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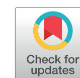
Research article

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 findings support the hypothesis that CtpF contributes to calcium efflux from mycobacterial cells. Accordingly, *Mtb* cells defective in *ctpF* (*Mtb*Δ*ctpF*) 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 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 of 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 maintain mycobacterial metal homeostasis during infection [7, 8, 9, 11, 12, 13, 14].

P-type ATPases are classified into five subfamilies (P_1–P_5), based on ionic specificity and structural characteristics [4, 6]: P_{1A}-type bacterial potassium transporters; P_{1B}-type heavy metal pumps; P_2-type alkaline/alkaline earth metal transporters; P_{3A}-type H^+ pumps; P_{3B}-type bacterial Mg^{2+} pumps; P_4-type putative lipid flippases; and the uncharacterized P_5-type ATPase pumps [4, 5, 6, 15]. Bioinformatic studies identified 12 P-type ATPases in the *Mtb* genome, namely: seven P_{1B}-type, four P_2-type, and one P_{1A}-type ATPases [16, 17, 18]. Regarding

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the functional characterization of these *Mtb* plasma membrane pumps, CtpA is a Cu^{+} 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 Cu^{+} pump [9], and CtpG is a Cd^{2+} transporter [20]. However, the ion specificity of 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, tetrahydrolipstatin [33], reactive nitrogen species (RNS), reactive oxygen species (ROS) [28, 29, 30, 34, 35], and under hypoxia [27, 29, 36, 37, 38]. This transcriptional behavior suggests that CtpF could be part of the strategies of the tubercle bacillus uses 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 together with the calcium accumulation in whole cells and plasma membrane vesicles from recombinant *M. smegmatis* overexpressing *Mtb* CtpF and *Mtb* cells defective in *ctpF* (*Mtb*Δ*ctpF*) confirmed that this transporter is associated with Ca^{2+} pumping from mycobacterial cells. In addition, *Mtb*Δ*ctpF* 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 7H11 agar plates supplemented with OADC and glycerol. For the experiments of bacterial tolerance to cations, the mycobacteria were grown in Sauton's medium (pH = 7.4) supplemented with 0.05 % 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 kanamycin (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].

Table 1
Bacterial strains and plasmids used in this study.

Strains	Relevant features	Reference
Mycobacterium tuberculosis		
H37Ra	Slow-growing attenuated strain, Amp^R, Chx^R, Cb^R	ATCC 25177
H37Ra:pJV53	Recombineering strain (with pJV53), Amp^R, Chx^R, Cb^R, Km^R	This study
H37RaΔctpF	Δ*ctpF*, gene replaced by a Hyg^R cassette	This study
Mycobacterium smegmatis		
mc^2155	Fast-growing, usually non-pathogenic *Mycobacterium*	ATCC 700084
Escherichia coli		
DH5α	*recA*-, *endA*-, Blue/white color screening with *lacZ*ΔM15	Thermo Fisher Scientific
TOP10	F– *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*D139 Δ(*ara*A-*leu*)7697 *gal*U *gal*K *rps*L *end*A1 *nup*G	Thermo Fisher Scientific
Plasmids		
pMV261	*E. coli-Mycobacterium* shuttle vector, *hsp*60 promoter, Km^R	[57]
pLNA29	*Mtb Rv1997* (*ctpF*) cloned into pMV261, KmR	This study
pJV53	Derivative of pLAM12 with *Che9c 60–61* genes under control of the acetamidase promoter	Gift from Unizar [43]
pYUB854	Hyg^R cassette is flanked by the γδ-res sites and by two MCSs for directional cloning of the homologous recombination substrates	Gift from Unizar [58]
pLNA22	607 bp upstream and 520 bp downstream of *Mtb Rv1997* (*ctpF*) in pYUB854	This study

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/FpmvHis-Rev (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 amplimer, flanked by the *BamHI* 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^2155 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 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 pGEM®-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* (*Mtb*Δ*ctpF*) were screened by colony PCR (primers listed in Table 2). Genomic DNA was isolated from the selected *Mtb* mutant to confirm the targeted gene

Table 2
List of oligonucleotides used in this study.

