The P-type ATPase CtpF is a plasma membrane transporter mediating calcium efflux in *Mycobacterium tuberculosis* cells

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**ABSTRACT**

Among the 12 P-type ATPases encoded by the genome of *Mycobacterium tuberculosis* (*Mtb*), CtpF responds to the greatest number of stress conditions, including oxidative stress, hypoxia, and infection. CtpF is the mycobacterial homolog of the sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) of higher eukaryotes. Its expression is regulated by the global regulator of latency, DosR. However, the role that CtpF plays in the mycobacterial plasma membrane remains unknown. In this study, different functional analyses showed that CtpF is associated with calcium pumping from mycobacterial cells. Specifically, *Mtb* CtpF expression in *Mycobacterium smegmatis* cells prevents Ca\(^{2+}\) accumulation compared with wild-type (WT) cells. In addition, plasma membrane vesicles from recombinant membranes, in which the direction of ion transport is inverted, accumulate more Ca\(^{2+}\) compared with vesicles obtained from the WT strain. This finding supports the hypothesis that CtpF contributes to calcium efflux from mycobacterial cells. Accordingly, *Mtb* cells defective in *ctpF* (*MtbΔctpF*) accumulate more Ca\(^{2+}\) compared with WT cells, while the Ca\(^{2+}\)-dependent ATPase activity is significantly lower in the mutant cells. Interestingly, the deletion of *ctpF* in *Mtb* impairs the tolerance of the bacteria to oxidative and nitrosative stress. Overall, our results indicate that CtpF is associated with calcium pumping from mycobacterial cells and the response to oxidative stress.

1. Introduction

Tuberculosis (TB) is produced by the acid-fast bacillus *Mtb* and is one of the top 10 causes of death worldwide. In 2017, there were 6.4 million new cases reported and 1.6 million deaths by TB [1]. The incidence of TB has increased as a result of the emergence of multidrug and extensively resistant (MDR and XDR) mycobacterial strains, *Mtb*-HIV coinfection, and the ineffectiveness of the Bacillus Calmette–Guérin (BCG) vaccine [1, 2]. Therefore, the search for alternative control strategies is a priority that relies on a better understanding of the molecular mechanism used by *Mtb* to succeed as an intracellular pathogen. In this sense, the role played by cell membrane proteins and transporters in the mycobacterial interaction with the host cell environment is pivotal. Previous studies have suggested the relevance of P-type ATPases in the mycobacterial physiology and host-pathogen interaction [3].

P-type ATPases are a large family of membrane proteins relevant for maintaining cellular homeostasis and generating appropriate electrochemical gradients for cell survival. These enzymes use the energy released by ATP hydrolysis to catalyze the transport of cations across the cell membrane [3, 4, 5, 6]. In fact, P-type ATPases are expressed during mycobacterial infection as a response to the toxicity produced by high levels of metals in human macrophages [7, 8, 9]. There are reports of diminished vacuolar concentration of Ca\(^{2+}\) (1.8 ± 1.3 mM) and K\(^{+}\) (19.5 ± 16.9 mM) in the early phagosome (first hour after phagocytosis) as compared with extracellular bacteria [7, 10]. However, the concentrations of Ca\(^{2+}\) (7.1 ± 3.3mM) and K\(^{+}\) (51.0 ± 28.6mM) as well as other metals such as copper, zinc, and iron are replenished or increased 24 h post-infection [7, 10]. Therefore, P-type ATPases, among other systems, play a critical role in maintaining mycobacterial metal homeostasis during infection [7, 8, 9, 11, 12, 13, 14].

P-type ATPases are classified into five subfamilies (P1–P5), based on ion specificity and structural characteristics [4, 6]: P1A-type bacterial potassium transporters; P1B-type heavy metal pumps; P2-type alkaline/ealkaline earth metal transporters; P3A-type H\(^{+}\) pumps; P3B-type bacterial Mg\(^{2+}\) pumps; P4-type putative lipid flippases; and the uncharacterized P5-type ATPase pumps [4, 5, 6, 15]. Bioinformatic studies identified 12 P-type ATPases in the *Mtb* genome, namely: seven P1B-type, four P2-type, and one P1A-type ATPases [16, 17, 18]. Regarding

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The functional characterization of these *Mtb* plasma membrane pumps, CtpA is a Ca\(^{2+}\) transporter \([19]\), CtpC transports Mn\(^{2+}\) and/or Zn\(^{2+}\) across the plasma membrane \([8, 13]\), CtpJ and CtpD are Fe\(^{2+}\), Co\(^{2+}\), and Ni\(^{2+}\) transporters \([12, 14]\), CtpV is a Ca\(^{2+}\) pump \([9]\), and CtpG is a Cd\(^{2+}\) transporter \([20]\). However, the ion specificity of the P\(_2\)-type ATPases and/or their possible roles in mycobacteria are unknown.

Unlike eukaryotic cells, there is not a wide variety of calcium-mediated processes in bacteria \([21]\). However, critical physiological processes such as cellular growth, motility, quorum sensing, sporulation, and the development of different bacterial structures are regulated by the cytosolic Ca\(^{2+}\) concentration in bacteria \([21]\). All forms of life, even mycobacteria, developed mechanisms such as passive and active transporters, ion channels, and non-protein channels to regulate calcium homeostasis \([21]\), including Ca\(^{2+}\)-ATPases that mediate calcium homeostasis \([22, 23]\).

*Mtb* CtpF is the mycobacterial P\(_2\)-type ATPase most closely related to the sarco/endoplasmic reticulum calcium ATPase (SERCA1a) from eukaryotes \([16]\). The expression of the ctpF gene is regulated by the global mycobacterial dormancy regulator DosR \([24, 25, 26, 27, 28, 29, 30, 31]\).

