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ORIGINAL PAPER

The P-type ATPase CtpG preferentially transports Cd^{2+} across the *Mycobacterium tuberculosis* plasma membrane

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Abstract

P_{1B}-type ATPases are involved in heavy metal transport across the plasma membrane. Some *Mycobacterium tuberculosis* P-type ATPases are induced during infection, suggesting that this type of transporter could play a critical role in mycobacterial survival. To date, the ion specificity of *M. tuberculosis* heavy metal-transporting P_{1B}-ATPases is not well understood. In this work, we observed that, although divalent heavy metal cations such as Cu^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+} Cd^{2+} and Pb^{2+} stimulate the ATPase activity of the putative P_{1B}-type ATPase CtpG in the plasma membrane, whole cells of *M. smegmatis* expressing CtpG only tolerate high levels of Cd^{2+} and Cu^{2+}. As indicator of the catalytic constant, Michaelis–Menten kinetics showed that CtpG embedded in the mycobacterial cell membrane has a V_{max}/K_m ratio 7.4-fold higher for Cd^{2+} than for Cu^{2+} ions. Thus, although CtpG can accept different substrates *in vitro*, this P-type ATPase transports Cd^{2+} more efficiently than other heavy metal cations across the mycobacterial plasma membrane.

Keywords *Mycobacterium tuberculosis* · Cell membrane · P-type ATPases · CtpG · Heavy metal tolerance

Introduction

Tuberculosis (TB) is caused by the acid-fast bacillus *Mycobacterium tuberculosis*, and is a major public health issue, with 8.6 new cases and 1.3 million people dying from TB around the world in 2014 (WHO 2015). Currently, a third of the world's population is infected with *M. tuberculosis*, which establishes a permanent infection reservoir and facilitates the spread of TB (WHO 2010). TB and HIV co-infection and the emergence of multi- and extensively drug-resistant TB (MDR- and XDR-TB) have delayed efficient TB control. Therefore, understanding the mechanisms used by tubercle bacilli for survival within host cells is essential for the development of new strategies to prevent latent and active *M. tuberculosis* infections.

Mycobacterium-infected cells increase toxic cation concentrations, which deleteriously react with mycobacterial lipids, proteins, and DNA (Agranoff and Krishna 1998). Increased concentrations of Cu^{2+}, Zn^{2+}, and Fe^{2+} produce an effective response mediated by inflammatory cytokines, such as TNF and INFɤ, inside phagosomes infected with *M. tuberculosis* (Wagner et al. 2005), indicating that high doses of heavy metals interfere with progression of infection. During infection, *M. tuberculosis* uses cell membrane transporters to avoid increased intracellular concentrations of heavy metal cations, such as Co^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+}, Zn^{2+}, $Mn^{2+}$and Cd^{2+}. Thus, mycobacterial cell membrane transporters are critical for ion homeostasis and bacterial survival inside macrophages (Raimunda et al. 2012).

Cadmium does not have apparent specific biological functions in bacteria, however, it competes with cell storages of Mn^{2+} and Zn^{2+}, and displaces Ca^{2+} and Zn^{2+} from proteins. In addition, Cd^{2+} interacts with nucleic acids, competes as a cofactor for proteins and makes bacteria susceptible to oxidative stress with the production of reactive oxygen species (Paulsen et al. 1996; Haney et al. 2005). In general, Cd^{2+} detoxification systems are activated by intracellular bacteria in response to the high concentration of this cation

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in phagocytic cells as a consequence of infection. P-type ATPase pumps, together with the cation diffusion facilitator (CDF) and resistance-nodulation-division (RND) transporter families are the main Cd^{2+} detoxification system in bacteria. These protein families are also used in bacteria to efflux other toxic cations such as Co^{2+}, Cu^{2+}, Ni^{2+}, Ag^{+}, Pb^{2+}. In some cases, genes encoding Cd^{2+} detoxification are the same as those used in Zn^{2+} detoxification (Paulsen et al. 1996; Haney et al. 2005). CDF transporters are found in bacteria, archaea, and eukarya, and correspond to polypeptides of 400 kDa approximately, which contain six transmembrane segments (TMS) and perform cation/H + exchange chemoprocessing in the periplasmic space, such as the case of CzcD protein from *Cupriavidus metallidurans* (Haney et al. 2005). Likewise, RND is a superfamily of bacterial transporters, which form part of a multimeric complex in the plasma membrane. An example is the CzcCBA complex, an antiporter cation/H^{+} of toxic ions, including Cd^{2+} that is transported externally from the cytoplasm of *C. metallidurans* (Paulsen et al. 1996).