Primer	Sequence (5′-3′)
F-RT Dir	CAGTGATCTTCGGTGTGGTG
F-RT Rev	GATTGAGCGTGAACGAGTCA
pMVComp Up	CAGCGAGGACAACTTGAGC
pMVComp Down	CGACTGCCAGGCATCAAATA
Fcm2 Dir	TTTTGGGATCCATTGTCGGCGTCAGTGTCTG
Fpmv His Rev	TTTTTAAGCTTCAATGATGATGATGATGATGTGGCGGTTGCGCCCGTA
pJV53dir	GTCAGTCACCAACCCTCCAC
pJV53rev	GAATCCTGCTTGGTGACAGC
ctpF_interno_dir	CTATGCACCCGACGTCCT
cpF_interno_rev	GAACCTGGTATCACGTTTTCG
Comp_Up-ctpF	TCGTCGAACACTCGTACCTG
Comp_Down_ctpF	CGTCCGCAACCTAGTTGAAT
primerpYUB854	GTGGCTCCCTCACTTTCTGG
Hyg_dir_out	ACTTCGAGGTGTTCGAGGAG
Adir2013	TTTTCTCGAGCGGATGGCAAGACC
Arev2013	TTTTGCTAGCGCGCGTTACCACC
Bdir2013	TTTTTCTAGATATCGGGGTGTGGGTGC
Brev2013	TTTTTCATGATACCACCAGCACGATCCAG

replacement by PCR, using primers matching within the Hyg^R 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 OD_{600} = 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–Zor–Selinger [45] or the BCA methods 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 OD_{600} = 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 final concentration of salts: 10 mM $CaCl_2$, 240 mM NaCl or 240 mM NaCl/40 mM KCl, followed by incubation at 37 °C for 1 h with agitation. Subsequently, cells were harvested and washed twice with washing buffer. Pellets were dried at 37 °C to constant weight and mineralized with 500 μL of HNO_3 (trace metal grade) for 1 h at 80 °C, followed by overnight incubation at 20 °C. Sample digestions were stopped by adding 30% H_2O_2 and were completed with 2% high purity HNO_3 in H_2O (18 MOhm*cm). The metal content of the samples was measured by flame absorption spectroscopy using a 300 Atomic Absorption Spectrometer (Perkin Elmer, USA). The calcium accumulation was normalized against the amount of calcium measured in the WT cells (dry mass) [9, 12, 13].

To assess the calcium accumulation inside membrane vesicles, 10 μg of protein (membrane vesicles) were mixed with reaction buffer (25 mM MOPS, 250 mM sucrose, 3 mM $MgSO_4$, 150 mM KCl, 0.05 % Brij-58, 3 mM Na_2ATP) and incubated at 37 °C for 1 min. Reactions were initiated by adding 100 μM Ca^{2+}, incubated at 37 °C for 30 min, stopped by filtration through 0.22 μM pore size filters (Millipore, USA), and the filters were washed two times with washing buffer (25 mM MOPS, 250 mM sucrose, 3 mM $MgSO_4$, 150 mM KCl, 0.05 % Brij-58, 1 mM EGTA). The filters were then mineralized as above. The metal content of the samples was 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 $MgCl_2$, 10 mM MOPS, pH = 7.4) and supplemented with 0.02 % Brij-58, 0.29 mM Ca^{2+} and 0.25 mM EGTA (final concentrations) to control the amount of free calcium. Maxchelator software was used to calculate the free Ca^{2+} [47]. The enzymatic reactions were initiated by adding 3 mM Na_2ATP and incubating at 37 °C for 30 min. The reactions were stopped by adding 100 μL of stopping solution (3 % ascorbic 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 OD_{690}. 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 diluted until OD_{600}=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: Ca^{2+} (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 OD_{600} 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 IC_{50} 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.

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 $OD_{600} = 0.05–0.06$. 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: H_2O_2 (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 OD_{600} 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 IC_{50} 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 Ca^{2+} P-type ATPase

Among the 12 ORFs that encode P-type ATPases in the *Mtb* genome, four of them have been classified as P_2-type ATPases: CtpE, CtpF, CtpI, 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 P_2-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 displays high homology with some prokaryotic organisms. In contrast, no X-ray structures of bacterial Ca^{2+} P-type ATPases have been resolved. There are only biochemical characterizations of some bacterial Ca^{2+} P-type ATPases from *Listeria monocytogenes* and *Streptococcus pneumoniae* [22, 50].

Members of the P_2-type ATPases group share the characteristic PEGL-motif and the conserved amino acids of the ion-binding pocket [51]. As shown in Fig. 1, CtpF is closely related to SERCA by sharing 8 of the 10 residues involved in calcium binding in SERCA [52]. The calcium coordination sites in SERCA are named Site I (Asn768, Glu771, Thr799, Asp800, Glu908) and Site II (Val304, Ala305, Ile307, Glu309, Asn796, Asp800) [52]. We observed that CtpF displays all of these residues, except for Ala305 that is substituted by a Gly residue and Asp800 that is substituted by an Ala residue (Fig. 1). Thus, even when the primary structures of CtpF (905 aa; 95 kDa) and SERCA (994 aa; 110 kDa) display just a 33% overall identity, they share an 80% identity with respect to the residues involved in Ca^{2+} coordination.

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 *Mtb*Δ*ctpF* cells. The latest were obtained by homologous recombination using the Che9c system (Fig. 2A) [43]. The allelic exchange replacement of the *ctpF* locus in the *MtbΔctpF* 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 γδ resolvase sites, allowing the further removal of the Hygromycin 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 Ca^{2+} than the control cells (WT transformed with empty pMV261). This suggests that CtpF may be involved in the active Ca^{2+} 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/40 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 *MtbΔctpF* strain. In agreement, *MtbΔctpF* cells accumulated 4-fold more Ca^{2+} 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 Ca^{2+}in the reaction milieu. As shown in Fig. 4B, vesicles obtained from recombinant *M. smegmatis* cells accumulated more Ca^{2+} than vesicles obtained from WT cells, confirming that CtpF transports the metal from the cytoplasm.

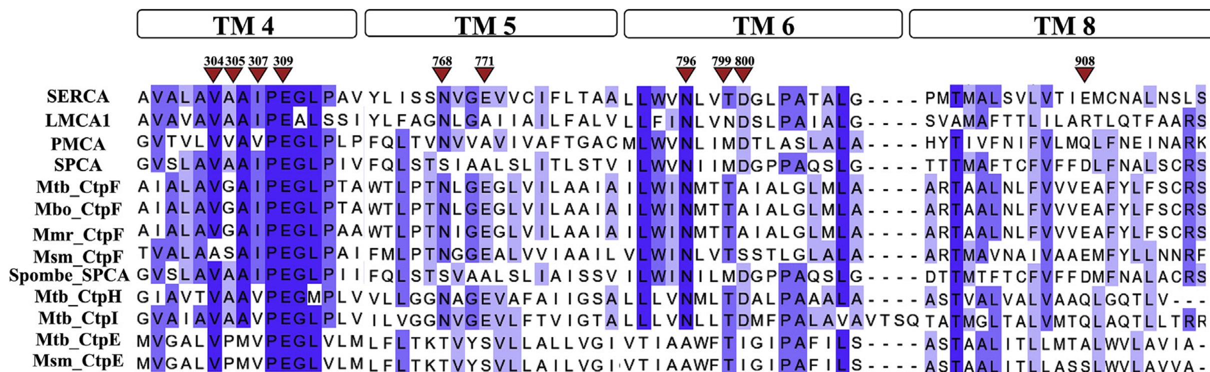

Fig. 1. *Mtb* CtpF possesses the characteristic Ca^{2+} binding residues of P-type ATPases. Multiple sequence alignment of 12 P_2-type ATPases in MEGA X using Muscle. All sequences were retrieved from UniProt. Jalview 2.10.5 was used to visualize the final result. The arrows show the 10 key calcium-binding 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.

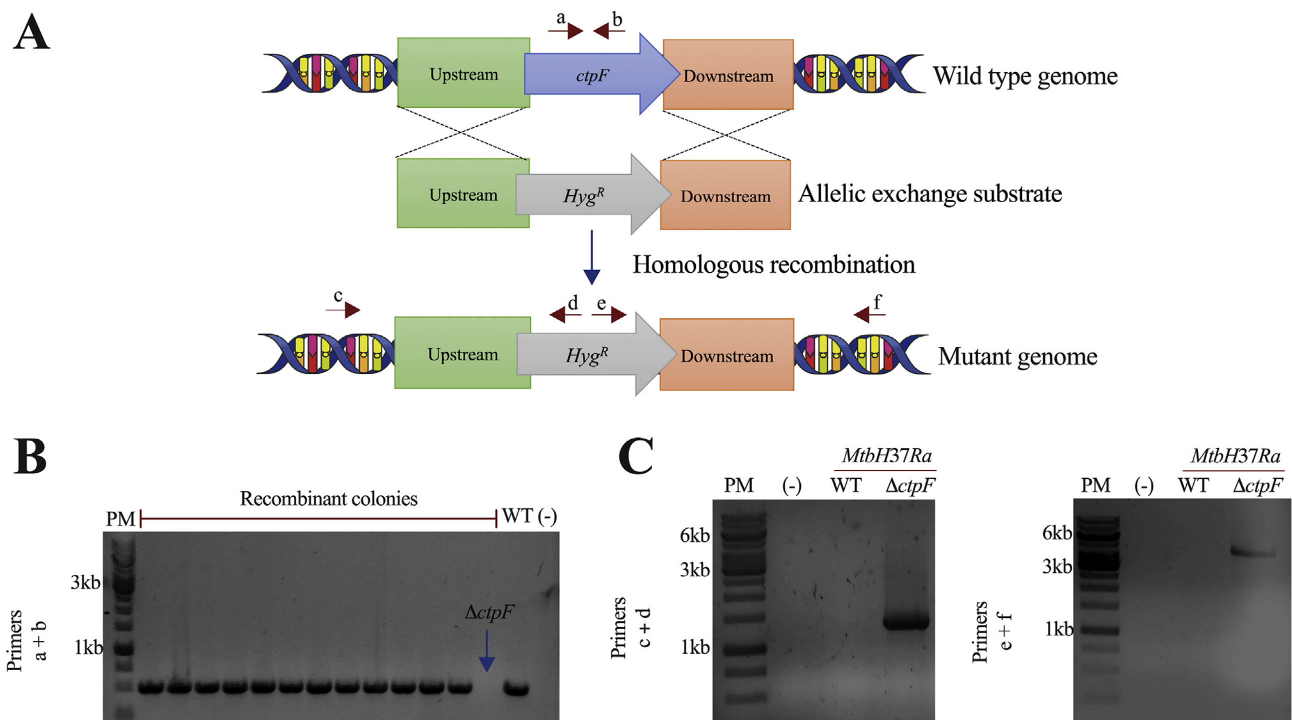

Fig. 2. Targeted allelic exchange of the *Mtb ctpF* locus. A) Schematic representation of the homologous recombination process to generate a *ctpF* knockout mutant of *Mtb*. B) Thirteen colonies were examined by PCR using primers a and b (508 pb for the WT locus and no product for the mutant). The recombinant strain showing the absence of the *ctpF* gene was selected and named *MtbΔctpF*. DNA from *Mtb* WT was used as control. C) A PCR analysis was performed using different combinations of primers c and d or e and f to confirm the homologous recombination in the target locus; no PCR product is expected from WT cells. Primers d and e are located within the Hyg^R cassette and only match in the chromosome of mutant strain.

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

We assessed the Ca^{2+}-dependent ATPase activity on plasma membrane vesicles obtained from mycobacterial cells. The enzyme reactions were supplemented with ATP as energy source, Mg^{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^{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^{2+} ATPase activity increased only 7 % in the *MtbΔctpF* 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^{2+}-dependent ATPase activity in vesicles from *MtbΔctpF* cells is in agreement with the proposed role of CtpF, as Ca^{2+}-efflux pump. Importantly, since the ATPase activity was measured on crude membrane extracts, part of the Ca^{2+}-stimulated ATPase activity in vesicles from *MtbΔctpF* cells should be attributed to other Ca^{2+}ATPases present in the mycobacterial plasma membrane. Corroborating CtpF is a Ca^{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^{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^{2+}, K^+, and Na^+ (Fig. 6). Assuming that CtpF is a potential calcium transporter, *MtbΔctpF* should be sensitive to high concentrations of calcium. As observed in Fig. 7, *MtbΔctpF* cells were more susceptible to Ca^{2+} than WT. Specifically, the IC_{50} value of calcium in *MtbΔctpF* cells (1.7 mM) was significantly lower than in WT cells (2.5 mM). Even though the tolerance of *MtbΔctpF* to Na^+ was significantly lower than WT, no difference in the tolerance to of K^+ 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 *MtbΔctpF* and *Mtb* WT to ROS/RNS stress conditions (Figs. 6 and 8). As observed in Fig. 8, *MtbΔctpF* cells were more sensitive to oxidative and nitrosative stresses compared to *Mtb* WT. With IC_{50} values of 1.6 mM H_2O_2 and 85 µM SNP for *MtbΔctpF* cells and 2.9 mM H_2O_2 and 214 µM SNP for *Mtb* WT, confirming that *MtbΔctpF* 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^{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

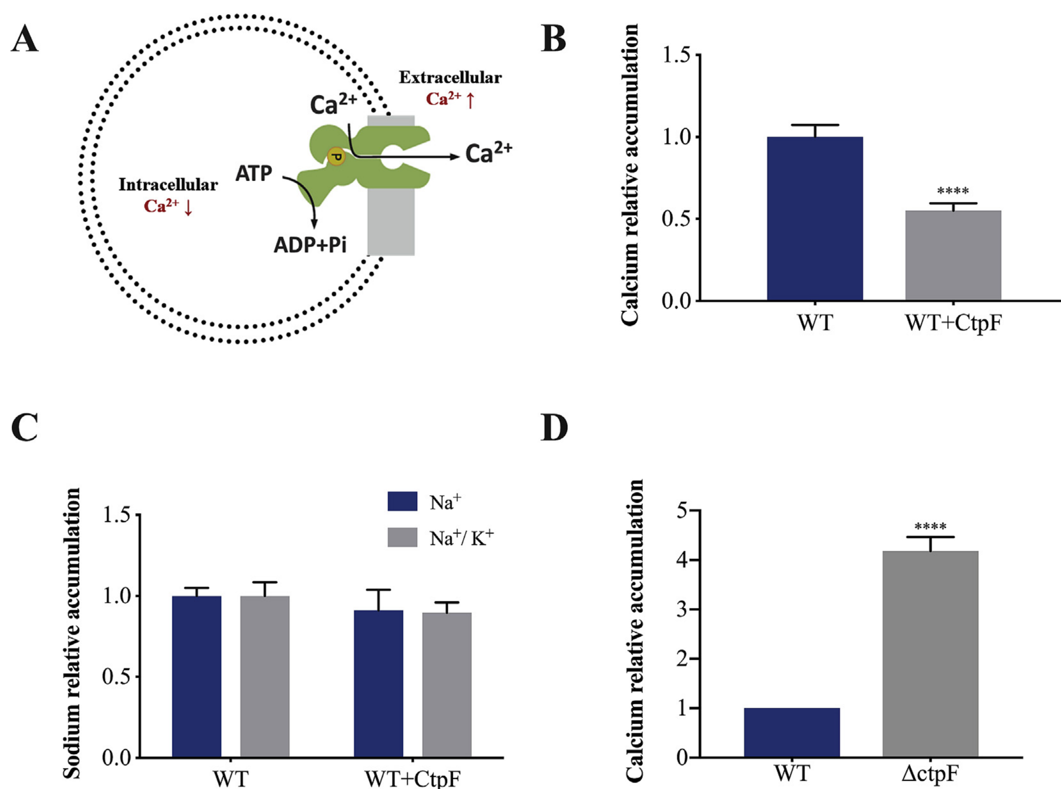

Fig. 3. Calcium accumulation in mycobacterial cells. A) Hypothesized function of CtpF in the mycobacterial plasma membrane. In the presence of a high extracellular calcium concentration, CtpF transports calcium against its concentration gradient. B) Calcium accumulation inside *M. smegmatis* WT and the 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, $****P < 0.0001$.

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 P_2-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

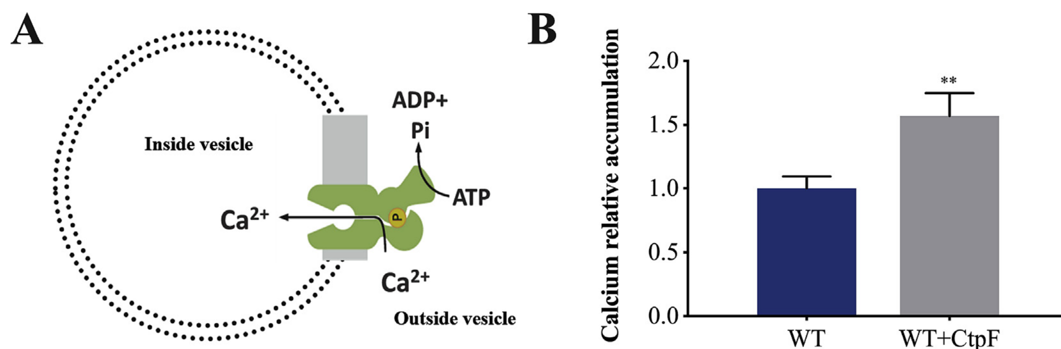

Fig. 4. Calcium accumulation in membrane vesicles obtained from *M smegmatis* cells. A) Model of inverted membrane vesicles where the cytoplasmic domains of CtpF are exposed to the reaction medium. B) Calcium accumulation in WT and recombinant membrane vesicles from cells expressing *ctpF*. All normalized 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, $**P < 0.01$.

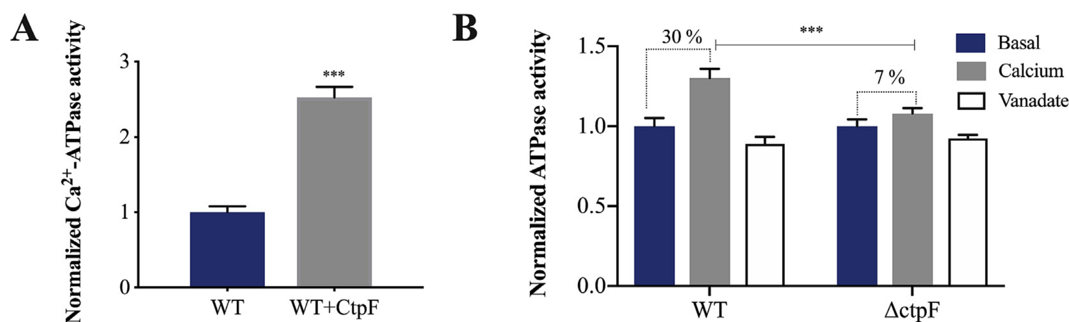

Fig. 5. Ca^{2+} P-type ATPase mediated by CtpF. A) Ca^{2+}-dependent ATPase activity of membrane vesicles of recombinant *M. smegmatis* normalized against the control strain (*M. smegmatis* WT). B) Ca^{2+}-ATPase activity of membrane vesicles of *Mtb* WT and *MtbΔctpF* against the basal ATPase activity. 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.