Interestingly, ctpF is activated under conditions similar to the phagosome environment and during infection \([28, 32]\). Speciﬁcally, ctpF responds when *Mtb* cells are treated with toxic substances, such as isoxyl, tetra-hydrodipristin \([33]\), reactive nitrogen species (RNS), reactive oxygen species (ROS) \([28, 29, 30, 34, 35]\), and under hypoxia \([27, 29, 36, 37]\).

The transcriptional behavior suggests that CtpF could be part of the strategies of the tubercle bacillus cells to face the environmental conditions encountered during infection \([39]\). However, the actual role of CtpF in mycobacterial ion homeostasis and its biology remains unknown. Which is the cation transported by CtpF? Are there any phenotypic consequences of ctpF overexpression and deletion in mycobacteria?

In this work, we assessed the role of CtpF in Ca\(^{2+}\) pumping from mycobacterial cells. Initially, the ATPase activity in the plasma membrane vesicles with the calcium accumulation in whole cells and plasma membrane vesicles from recombinant *M. smegmatis* overexpressing *Mtb* CtpF and *Mtb* cells defective in ctpF (*MtbΔctpF*) conﬁrmed that this transporter is associated with Ca\(^{2+}\) pumping from mycobacterial cells. In addition, *MtbΔctpF* cells were more susceptible to oxidizing agents, suggesting a link between Ca\(^{2+}\) transport and the mycobacterial response to oxidative stress.

## 2. Materials and methods

### 2.1. Bacterial strains and growth conditions

The bacterial strains, plasmids, and primers used in this study are listed in Tables 1 and 2. *Mtb* strains were grown in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose-catalase (OADC) (50 μg/mL oleic acid, 0.5 % Bovine albumin Fraction V, 0.2 % dextrose and 0.004 % catalase) and 0.5 % glycerol at 37 °C with gentle agitation (80 rpm) until OD\(_{600}\) = 0.5–0.8, or on 7H10 and H11 agar plates supplemented with oleic acid and glycerol. For the experiments of bacterial tolerance to cations, the mycobacteria were grown in Sauton’s medium (pH = 7.4) supplemented with 0.5 % Tween 80 and 0.2 % glucose at 37 °C and 80 rpm. *Escherichia coli* DH5α and TOP10, used for plasmid propagation, were cultured at 37 °C in LB broth with agitation (180 rpm) or on LB agar plates. When required, 7H9, 7H10, and 7H11 were supplemented with 20 μg/mL kanamycin (Kan), 100 μg/mL hygromycin (Hyg), while LB was supplemented with 100 μg/mL ampicillin (Amp), 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 80 μg/mL X-gal, or 20 μg/mL camcyanin (Kan). *Mtb* genomic DNA was isolated as previously reported \([40]\).

### 2.2. Bioinformatic analysis

The amino acid sequence of CtpF was pairwise aligned with 12 P\(_2\)-type Ca\(^{2+}\)-ATPase sequences retrieved from UniProt and the alignments were visualized using Jalview 2.10.5 software \([41]\).

### 2.3. *Mtb* ctpF gene cloning and expression in *M. smegmatis*

The ctpF gene (*Rv1997*) was amplified by PCR from the template genomic DNA of *Mtb* H37Ra using the primer pair Fcm2Dir/FpmvHisRev (Table 2) and the Phusion DNA Polymerase (Thermo Scientific). The reverse primer added a His\(_{6}\)-tag to the C-term of the protein. The amplified, flanked by the BarnHI and HindIII restriction sites, was cloned into the shuttle vector pMV261 to obtain the pLNA29 plasmid, whose integrity was confirmed by PCR, restriction mapping, and DNA sequencing. *M. smegmatis* mc\(_{155}\) cells were transformed with the pLNA29 plasmid and the transformant colonies were verified by colony PCR. Recombinant colonies were grown in LB-Kan at 37 °C with agitation at 180 rpm until an OD\(_{600}\) = 0.5–0.6. Protein expression was induced by heat shock at 45 °C for 1 h \([42]\). Protein extracts were analyzed in 10% polyacrylamide gels (SDS-PAGE) and immunostaining dot-blots using rabbit anti- His\(_{6}\) polyclonal primary antibody and goat anti-rabbit secondary antibody HRP-conjugate (Thermo Scientific, USA).

### 2.4. Construction of the ctpF-defective *Mtb* strain

The deletion of the ctpF gene in *Mtb* was performed using the Che9c recombineering system \([43]\). Briefly, the allelic exchange substrate (AES) was generated by separately cloning 500 bp of the upstream and downstream sequences of the ctpF gene into the pYUB854 vector flanking a hygromycin-resistance cassette. The upstream (section A) and downstream (section B) regions of the ctpF gene were amplified by PCR and separately cloned into the pGEM8-T Easy vector (Promega). The A and B sections were subcloned into pYUB854 to generate the pLNA22 plasmid, from which the AES was released by restriction enzymes digestion. Simultaneously, the recombineering strain (*Mtb* transformed with the pJV53 plasmid) was cultured in 7H9 supplemented with 0.2 % succinate, 0.05 % Tween 80 and Kan, until an OD\(_{600}\) = 0.5–0.8. Cells were then supplemented with 0.2 M glycine and induced with 0.2% acetamide. The Recombineering cells were electroporated with 100 ng AES and plated on 7H11-OADC-Kan-Hyg \([43]\). *Mtb* colonies defective in ctpF (*MtbΔctpF*) were screened by colony PCR (primers listed in Table 2). Genomic DNA was isolated from the selected *Mtb* mutant to confirm the targeted gene

| Strains | Relevant features | Reference |
|---------|------------------|-----------|
| **Mycobacterium tuberculosis** | Slow-growing attenuated strain, Amp\(^{\beta}\), Chx\(^{\beta}\), Ga\(^{\beta}\) | ATCC 25177 |
| H37Ra | Recombining strain with (pJV53), Amp\(^{\beta}\), Chx\(^{\beta}\), Co\(^{\beta}\), Km\(^{\beta}\) | This study |
| H37RaΔctpF | ActcP, gene replaced by a Hyg\(^{\beta}\) cassette | This study |
| **Mycobacterium smegmatis** | Fast-growing, usually non-pathogenic | ATCC 700084 |
| mc\(_{155}\) |  | |

**Table 1** Bacterial strains and plasmids used in this study.
replacement by PCR, using primers matching within the Hygβ cassette and flanking the sections A and/or B of the AES (primers listed in Table 2). Finally, the PCR products were sequenced to confirm the integrity of the targeted gene replacement.

2.5. Preparation of plasma membrane vesicles

Cultures of mycobacterial cells (5 L) were grown until OD600 = 0.5–0.8. The entire procedure was performed at 4 °C. Cells were harvested by centrifugation and washed twice with buffer A (10 mM MOPS, 0.08 g/mL sucrose, pH = 7.4), resuspended in lysis buffer (10 mM MOPS, 1 mM EDTA, 0.3 mM PMFS, pH = 7.4), and mechanically lysed with a Mini Bead Beater (Biospec) by eight 1-minute cycles. Cellular debris were removed by centrifugation at 25,000 x g for 30 min in a Megafuge 16R Centrifuge (Thermo Scientific). Supernatants (membrane and cytoplasmic fractions) were isolated by centrifugation at 100,000 x g for 90 min in a Sorvall WX Floor Ultra Centrifuge (Thermo Scientific). The remaining supernatant was discarded and the pellet containing the membrane fraction was resuspended in buffer A [44]. The protein concentration was assessed with the Bradford method or the BCA method using the Pierce BCA Protein Assay Kit (Thermo scientific). The protein extracts were finally adjusted to 1 mg/mL, aliquoted, and stored at -20 °C until use.

2.6. Metal accumulation assays

Cultures of mycobacterial cells were grown until OD600 = 0.5–0.8. Then, the cells were harvested and washed three times with washing buffer (10 mM MOPS, 140 mM choline chloride, 0.5 mM DTT, 250 mM sucrose). The cell pellet was resuspended in 5 mL in washing buffer and separately supplemented with cations using the following reaction buffer (25 mM MOPS, 3 mM MgSO4, 150 mM KCl, 0.05 % Brij-58, 3 mM Na2ATP) and incubated at 37 °C for 1 min. Reactions were initiated by adding 100 μM Ca2+, incubated at 37 °C for 30 min, stopped by filtration through 0.22 μM pore size filters (Millipore, USA), and the filters were washed twice with washing buffer (25 mM MOPS, 250 mM sucrose, 3 mM MgSO4, 150 mM KCl, 0.05 % Brij-58, 3 mM Na2ATP) and incubated at 37 °C for 1 min. The samples were measured by flame absorption spectroscopy using a contrAA 700 Jena Analytik Atomic Absorption Spectrometer.

2.7. ATPase activity assays

The ATPase activity of the plasma membrane vesicles was measured according to the Fiske-Subbarow method with modifications, as previously described [19, 20, 46]. Enzymatic reactions (final volume 50 μL) were performed in 96-well plates using 10 μg of protein in reaction buffer (3 mM MgCl2, 10 mM MOPS, pH = 7.4) and supplemented with 0.02 % Brij-58, 0.29 mM Ca2+ and 0.25 mM EGTA (final concentrations) to control the amount of free calcium. Maxichelator software was used to calculate the free Ca2+ [47]. The enzymatic reactions were initiated by adding 3 mM Na2ATP and incubating at 37 °C for 30 min. The reactions were stopped by adding 100 μL of stopping solution (3 % acetic acid, 0.5 % ammonium molybdate, 3 % SDS and 2 M HCl). Subsequently, the samples were kept 10 min at 4 °C and then 150 μL of stabilizing solution (3.5 % bismuth citrate, 3.5 % sodium citrate, 2 M HCl) were added. Afterward, the samples were incubated at 37 °C for 10 min. Finally, the Pi released was quantified by measuring the OD405 nm. The ATPase activity is reported as Pi nmol produced by mg protein by min of reaction (nmol Pi. mg⁻¹.min⁻¹) from three independent experiments [19, 20, 46].

2.8. Mycobacterial tolerance to metal cations

The culture was harvested and washed three times with Sauton's medium (pH = 7.4) supplemented with 0.05 % Tween 80 and 0.2 % glucose. The cell pellet was resuspended in Sauton's medium and dialyzed until OD600=0.05–0.06. Subsequently, 100 μL of bacterial suspension were separately mixed in 96-well plates with 100 μL of serial dilutions of cations in a range of previously determined concentrations: Ca2+ (0.4 mM - 9.5mM), Na+ (10 mM–150 mM) and K+ (5 mM–150 mM).

Cultures were incubated at 37 °C for 21 days at 80 rpm and the final OD600 of cultures was measured in an iMARKTM Microplate Reader (Bio-Rad, CA, USA). Cells grown in the same medium without cation or supplemented only with 10 μg/mL isoniazid were considered controls for 100 % and 0 % growth, respectively [19, 20]. The IC50 was determined using GraphPad Prism 8.0 software using a nonlinear regression with log (inhibitor) vs. normalized response-Variable slope. Each experiment was assessed in triplicate from three biological replicates.

Table 2

| Primer | Sequence (5’-3’) |
|--------|----------------|
| F-RT Dir | CAGTAGATCCTGGTGTGGTGG |
| F-RT Rev | GATTGACGGAGAAGAGTCA |
| pMVComp Up | CAGGGAGGAGACATGGGAC |
| pMVComp Down | CGACTGCCAGGTCATAAATA |
| Fcm2 Dir | TTTTGGATATCATGGTGCTAGTCTG |
| Fpv/His Rev | TTTTGAAGCCTCGAATGATGATGATGTGGCGGTTGCGCCCGTA |
| pVS3dir | GTCACTTAGACACCTCCTC |
| pVS5rev | GAATCTGCGTGTCAGACCC |
| cpf_interno_dir | TTATGCACCCGACGTCCT |
| cpf_interno_rev | CTATGCACCCGACGTCCT |
| Comp.Up-cpf | TCGTGGAAACTCTGTTAGTC |
| Comp.Down_cpf | CGTCGCCGCGAGGCTTACAG |
| primersYUB854 | GTCGCCGCGAGGCTTACAG |
| Hyg_dir/out | ACGCGAGGACAACTTGAGC |
| Adir2013 | TTGTCTGAGGCGAGTGGCGAGACC |
| Arev2013 | TTGTCTGAGGCGAGTGGCGAGACC |
| Bdir2013 | TTGTCTGAGGCGAGTGGCGAGACC |
| Brev2013 | TTGTCTGAGGCGAGTGGCGAGACC |
2.9. Oxidative and nitrosative stress assays

Cultures of mycobacterial cells were harvested and washed three times with 7H9 supplemented with oleic acid—albumin—dextrose (OAD) (50 µg/mL oleic acid, 0.5 % Bovine albumin Fraction V and 0.2 % dextrose) and 0.05 % Tween 80. The cell pellet was resuspended in 7H9-OAD and diluted until an OD600 = 0.05–0.06. Subsequently, 100 µL of bacterial suspension were separately mixed in 96-well plates with 100 µL of serial dilutions of redox agents in a range of previously determined concentrations; H2O2 (0.5 mM–25 mM) and sodium nitroprusside (SNP) (0.01 mM–1 mM) [29, 34, 48]. Cultures were incubated at 37 °C for 8 days at 80 rpm and the final OD600 of cultures were measured in an iMARKTM Microplate Reader (Bio-Rad, CA, USA). Cells grown in the same medium without an oxidant agent or supplemented only with 10 µg/mL isoniazid were considered controls for 100 % and 0 % growth, respectively. The IC50 was determined in GraphPad Prism 8.0 software using nonlinear regression with log (inhibitor) vs. normalized response -Variable slope. The results are representative of three independent experiments.

3. Results

3.1. Mtb CtpF encodes a putative Ca2⁺ P-type ATPase

Among the 12 ORFs that encode P-type ATPases in the Mtb genome, four of them have been classified as P2-type ATPases: CtpE, CtpF, CtpL, and CtpH [16]. This subclass of membrane transporters includes calcium transport-associated enzymes such as SERCA, PMCA1, SPCA and LMCA1, together with Na+/K⁺-ATPases [49]. SERCA is the best structurally characterized P2-type ATPase, and multiple conformations comprising its entire catalytic cycle have been resolved and reported in the Protein Data Bank. In addition, SERCA is highly conserved in higher eukaryotes and the entire catalytic cycle have been resolved and reported in the Protein Data Bank. As shown in Fig. 1, CtpF is closely related to SERCA by sharing 8 of the 10 calcium coordination sites in SERCA, 8 of which are conserved in CtpF. Blocks in top represent the transmembrane segments (TM) were the cation binding amino acids are located.

3.2. CtpF prevents calcium accumulation in mycobacterial cells

In order to determine if CtpF is involved in calcium transport across the mycobacterial cell membrane, we evaluated calcium accumulation in recombinant obtained from M. smegmatis cells expressing CtpF, Mtb WT and ΔctpF cells. The latest were obtained by homologous recombination using the Can cassette system (Fig. 2A) [43]. The allelic exchange replacement of the ctpF locus in the ΔctpF mutant cells was confirmed by PCR (Fig. 2B and C). Nucleotide sequencing of the amplimers showed that the Hyg cassette indeed inserted into the ctpF gene, by showing the AES insertion into the desired site of the Mtb genome and the presence of the β-gal resolvase sites, allowing the further removal of the Hygmycin resistance cassette to eliminate the antibiotic resistance gene [43].

When exposing the M. smegmatis cells expressing CtpF to a high calcium concentration in the extracellular medium (10 mM) during 1 h, the recombinant cells accumulate 2-fold less Ca2⁺ than the control cells (WT transformed with empty pMV261). This suggests that CtpF may be involved in the active Ca2⁺ transport from mycobacterial cells against the concentration gradient (Figs. 3A and 3B). To confirm that it was an ion-specific transport, this experiment was also performed using 240 mM sodium and 240 mM sodium/potassium treatment. No significant differences were observed between recombinant and control cells under those conditions (Fig. 3C). To further confirm that CtpF is associated with calcium efflux from mycobacterial cells, we determined the calcium accumulation in the ΔctpF strain. In agreement, ΔctpF cells accumulated 4-fold more Ca2⁺ than the Mtb WT cells (Fig. 3D). These results are consistent with the reduced calcium accumulation observed for the recombinant strain and supports the hypothesis that CtpF is a calcium efflux pump (Fig. 3A).

3.3. CtpF promotes calcium accumulation in right-side-out mycobacterial plasma membrane vesicles

To further confirm that CtpF transports calcium from inside mycobacterial cells against the concentration gradient, we measured calcium accumulation in an inverted membrane vesicle model supplemented with Brij 58 to obtain an "inside-out" configuration (Fig. 4A) [44]. In this experimental model, the cytoplasmic domains of CtpF are exposed to the reaction medium, the direction of transport is inverted, and calcium accumulation depends on the availability of ATP and Ca2⁺ reaction medium, the direction of transport is inverted, and calcium accumulation depends on the availability of ATP and Ca2⁺. No significant differences were observed between recombinant and control cells under those conditions (Fig. 3C). To further confirm that CtpF is associated with calcium efflux from mycobacterial cells, we determined the calcium accumulation in the ΔctpF strain. In agreement, ΔctpF cells accumulated 4-fold more Ca2⁺ than the Mtb WT cells (Fig. 3D). These results are consistent with the reduced calcium accumulation observed for the recombinant strain and supports the hypothesis that CtpF is a calcium efflux pump (Fig. 3A).

![Fig. 1. Mtb CtpF possesses the characteristic Ca2⁺ binding residues of P-type ATPases. Multiple sequence alignment of 12 P2-type ATPases in MEGA X using Muscle. All sequences were retrieved from UniProt. Jhalview 2.1.0.5 was used to visualize the final result. The arrows show the 10 key calcium-binding residues of SERCA, 8 of which are conserved in CtpF. Blocks in top represent the transmembrane segments (TM) were the cation binding amino acids are located.](image-url)
In agreement, Ca\textsuperscript{2+} (2.5-fold) than in vesicles obtained from assessed the tolerance of contribute to restoring intracellular calcium levels in and Na\textsuperscript{+} activity was higher in vesicles from M. smegmatis vesicles expressing CtpF. As expected, the Ca\textsuperscript{2+} was higher in vesicles from M. smegmatis cells expressing CtpF (2.5-fold) than in vesicles obtained from M. smegmatis WT cells (Fig. 5A). In agreement, Ca\textsuperscript{2+} ATPase activity increased only 7% in the Mtb\Delta ctpF plasma membrane vesicles, when the increment was 30% in vesicles obtained from the Mtb WT cells, compared to the basal ATPase activity (Fig. 5B). This decreased Ca\textsuperscript{2+}-dependent ATPase activity in vesicles from Mtb\Delta ctpF cells is in agreement with the proposed role of CtpF, as Ca\textsuperscript{2+}-efflux pump. Importantly, since the ATPase activity was measured on crude membrane extracts, part of the Ca\textsuperscript{2+}-stimulated ATPase activity in vesicles from Mtb\Delta ctpF cells should be attributed to other Ca\textsuperscript{2+} ATPases present in the mycobacterial plasma membrane. Corroborating CtpF is a Ca\textsuperscript{2+} P-type ATPase, its activity was susceptible to vanadate (Fig. 5B), a known inhibitor of this kind of transporters [44, 46, 53].

3.4. Ca\textsuperscript{2+} ions stimulate the CtpF ATPase activity

We assessed the Ca\textsuperscript{2+}-dependent ATPase activity on plasma membrane vesicles obtained from mycobacterial cells. The enzyme reactions were supplemented with ATP as energy source, Mg\textsuperscript{2+} as cofactor, and Brij-58. Then, the ATPase activity was measured by quantifying the Pi released from ATP hydrolysis [44, 46]. As expected, the Ca\textsuperscript{2+}-ATPase activity was higher in vesicles from M. smegmatis cells expressing CtpF (2.5-fold) than in vesicles obtained from M. smegmatis WT cells (Fig. 5A). In agreement, Ca\textsuperscript{2+} ATPase activity increased only 7% in the Mtb\Delta ctpF plasma membrane vesicles, when the increment was 30% in vesicles obtained from the Mtb WT cells, compared to the basal ATPase activity (Fig. 5B). This decreased Ca\textsuperscript{2+}-dependent ATPase activity in vesicles from Mtb\Delta ctpF cells is in agreement with the proposed role of CtpF, as Ca\textsuperscript{2+}-efflux pump. Importantly, since the ATPase activity was measured on crude membrane extracts, part of the Ca\textsuperscript{2+}-stimulated ATPase activity in vesicles from Mtb\Delta ctpF cells should be attributed to other Ca\textsuperscript{2+} ATPases present in the mycobacterial plasma membrane. Corroborating CtpF is a Ca\textsuperscript{2+} P-type ATPase, its activity was susceptible to vanadate (Fig. 5B), a known inhibitor of this kind of transporters [44, 46, 53].

3.5. CtpF confers Ca\textsuperscript{2+} tolerance to mycobacterial cells

Since Mtb activates P-type ATPases to face high concentrations of metals inside macrophages [7, 8, 9], it is possible that CtpF may contribute to restoring intracellular calcium levels in Mtb. Therefore, we assessed the tolerance of Mtb cells to different concentrations of Ca\textsuperscript{2+}, K\textsuperscript{+}, and Na\textsuperscript{+} (Fig. 6). Assuming that CtpF is a potential calcium transporter, Mtb\Delta ctpF should be sensitive to high concentrations of calcium. As observed in Fig. 7, Mtb\Delta ctpF cells were more susceptible to Ca\textsuperscript{2+} than WT. Specifically, the IC\textsubscript{50} value of calcium in Mtb\Delta ctpF cells (1.7 mM) was significantly lower than in WT cells (2.5 mM). Even though the tolerance of Mtb\Delta ctpF to Na\textsuperscript{+} was significantly lower than WT, no difference in the tolerance to K\textsuperscript{+} was observed (Fig. 7). These results reinforce the idea that CtpF contributes to maintaining the physiological level of calcium in the mycobacterial cytosol.

3.6. CtpF is associated with the oxidative stress response in mycobacterial cells

Being part of the DosRS regulon [24, 25, 26, 27, 28, 29, 30, 31], we suspected a possible association between the transcriptional response of ctpF and oxidative/nitrosative stress. Testing this, we evaluated the sensitivity of Mtb\Delta ctpF and Mtb WT to ROS/RNS stress conditions (Figs. 6 and 8). As observed in Fig. 8, Mtb\Delta ctpF cells were more sensitive to oxidative and nitrosative stresses compared to Mtb WT. With IC\textsubscript{50} values of 1.6 mM H\textsubscript{2}O\textsubscript{2} and 85 μM SNP for Mtb\Delta ctpF cells and 2.9 mM H\textsubscript{2}O\textsubscript{2} and 214 μM SNP for Mtb WT, confirming that Mtb\Delta ctpF cells displayed hyper-susceptibility to ROS/RNS stresses compared to the WT strain.

4. Discussion

Calcium is a pivotal messenger for different physiological processes and signaling cascades in bacteria [50, 54]. Calcium is directly involved in membrane transport mechanisms, chemotaxis, cell division, and differentiation processes [54, 55]. The intracellular calcium content in bacteria increases when cells are surrounded by natural environments containing high doses of this metal (millimolar calcium concentrations). However, this increase in calcium levels should be transient in order to maintain bacterial viability [50, 54]. Therefore, a calcium homeostatic system is essential for bacterial survival [50, 54]. In this sense, Ca\textsuperscript{2+} P-type ATPases may play a relevant essential role in bacterial integrity.

CtpF is the most closely related mycobacterial transporter to the well-studied calcium eukaryotic transporter SERCA. Accordingly, our bioinformatics predictions showed that both proteins share 8 of 10 amino acids from the calcium coordination sites. Since other bacterial calcium...
ATPases also share key calcium-binding residues with SERCA, such as LMCA1 from *L. monocytogenes* [22], we hypothesized that CtpF is a Ca\(^{2+}\)-transporting P-type ATPase. Testing this, calcium accumulation experiments were conducted on whole mycobacterial cells and plasma membrane vesicles. *M. smegmatis* was used as an expression host for CtpF since this environmental species is an easier-to-handle model with similar envelope and membrane properties to *Mtb* [56]. The calcium accumulation experiments indicated that *M. smegmatis* plasma membrane with *Mtb* CtpF embedded responded to increased extracellular calcium concentration. Even though, this could be attributable not only to P-type ATPases but also to the activity of different metal transport systems [21], we consider that this reduced calcium accumulation in *M. smegmatis* recombinant cells to the presence of CtpF in the membrane, which is the relevant difference between the recombinant and control cells. To further discard that CtpF is a sodium/potassium P2-type ATPase [46], we tested sodium accumulation. The results showed that there is no significant difference in the accumulation of sodium between recombinant and control cells. These results suggest a calcium efflux activity mediated by CtpF.

Further evidence for CtpF-mediated calcium transport to the extracellular medium was provided by experiments using a membrane vesicle model, in which, hypothetically, the direction of calcium transport by CtpF is reversed [44]. This approach was complemented by measuring ATPase activity. As expected, calcium accumulation and Ca\(^{2+}\)-dependent

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**Fig. 3.** Calcium accumulation in mycobacterial cells. A) Hypothesized function of CtpF in the mycobacterial plasma membrane. In the presence of a high extracellular calcium concentration, CtpF transports calcium against its concentration gradient. B) Calcium accumulation inside *M. smegmatis* WT and the recombinant cells expressing CtpF. The amount of calcium accumulated was internally normalized against the amount of calcium measured in the dry mass of pellet from the WT strain. C) Sodium accumulation in the absence and presence of potassium in the WT and recombinant strains normalized with respect to the metal accumulation in the WT strain. D) Calcium accumulation in *Mtb* cells. The amount of calcium accumulated was internally normalized against the amount of calcium measured in the dry mass of pellet from the WT strain. The data shown are representative of three independent experiments. Unpaired two-tailed *t* test, ****P < 0.0001.

**Fig. 4.** Calcium accumulation in membrane vesicles obtained from *M. smegmatis* cells. A) Model of inverted membrane vesicles where the cytoplasmic domains of CtpF are exposed to the reaction medium. B) Calcium accumulation in WT and recombinant membrane vesicles from cells expressing ctpF. All normalized against the calcium accumulation in membrane vesicles of the WT strain. Data corresponds to mean ± SEM from three independent replicates. Unpaired two-tailed *t* test, **P < 0.01.
ATPase activity were higher in the membrane vesicles from the recombinant cells compared to control cells. This validates the hypothesis that the direction of transport was inverted in plasma membrane vesicles. The mutant phenotype of the \( \textit{Mtb} \Delta \text{ctpF} \) corroborates that CtpF is a calcium efflux pump. This is: 1) \( \textit{Mtb} \Delta \text{ctpF} \) cells have an impaired capacity to restore cytoplasmic calcium levels upon extracellular Ca\(^{2+}\) exposure; 2) Vesicles isolated from \( \textit{Mtb} \Delta \text{ctpF} \) exhibited a reduced Ca\(^{2+}\)-dependent ATPase activity and 3) \( \textit{Mtb} \Delta \text{ctpF} \) cells are more susceptible to Ca\(^{2+}\) than

Fig. 5. Ca\(^{2+}\)-P-type ATPase mediated by CtpF. A) Ca\(^{2+}\)-dependent ATPase activity of membrane vesicles of recombinant \textit{M. smegmatis} normalized against the control strain (\textit{M. smegmatis} WT). B) Ca\(^{2+}\)-ATPase activity of membrane vesicles of \textit{Mtb} WT and \textit{Mtb}ΔctpF against the basal ATPase activity. The dotted lines represent the percentage of the ATPase activity stimulated by Ca\(^{2+}\). Values correspond to the mean ± SEM from three independent replicates. Unpaired two-tailed \( t \) test, ***\( P < 0.001 \).

Fig. 6. Growth of \textit{Mtb} cells in presence of cations and oxidizing agents. \textit{Mtb} strains were grown in Sauton's media supplemented with varying concentrations of A) CaCl\(_2\). B) NaCl. C) KCl. 7H9-OAD media supplemented with varying concentrations of D) H\(_2\)O\(_2\). E) SNP. The OD\(_{600}\) of mycobacteria growing in absence of cations or oxidizing agents was considered as positive control (100% of cell growth).
indeed, S. pneumoniae proteins, and 4) restoration of the calcium concentration by inducing a metal influx system to maintain calcium homeostasis when the tubercle bacillus is surviving in highly enriched extracellular environment against the concentration gradient. There are reports that intracellular calcium accumulation in S. pneumoniae activates molecular systems in response to oxidative stress; indeed, S. pneumoniae defective in Ca\(^{2+}\)-ATPase is more sensitive to oxidative stress compared to WT strain [50]. In agreement, our data showing the hypersensitivity to oxidizing agents of the \(\Delta ctpF\) strain to oxidative stress, suggest a link of the efflux pump function of CtpF with more complex cellular processes, as the response to toxic substances encountered during infection, or bacterial signaling in other to respond to the arsenal of the host cell. In conclusion, our experiments indicate that CtpF transports calcium from mycobacterial cells to the extracellular environment against the concentration gradient.

5. Conclusions

CtpF resembles SERCA, the well-studied P2-type Ca\(^{2+}\)-ATPase in higher eukaryotes. Our data show that CtpF is in fact a bacterial Ca\(^{2+}\)-ATPase. The expression of CtpF in M. smegmatis allows recombinant cells to withstand a transient increase in calcium concentration. Accordingly, \(\Delta ctpF\) cells exhibit higher calcium accumulation and increased susceptibility to the cation. The increased susceptibility of the \(\Delta ctpF\) strain to oxidative stress, suggest a link of the efflux pump function of CtpF with more complex cellular processes, as the response to toxic substances encountered during infection, or bacterial signaling in other to respond to the arsenal of the host cell. In conclusion, our experiments indicate that CtpF transports calcium from mycobacterial cells to the extracellular environment against the concentration gradient.

Fig. 7. Tolerance of Mtb cells to metal cations. Mycobacterial cells were grown in Sauton media supplemented with varying concentrations of CaCl\(_2\), KCl, and NaCl. OD\(_{600}\) of cultures supplemented without cations were considered as 100% of cell growth. Values represent the IC\(_{50}\). Data are mean ± SEM from three independent experiments. Unpaired two-tailed \(t\) test, *\(P < 0.05\), **\(P < 0.01\).

Fig. 8. Response of Mtb WT and \(\Delta ctpF\) to oxidative and nitrosative stresses. Bacteria were grown in 7H9-OAD media supplemented with varying concentrations of H\(_2\)O\(_2\) and SNP. OD\(_{600}\) of cultures in absence of oxidant agent are considered the 100%. Values represent the IC\(_{50}\). Data are mean ± SEM from three independent experiments. Unpaired two-tailed \(t\) test, ****\(P < 0.0001\).

WT cells. Therefore, CtpF plays an important role in calcium homeostasis by preventing a toxic metal overload and favoring mycobacterial survival under infection conditions.

Undoubtedly, \(\Delta\) requires an efflux system to maintain calcium homeostasis when the tubercle bacillus is surviving in highly enriched calcium environments, such as lungs and mucous membranes [50]. The deletion of P-type ATPases in mycobacteria is known to cause unbalanced cation transport across the plasma membrane and impaired capacity to respond to toxic substances [8, 9, 12, 13, 14]. It is likely that different concerted cellular events contribute to maintaining the optimal levels of intracellular calcium, including: 1) calcium influx; 2) an increase in the intracellular calcium concentration; 3) calcium binding of target proteins, and 4) restoration of the calcium concentration by inducing a metal influx mechanisms, in which CtpF could be relevant.

There are reports that intracellular calcium accumulation in higher eukaryotes. Our data show that CtpF is in fact a bacterial Ca\(^{2+}\)-ATPase. The expression of CtpF in M. smegmatis allows recombinant cells to withstand a transient increase in calcium concentration. Accordingly, \(\Delta ctpF\) cells exhibit higher calcium accumulation and increased susceptibility to the cation. The increased susceptibility of the \(\Delta ctpF\) strain to oxidative stress, suggest a link of the efflux pump function of CtpF with more complex cellular processes, as the response to toxic substances encountered during infection, or bacterial signaling in other to respond to the arsenal of the host cell. In conclusion, our experiments indicate that CtpF transports calcium from mycobacterial cells to the extracellular environment against the concentration gradient.

Declarations

Author contribution statement

Milena Maya-Hoyos, Cristian Rosales: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Lorena Novoa-Aponte: Conceived and designed the experiments; Wrote the paper.

Eliana Castillo: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Carlos Yesid Soto: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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References

[1] World Health Organization, Global Tuberculosis Report 2018, 2018.
[2] P. Andersen, T.J. Scriba, Moving tuberculosis vaccines from theory to practice, Nat. Rev. Immunol. 19 (2019) 550–562.
[3] L. Yatime, M.J. Buch-Pedersen, M. Mugaaga, J.P. Morth, A.M.L. Winther, B.P. Pedersen, C. Olsen, J.P. Andersen, B. Vilsen, B. Schiott, M.G. Palmgren, J.V. Müller, P. Nissen, N. Fedossova, P-type ATPases as drug targets: tools for medicine and science, Biochim. Biophys. Acta Bioenerg. 1787 (2009) 207–220.
[4] J.P. Morth, B.P. Pedersen, M.J. Buch-Pedersen, J.P. Andersen, B. Vilsen, M.G. Palmgren, P. Nissen, A structural overview of the plasma membrane Na\(^+\)-,K\(^+\)-ATPase and H\(^+\)-ATPase ion pumps, Nat. Rev. Mol. Cell Biol. 12 (2011) 60–70.
[5] M. Bublitz, H. Poulsen, J.P. Morth, P. Nissen, In and out of the cation pumps: P-Type ATPase structure revisited, Curr. Opin. Struct. Biol. 20 (2010) 431–439.
[6] M.G. Palmgren, P. Nissen, P-type ATPases, Annu. Rev. Biophys. 40 (2011) 243–266.
[7] T. Soldati, O. Neyrolles, Mycobacteria and the intraphagosomal environment: take it with a pinch of salt(s)!, Traffic 13 (2012) 1042–1052.
H.D. Park, K.M. Guinn, M.I. Harrell, R. Liao, M.I. Voskuil, M. Tompa, D. Schnappinger, S. Ehrt, M.I. Voskuil, Y. Liu, J.A. Mangan, I.M. Monahan, M. Maya-Hoyos et al. Heliyon 5 (2019) e02852