There are also detoxifying proteins, such as metallothioneins that sequester heavy metal cations and increasing bacterial tolerance to these toxic substances. For instance, the bacterial cytoplasmic metallothioneins, such as SmtA from *Synechococcus* and *P. putida* (Olafson et al. 1988; Blindauer et al. 2002) and metallo-chaperones (Blencowe and Morby 2003) detoxify bacteria from toxic metals such as Cd^{2+} and Zn^{2+}.

P-type ATPases responsible for Cd^{2+} detoxification belong to the $Zn^{2+}/Cd^{2+}/Pb^{2+}$ translocating P_{1B}-type ATPases (Argüello 2003). P_{1B}-type, as well as the other P-type ATPases, use the energy released in ATP hydrolysis to pump heavy metal cations (Argüello et al. 2007). The key steps in this process include: (1) metal binding on the cytosolic ATPase portion, (2) metal transfer to the ATPase transmembrane segment (TMS), (3) ATP hydrolysis and enzyme autophosphorylation of a conserved arginine residue, (4) metal release in the extracellular side, and (5) subsequent enzyme dephosphorylation to reinitiate the cycle. Specifically, P_{1B}-type ATPases, also known as CPx-type ATPases, transport toxic heavy metal cations to the cellular periplasm (Sharma et al. 2006). The ion specificity of P_{1B}-type ATPases is given by the metal binding domain that is located in the N-terminal cytoplasmic region (Argüello 2003). P_{1B}-type ATPases are present in many bacterial species, including *Escherichia coli* (ZntA), *Staphylococcus aureus* (CadA), *Pseudomonas putida*, and *C. metllidurans*, among others (Nucifora et al. 1989; Rensing et al. 1997; Hu and Zhao 2007; Scherer and Nies 2009).

Interestingly, tubercle bacilli contain a high number of heavy metal P_{1B}-type ATPase transporters compared to other intracellular pathogens, suggesting that these transporters could be critical for mycobacterial survival (Novoa-Aponte et al. 2012). *M. tuberculosis* contains twelve open-reading frames annotated as P-type ATPases; seven of which are putative P_{1B}-type ATPases (CtpA, CtpB, CtpC, CtpD, CtpG, CtpJ, and CtpV). They are responsible for catalyzing the translocation of heavy metals across the cell membrane (Novoa-Aponte et al. 2012). The role of putative mycobacterial P_{1B}-type ATPases for dealing with high doses of heavy metal ions is not well understood. However, it is known that P_{1B}-type ATPases, such as CtpC and CtpD, are required for murine infection with *M. tuberculosis* (Sassetti and Rubin 2003); CtpA and CtpC are overexpressed during the infection processes (Botella et al. 2011); and CtpV is needed for *M. tuberculosis* virulence (Ward et al. 2010; Raimunda et al. 2014). In addition, we recently described that CtpA is stimulated by high doses of Cu^{+} in the mycobacterial plasma membrane (León-Torres et al. 2015).

In particular, CtpG is an interesting heavy metal transporter. This putative P-type ATPase has been associated with different mechanisms used for *M. tuberculosis* survival, for example, with a role in Zn^{2+} poisoning in human macrophages and survival in human phagocytic cells (Botella et al. 2011). Furthermore, CtpG is activated in response to the oxidizing agent diamide in oxidative stress processes (Hampshire et al. 2004) and to *M. tuberculosis* starvation (Betts et al. 2002; