not show any significant
10 similarity to other protein domains present in the databases, being exclusive to MceG orthologs (Fig. 7 and
11 Fig. S1). The peculiar structure of MceG can be observed in its 3D-model showing an N-terminal domain
12 that can be folded as the conventional ABC-ATPases (Schmitt *et al.*, 2002) and the additional C-terminal
13 domain (residues 250-360) that cannot be modelled as no homologous structures are available in the
14 databases (Fig. S2).

15 In other ABC transport systems such as the maltose uptake system of *Escherichia coli*, the
16 ATPase component (Malk) plays a regulatory role in the induction of catabolic genes (Bukau *et al.*, 1986;
17 Reyes *et al.*, 1988). In contrast to these data, the Δ MceG mutant shows very similar levels of a *mce4*
18 operon gene (*MSMEG_5899*) compared to the WT when growing in glycerol. In both cases the expression
19 levels in the exponential phase were 3-fold higher than in stationary phase (Fig. 8), indicating that the lack
20 of this protein doesn't alter the levels of the *mce* transcripts. However, previous studies performed with the
21 MceG protein of *M. tuberculosis*, showed that tagging MceG in its C-terminal end resulted in a non-
22 functional protein unable to complement the Δ MceG mutant (data not shown), indicating the importance of
23 this region in the ATPase function. Therefore, to help identify the essential regions of the C-terminal
24 domain in mediating MceG function, we tested the complementing ability of a series of C-terminal
25 truncations of ATPase, namely ATPase322, ATPase292 and ATPase258 where the number corresponds
26 to the final amino acid residue (Fig. 9A), when compared to the full length ATPase (360aa). All these
27 constructs carried an N-terminal tag (FLAG-mVenus FP) to help verify that the truncated proteins are
28 stably expressed in the Δ MceG mutant. Upon transformation into Δ MceG mutant, we found that the N-

terminally tagged wild-type MceG is able to complement the Δ MceG mutant and imparts wild-type levels of growth in cholesterol demonstrating that the N-terminal tags do not disrupt its function. However, strains complemented with the truncated MceG proteins showed a growth defect in cholesterol that was proportional to the length of the C-terminal truncation (Fig. 9B). The ATPase258 mutant cannot consume all the cholesterol present in the medium since it is unable to provide enough energy to the transport system to uptake cholesterol below a certain concentration. The wild-type and all truncated MceG proteins were expressed at similar levels as assessed by western blotting with anti-FLAG antibodies (Fig. 10A), and shared a similar cellular distribution as assessed by the cellular localization of fluorescently-labelled fusion proteins (Fig. 10B). This result confirms that they are expressed at similar levels and suggests that they are associated with similar protein complexes. In addition, the complemented mutants showing a poor growth in cholesterol also displayed a clumping phenotype similar to that observed for the Δ MceG mutant (Fig. 10C). These results strongly suggest that the C-terminal domain of MceG plays an important role not only in the Mce4-dependent cholesterol uptake process, but also in other cell envelope homeostasis functions mediated by the Mce systems.

Discussion

The four *mce* loci of *M. tuberculosis* have garnered significant interest due to their demonstrated role in pathogenesis (Shimono *et al.*, 2003; Senaratne *et al.*, 2008). In *M. smegmatis*, the *mce* loci consist of 6 homologous operons (10-13 genes) named *mce1*, *mce2*, *mce3*, *mce4*, *mce5*, *mce5bis* and *mce7* that likely arose by duplications of a single ancestral locus (Cole *et al.*, 1998). While these systems appear to have distinct functions, existing evidence suggests that they all share a common ATPase subunit, MceG (Joshi *et al.*, 2006). Reciprocal BLAST and synteny analyses of mycobacterial genomes suggested that the *MSMEG_1366* gene is the most likely ortholog of the MceG ATPase of *M. tuberculosis* in *M. smegmatis*. In this study we provide evidence that this protein is the functional ortholog of MceG and additional support for the hypothesis that this protein is necessary for the function of multiple Mce systems.