S.L. Kendall, F. Movahedzadeh, S.C.G. Rison, L. Wernisch, T. Parish, K. Duncan, J. Bacon, B.W. James, L. Wernisch, A. Williams, K.A. Morley, G.J. Hatch, P.a. Pulido, L. Novoa-Aponte, N. Villamil, C.Y. Soto, The DosR dormancy regulator is a transcription factor that mediates adaptation of Mycobacterium tuberculosis within macrophages insights into the inner cell membrane, Biometals 28 (2015) 713–724.

M.U. Shiloh, P. Manzanillo, J.S. Cox, Mycobacterium tuberculosis senses host-derived carbon monoxide during macrophage infection, Cell Host Microbe 3 (5) (2008) 323–330.

A. Kumar, J.S. Deshane, D.K. Grossman, S. Bolinett, B.S. Yan, I. Kramnik, A. Agarwal, A.J.C. Steyn, Heme oxygenase-1-derived carbon monoxide mediates the Mycobacterium tuberculosis dormancy program, J. Biol. Chem. 283 (2008) 18032–18039.

G.K. Schoolnik, D.R. Sherman, Rv3133c/dosR is a transcription factor that mediates adaptation of Mycobacterium tuberculosis in response to anti-bacterial compounds, Tuberculosis 84 (3–4) (2004) 253–274.

H. Ohno, G. Zhu, V.P. Mohan, D. Chu, S. Kohno, W.R. Jacobs, J. Chan, The effects of reactive nitrogen intermediates on gene expression in Mycobacterium tuberculosis, Cell Microbiol. 5 (9) (2003) 1403–1408.

S.H. Cho, D. Goodlett, S. Franzblau, ICAT-based Comparative Proteomic Analysis of Non-replicating Persistent Mycobacterium tuberculosis, Tuberculosis 86 (6) (2006) 445–460.

D.G.N. Muttucumaru, G. Roberts, J. Hinds, R.A. Stabler, T. Parish, Gene Expression Profile of Mycobacterium tuberculosis in a Non-replicating State, Tuberculosis 84 (3–4) (2004) 239–246.

J.C. Betts, N.G. Stoker, The Mycobacterium tuberculosis dosRS two-component system is induced by multiple stresses, Tuberculosis 84 (3–4) (2004) 247–255.

T. Padilla-Benavides, J.E. Long, D. Raimunda, C.M. Sassetti, J.M. Argüello, A novel P1B-type Mo2+–transporting ATPase is required for secreted protein metallation in mycobacteria, J. Biol. Chem. 288 (2013) 11324–11347.

D. Raimunda, J.E. Long, T. Padilla-Benavides, C.M. Sassetti, J.M. Argüello, Differential roles for the Ca2+/Ni2+–transporting ATPases, CtpD and CtpE, in Mycobacterium tuberculosis virulence, Mol. Microbiol. 91 (4) (2015) 1847–1864.

S. Jayashree, H. Zhenying, D. Nithya, G. Prathamesh, A. Lokesh, S.S. Suresh, A Simple and Effective Assay for In Vivo Detection of Novel Ca2+–ATPase Transporters in Mycobacterial Cells, PLoS ONE 14 (9) (2019) e0222757.

M. Lajoie, S. Garcia-Franco, G. Schuster, D. Schnappinger, D. Tompkins, B. Niazi, S. Ehrt, L. Wernisch, A. Williams, K.A. Morley, G.J. Hatch, P.a. Pulido, L. Novoa-Aponte, N. Villamil, C.Y. Soto, An unexpected role for a mycobacterial P-type ATPase, is stimulated by copper (I) in the mycobacterial plasma membrane, Arch. Microbiol. 200 (3) (2018) 483–500.

J. Immunol. 174 (3) (2005) 1491–1500.

M. Lajoie, S. Garcia-Franco, G. Schuster, D. Schnappinger, D. Tompkins, B. Niazi, S. Ehrt, L. Wernisch, A. Williams, K.A. Morley, G.J. Hatch, P.a. Pulido, L. Novoa-Aponte, N. Villamil, C.Y. Soto, An unexpected role for a mycobacterial P-type ATPase, is stimulated by copper (I) in the mycobacterial plasma membrane, Arch. Microbiol. 200 (3) (2018) 483–500.

J. Immunol. 174 (3) (2005) 1491–1500.

D. Schnappinger, D. Tompkins, B. Niazi, S. Ehrt, L. Wernisch, A. Williams, K.A. Morley, G.J. Hatch, P.a. Pulido, L. Novoa-Aponte, N. Villamil, C.Y. Soto, An unexpected role for a mycobacterial P-type ATPase, is stimulated by copper (I) in the mycobacterial plasma membrane, Arch. Microbiol. 200 (3) (2018) 483–500.

J. Immunol. 174 (3) (2005) 1491–1500.

D. Schnappinger, D. Tompkins, B. Niazi, S. Ehrt, L. Wernisch, A. Williams, K.A. Morley, G.J. Hatch, P.a. Pulido, L. Novoa-Aponte, N. Villamil, C.Y. Soto, An unexpected role for a mycobacterial P-type ATPase, is stimulated by copper (I) in the mycobacterial plasma membrane, Arch. Microbiol. 200 (3) (2018) 483–500.

J. Immunol. 174 (3) (2005) 1491–1500.