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 *MtbΔctpF* corroborates that CtpF is a calcium efflux pump. This is: 1) *MtbΔctpF* cells have an impared capacity to restore cytoplasmic calcium levels upon extracellular Ca^{2+} exposure; 2) Vesicles isolated from *MtbΔctpF* exhibited a reduced Ca^{2+}-dependent ATPase activity and 3) *MtbΔctpF* cells are more susceptible to Ca^{2+} than

Fig. 6. Growth of *Mtb* cells in presence of cations and oxidizing agents. *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_2O_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).

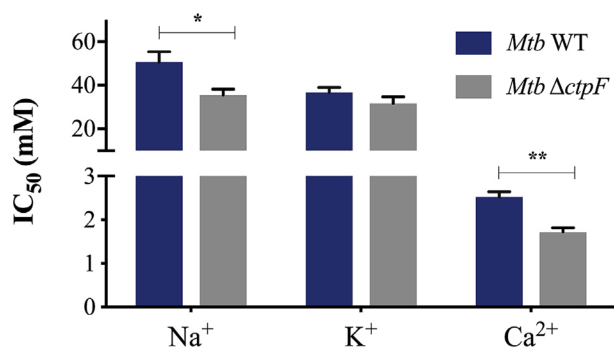

Fig. 7. Tolerance of *Mtb* cells to metal cations. Mycobacterial cells were grown in Sauton media supplemented with varying concentrations of $CaCl_2$, KCl, and NaCl. OD_{600} of cultures supplemented without cations were considered as 100% of cell growth. Values represent the IC_{50}. Data are mean ± SEM from three independent experiments. Unpaired two-tailed *t* test, *P < 0.05, **P < 0.01.

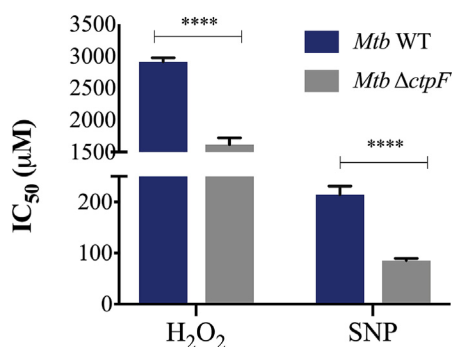

Fig. 8. Response of *Mtb* WT and *MtbΔctpF* to oxidative and nitrosative stresses. Bacteria were grown in 7H9-OAD media supplemented with varying concentrations of H_2O_2 and SNP. OD_{600} of cultures in absence of oxidant agent are considered the 100%. Values represent the IC_{50}. Data are mean ± SEM 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, *Mtb* 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 efflux mechanisms, in which CtpF could be relevant.

There are reports that intracellular calcium accumulation in *S. pneumoniae* activates molecular systems in response to oxidative stress; indeed, *S. pneumonia* defective in Ca^{2+}-ATPase is more sensitive to oxidative stress compared with the WT strain [50]. In agreement, our data showing the hypersensitivity to oxidizing agents of the *MtbΔctpF* strain suggest a correlation between calcium pumping and oxidative stress response in mycobacteria. This is in agreement with *ctpF* expression being regulated by the dormancy regulator DosR in response to low levels of O_2 and nitric oxide exposure [26, 27, 29, 34, 38]. We speculate that CtpF is activated in response redox stress, a characteristic stress condition faced by pathogens during infection. This implies a critical function of CtpF in *Mtb*; however, experiments evaluating the importance of CtpF during infection are necessary to further confirm this hypothesis.

5. Conclusions

CtpF resembles SERCA, the well-studied P_2-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, *MtbΔctpF* cells exhibit higher calcium accumulation and increased susceptibility to the cation. The increased susceptibility of the *MtbΔctpF* 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, materials, 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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