The bioinformatic analysis of the promoter region of *mceG* in *M. smegmatis* showed that this gene is expressed as a single transcriptional unit and does not contain any evident regulatory sequence in

1 its vicinity that can be recognized by KstR or KstR2 regulators. However, the expression analysis
2 performed using RT-qPCR revealed an upregulation of the *mceG* gene in the presence of cholesterol
3 compared to glycerol during the exponential growth phase. In agreement with this observation, Uhia *et al.*
4 (2012) observed that although 89 genes are overexpressed in *M. smegmatis* in the presence of
5 cholesterol, only 52 have KstR or KstR2 operator sequences. Thus, a significant number of genes (37
6 genes) that are induced in the presence of cholesterol are likely induced by other mechanisms.

7 Our results confirmed that MceG is the only ATPase able to provide the energy required by the
8 Mce4 transport proteins in *M. smegmatis* given that in the absence of this enzyme the cells were not able
9 to use cholesterol and other related sterols as the only carbon and energy source. The absolute inability of
10 the Δ MceG mutant of *M. smegmatis* to utilize cholesterol is somewhat different from the behaviour of the
11 equivalent Δ MceG mutant of *M. tuberculosis* that is still able to grow on cholesterol although at a lower
12 rate (Pandey and Sassetti, 2008). While this could suggest that the pathogenic mycobacteria have
13 developed alternative active mechanisms or alternative ATPases to replace MceG to uptake cholesterol,
14 we cannot rule out other explanations, such as different passive diffusion of cholesterol through the cell
15 membrane of *M. smegmatis* and *M. tuberculosis*.

16 The morphological studies that we have carried out with the Δ MceG mutant showed a high
17 clumping phenotype and an altered colony morphology in the mutant compared to the *M. smegmatis* wild-
18 type strain. These findings correlate with the phenotypes observed in the six *mce* deletion mutant of *M.*
19 *smegmatis* (Klepp *et al.*, 2012), suggesting that MceG in *M. smegmatis* is the only ATPase used by all the
20 Mce transport systems. These phenotypes are likely due to alterations in the cell envelope, as *mce*
21 operons have been implicated in cell wall remodelling processes in *M. tuberculosis* (Dunphy *et al.*, 2010;
22 Marjanovic *et al.*, 2011; Santangelo *et al.*, 2009). Our study furthers these observations by discovering that
23 MceG is specifically required for cell envelope homeostasis under metabolic stress conditions. This result
24 agrees with the previous observations that suggested that the nutrient limited conditions (Kumar *et al.*,
25 2005) and the stringent response induction (Dahl *et al.*, 2003) promote the expression of different Mce
26 systems in *M. tuberculosis*. In addition, the study of Santangelo *et al.* (2016) concludes that the lack of
27 Mce4 proteins abrogates the recycling of certain undefined lipids that trigger a redox stress response.
28 More generally, these observations are consistent with the proposed function of the Mla transport system

1 of *E. coli* (Malinverni *et al.*, 2009). This ABC transporter is distantly related to Mce systems but it
2 contributes to the maintenance of outer membrane lipid asymmetry, which is required for adapting *E. coli*
3 to membrane stress (Malinverni *et al.*, 2009). Taken together, these observations suggest that Mce
4 systems may be specifically required for maintaining cell envelope composition during environmental
5 stresses.

6 The MceG protein possesses two different domains, the N-terminal one that contains the catalytic
7 residues needed for the ATPase function and an atypical C-terminal extension. C-terminal extensions of
8 other ABC transporter related ATPases can serve regulatory functions through interacting with
9 transcription factors (Joly *et al.*, 2004; Richet *et al.*, 2005) or binding to compounds that reversibly inhibit
10 transport (Gerber *et al.*, 2008). This phenomenon has been most clearly described for the maltose
11 transporter in *E. coli* (Böhm *et al.*, 2002) in which the MalK ATPase is bound to the transcriptional activator
12 MalT through its C-terminal domain. MalT is released upon ATP hydrolysis and activates transcription of
13 the *mal* operon. In contrast to these data, the *M. smegmatis* Δ MceG mutant does not show a constitutive
14 expression of the *mce4* genes compared to the wild-type strain (Fig. 8), suggesting that the lack of this
15 protein does not alter the expression of the cholesterol catabolic regulon. In addition, the overproduction of
16 the MceG ATPase in *M. smegmatis* (WT-C strain) did not show any effect on the growth in cholesterol,
17 differing from the results obtained in *E. coli* where the overproduction of the MalK ATPase inhibits maltose
18 uptake (Reyes *et al.*, 1988).

19 Despite the lack of an obvious regulatory function, the conservation of the C-terminal domain of
20 MceG among actinobacterial orthologs suggested an important functional role. Indeed, we found that in *M.*
21 *smegmatis* the C-terminal domain was important not only for cholesterol utilization but also for the
22 phenotypes associated with cell wall remodelling. The only truncation mutant that retained wild-type
23 activity was a deletion of the last 38 amino acids. These last 38 amino acids constitute the region with the
24 lowest similarity between the different MceG ATPases (Fig. S3) and therefore appears to be less
25 essential.

26 Summarizing, our studies using different experimental approaches have identified the
27 MSMEG_1366 (*mceG*) gene as coding for a functional MceG ATPase in *M. smegmatis*. Deletion of this
28 gene, or just its unique C-terminal domain produced phenotypes consistent with a complete loss in Mce

functions. Thus, as it has been hypothesized in *M. tuberculosis*, the activity of the multiple Mce systems of mycobacteria relies on this single ATPase. As a result, diverse cellular processes, including sterol uptake and cell wall homeostasis are perturbed in the absence of this protein. The singular role for this protein in such a wide variety of critical functions suggests that it may represent an attractive target for new anti-mycobacterial therapies.

Experimental procedures

Bacterial strains and culture conditions

The strains as well as the plasmids used in this work are listed in Table S1. *M. smegmatis* mc²155 strain was grown at 37°C in an orbital shaker at 250 rpm. Middlebrook 7H9 broth medium (Difco) without albumin-dextrose supplement and without glycerol was used as minimal medium. As carbon source 1.8 mM cholesterol (Sigma), 1.8 mM cholest-4-en-3-one (Sigma), 1 mM phytosterols (Gadea Pharma) or a mixture of 2-10 mM glycerol-1.8 mM cholesterol, all of them dissolved in 3.6% Tyloxapol (Sigma), was added. Due to the low solubility of steroids, stock solutions were warmed at 80°C with agitation and then autoclaved. As rich medium, 7H9 broth containing 0.2% glycerol, 10% albumin-dextrose supplement and 0.05% Tween 80 or 7H10 agar (Difco) plates supplemented with 0.5% glycerol and 10% albumin-dextrose were used. Gentamycin (5 µg ml⁻¹) and kanamycin (25 µg ml⁻¹) were used for selection of mycobacteria. Ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹) were used for plasmid selection and maintenance in *Escherichia coli* DH10B strain.

Gene deletions and complementation

The knock-out strain ΔMceG (ΔMSMEG_1366) was constructed by homologous recombination using the plasmid pJQ200x (Jackson *et al.*, 2001). The strategy used has been previously described by García-Fernández *et al.*, (2013). The primers used are listed in Table S2. For complementation, the MSMEG_1366 gene was amplified by PCR using primers pMV1366_F and pMV1366_R (Table S2). The resulting amplicon was digested with the appropriate restriction enzymes and cloned into the expression vector pMV261 (Table S1) under the control of *hsp60* promoter, yielding pMV1366 that was introduced in the mutant strain by electroporation to generate the complemented strain ΔMceG-C.

Generation of the MceG truncated versions

N-terminal FLAG-Venus tagged ATPase constructs (full-length and truncations 322, 292 and 258) were cloned downstream of a p46 promoter in the mycobacterial expression plasmid pDE43-MEK vector using the Multisite-Gateway Cloning Technology (Invitrogen) to yield pMEKVenus_ATPase-FL, pMEKVenus_ATPase322, pMEKVenus_ATPase292 and pMEKVenus_ATPase258. The full length version is 360 amino acids long and each truncation carries C-terminal deletion after the indicated amino acid position. Each one of these vectors was electroporated independently into competent cells of Δ MceG to identify the length of the C-terminal tail required for complementation of the Δ MceG phenotype.

RNA extraction and real-time PCR (RT-PCR)

RNA for RT-qPCR was extracted from the *M. smegmatis* mc²155 strain grown to logarithmic phase (OD₆₀₀ 0.8-0.9 in medium containing 18 mM glycerol or 0.5–0.6 in medium containing 1.8 mM cholesterol) or in stationary phase (OD₆₀₀ 3-3.2 in medium containing 18 mM glycerol or 1.6–1.8 in medium containing 1.8 mM cholesterol). The culture was centrifuged and the pellet resuspended in 1 ml of Kirby solution (1% SDS, 0.2 M EDTA) with lysozyme (15 mg ml⁻¹) (Sigma). The mixes were incubated at room temperature for 5 min and then transferred to Falcon tubes containing solid glass beads (150-212 μ m diam., Sigma). 200 μ l of PCIA (phenol-chloroform-isoamyl alcohol acid) and 800 μ l of buffer RLT (Qiagen) were added to each sample and several steps of 5 min agitation were performed. The cell lysates were recovered by centrifugation and the supernatant was added to new Falcon tubes with 700 μ l of absolute ethanol (Merck). RNA was purified from the lysate using the RNeasy kit (Qiagen) according to the manufacturer's instructions. RNA was treated with the DNase I and Removal treatment kit (Ambion) according to manufacturer's instructions and the final yield was measured using a Nanodrop (Nanodrop technologies). Reverse transcription was performed with 1 μ g of purified RNA using the transcriptor first strand cDNA synthesis kit (Roche). Real time quantitative polymerase chain reactions for the analysis of the expression of single genes were performed using a LightCycler® 480 instrument (Roche). Reactions contained 5 μ l of a 1:5 cDNA dilution, 0.25 μ l of each primer (Table S2) and 10 μ l of LightCycler® 480 SYBR Green I Master (Roche) in a total volume of 20 μ l. The reactions were denatured at 95 °C for 5 min before cycling

for 50 cycles of 95 °C for 10 s, 62 °C for 10 s, and 72 °C for 10 s. Each gene was measured in triplicate in three independent experiments. The relative amount of mRNA for each gene was determined using the mRNA levels of *sigA* (*MSMEG_2758*) as internal control (Gomez *et al.*, 2000).

Western blot analysis

Mycobacteria were lysed by bead beating in 50 mM Hepes buffer (pH 7.5) containing 50 mM NaCl with protease inhibitor (Roche). Lysates were analyzed by SDS/PAGE (Bio-Rad) on a 12.5% gel under reducing conditions. Electrophoretic transfer of proteins from gels to nitrocellulose membrane was performed using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). Membranes were blocked overnight in blocking buffer (PBS containing 2% skimmed milk), washed three times with TBS buffer (10 mM Tris buffer (pH 7.6), 0.9% (w/v) NaCl, and 0.1% (v/v) Tween-20), and incubated with a 1:1000 dilution of anti-FLAG antibodies (Sigma) for 1 h. The membrane was washed 3 times with TBS and incubated with a 1:5000 dilution of HRP-conjugated anti-rabbit antibodies (GE Healthcare) for 1 h. After additional washing, blots were developed using the ECL Plus Western blotting detection reagents, as per manufacturer recommendations (GE Healthcare).

Macroscopic studies

Bacterial clumping was analyzed by culturing the different strains in minimal medium with 18 mM glycerol devoid of detergents. Colony morphology was analyzed by plating approximately 100 colony forming units (CFU) of the parental and the mutant strains on 7H10 solid agar medium. Congo Red (1 µg ml⁻¹), an azo-dye with a well-known affinity for lipids and lipoproteins, was added to improve visualization of colony morphology alterations as described by Cangelosi *et al.* (1999). Plates were incubated at 37 °C for 3 days and the colony morphology was inspected either by naked eye or under a binocular scope at low magnification.

Cholesterol uptake assay

Cholesterol uptake was directly measured in resting cell suspensions. Cells were grown to mid-log phase on 18 mM glycerol, washed, and resuspended at a final cell density of A600 = 8 in 7H9 (minimal medium)

1 buffer. Aliquots of 0.5 ml of the cells were placed in 1.5 ml vials, and 0.01 $\mu\text{Ci ml}^{-1}$ of [4- ^{14}C]cholesterol (53
2 mCi/mmol, PerkinElmer Life Sciences) and 0.03 mM of non labelled cholesterol were added. The vials
3 were incubated for different times at 37°C. Cholesterol uptake was stopped by adding 5 ml of cold PBS
4 buffer and collecting the cells immediately after on a 0.45- μm Millipore nitrocellulose filter (Fisher
5 Scientific). Filtered cells were washed twice with 10 ml of PBS buffer with 5% Tween 20 (Fisher) and 10 ml
6 of 50% ethanol. The filters were dried and placed in Beckman Ready-Safe scintillation mixture (Beckman
7 Coulter) and counted in a Beckman LS-600IC scintillation counter to determine the amount of cholesterol
8 taken up by the cells. Where indicated, 2.0 mM dicyclohexylcarbodiimide (DCCD) was added to cell
9 suspensions 10 min prior to the addition of the labeled cholesterol.

11 *Mycolic acid (MAMEs) extraction and TLC analysis*

12 Wild-type and ΔMceG strains were cultured in minimal media with 18 mM glycerol and 0.05% Tween80 in
13 normal conditions. Low aeration conditions were performed by culturing the cells in 50 ml Falcon tubes
14 with 45 ml of 7H9 medium that were parafilm-sealed and cultured at 37 °C in static conditions. After 4 days
15 of incubation, the cells were used to inoculate minimal media with 18 mM glycerol and normal aeration
16 conditions. For the stringent response induction, DL-norvaline (0.5 mg ml $^{-1}$) (Sigma) was added when the
17 cells growing in minimal media with 18 mM glycerol reached an OD $_{600}$ of 0.6. In each case, 50 ml aliquots
18 were collected at different growth phases, washed with PBS and kept at -80 °C. For the MAMEs
19 extraction, frozen samples were lyophilized and 25 mg of dry weight were transferred to Pyrex glass tubes
20 with PTFE caps (Supelco). MAMEs extraction was performed as previously described by Slayden and
21 Barry, 2001. TLC of MAMEs was performed on Silica Gel-60 plates (Meck) and developed a total of 5
22 times using petroleum ether/diethyl ether (95:5, v/v). Individual MAMEs were visualized by charring at 100
23 °C using 5% molybdophosphoric acid in ethanol (Besra, 1998).

25 *Bioinformatic analysis*

26 The local sequence alignment was performed using ClustalW2
27 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). Homology search was carried out using pBLAST in NCBI
28 database using the rv0655 protein as query sequence. Structure prediction of the protein was carried out

using SWISS-MODEL workspace (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1&userid=USERID&token=TOKEN) (Arnold *et al.*, 2006).

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Conflict of interest

None declared

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Figure legends

Fig. 1. Schematic representation of the *mceG* genomic regions in *M. smegmatis* mc²155, *M. tuberculosis* H37Rv and *R. jostii* RHAI.

Fig. 2. Differential expression of *mceG* gene. Expression of the *MSMEG_1366* gene in exponential (black bars) or in early stationary (grey bars) growth phase in the wild-type strain cultured in cholesterol relative to glycerol. Transcription levels were measured using RT-qPCR as described in Experimental procedures. The values indicate the ratios of mRNA levels observed for the strain growing on cholesterol relative to glycerol, and normalized to *sigA* levels. Data represent averages of three independent experiments, error bars indicate $\pm$ standard deviation and asterisks indicate that results are statistically significant (one-way ANOVA with a Student-Newman-Keuls test; ** $P < 0.01$; *** $P < 0.001$).

Fig. 3. Growth analysis of mycobacterial strains. Growth curves of wild-type, Δ MceG, Δ MceG (pMV261), WT-C and Δ MceG-C strains on 1.8 mM cholesterol as the sole carbon and energy source (A). Growth curves of WT, Δ MceG and Δ MceG-C strains on 1.8 mM of cholest-4-en-3-one (B) or on 1 mM of phytosterols (C) or of WT and Δ MceG Δ KstR strains on 1.8 mM cholesterol (D) as the sole carbon and energy source. Growth was monitored by measuring the OD at 600 nm. Data represent averages of three independent experiments.

Fig. 4. Radioactive cholesterol uptake by wild-type and Δ MceG strains. DCCD refers to measurements obtained in the presence of the ATPase inhibitor. Data represent averages of two independent experiments.

Fig. 5. Phenotypic studies of the wild-type, Δ MceG and Δ MceG-C strains. (A) Aggregation assay in 18 mM glycerol medium devoid of detergents. (B) Colony morphology studies made in 7H10 plates containing 1 μ g ml⁻¹ Congo Red dye.

Fig. 6. TLC analysis of MAMEs from glycerol-grown *M. smegmatis* Δ MceG and wild-type strains. The strains were cultured under normal aeration conditions (A), under low aeration (B) or in the presence of D,L-norvaline (C) and the MAMEs extracted as described in Experimental Procedures.

Fig. 7. Analysis of the ABC domains present in different ATPases. Scheme showing the ATPase domains present in MalK as reference ABC ATPase with a C-terminal regulatory domain (A), the MceG ATPase (B) and HisP as reference ABC ATPase (C).

Fig. 8. Expression studies in the Δ MceG strain. Differential expression of the *MSMEG_5899* (belonging to the *mce4* operon) and *mceG* genes in exponential (black bars) or in early stationary growth phase (grey bars) in the Δ MceG and wild-type strains cultured in glycerol. Transcription levels were measured using RT-qPCR as described in Experimental procedures. The values indicate the ratios of mRNA levels observed for the strain growing on glycerol and normalized to *sigA* levels. Data represent averages of three independent experiments and error bars indicate $\pm$ standard deviation

Fig. 9. Role of the C-terminal domain of MceG in cholesterol metabolism. (A) Schematics of C-terminal truncations: The dark grey bar indicates the conserved N-terminal domain of ATPase and is retained in all constructs, The variable length C-terminal light grey bars indicate C-terminal truncations. (B) Growth of the Δ MceG mutant expressing the truncated versions of the protein in 1.8 mM of cholesterol as the sole carbon source. The Δ MceG mutant is shown in black circles, the white square indicates the baseline OD obtained with the uninoculated cholesterol containing medium. Data represent averages of three independent experiments.

Fig. 10. Analysis of the MceG C-terminal function in *M. smegmatis* mc²155. (A) The MceG proteins expressed by the Δ MceG strain complemented with pMEKVenus_ATPase-FL, pMEKVenus_ATPase322, pMEKVenus_ATPase292 and pMEKVenus_ATPase258 were subjected to SDS-PAGE and Western blot analysis using anti-FLAG antibodies. The Δ MceG strain complemented with pMEKVenus_ATPase lacking the FLAG epitope was used as negative control. (B) Localization of mVenus-tagged ATPase reporter in the Δ MceG strain expressing full length (ATPase) and a truncated version (ATPase258) of MceG. Differential interference contrast (DIC) and FITC channel images were captured using a Delta Vision Microscope (Applied Precision) and deconvolved using softWoRx (Applied Biosystems). (C) Aggregation studies of Δ MceG, ATPase (Δ MceG (pMEKVenus_ATPase-FL)), 322 (Δ MceG (pMEKVenus_ATPase322)), 292 (Δ MceG (pMEKVenus_ATPase292)) and 258

- 1 (Δ MceG (pMEKVenus_ATPase258)) strains cultured in 18 mM glycerol containing medium devoid of any
- 2 detergents.
- 3

Supplementary Material

Table S1. Bacterial strains and plasmids used in this study.

Table S2. Primers used in this study.

Table S3. Genomic localization of MceG orthologues in mycobacteria. The genes found to be common for all genomic regions are highlighted in bold.

Table S4. Genomic localization of MceG orthologues in Actinobacteria other than mycobacteria. The genes found to be common for all genomic regions are highlighted in bold.

Fig. S1. Analysis of the ABC domains present in different ATPases. Clustal analysis performed with the MalK, MceG and HisP sequences to highlight the differences in their C-terminal domains. The characteristic motifs found in all ABC ATPases are highlighted.

Fig. S2. Homology modelling of MceG in *M. smegmatis*. (A) 3D-model of MceG using the chain D of the MalK ATPase of *E. coli* as template. (B) 3D-structure of MalK.

Fig. S3. Clustal analysis of different MceG orthologues. Sequence comparison of the MceG proteins present in *S. coelicolor*, *M. smegmatis*, *M. tuberculosis*, *M. bovis*, *M. leprae*, *N. farcinica* and *R. jostii*. The last amino acids present in the ATPase258, ATPase292 and ATPase322 truncated versions are highlighted.

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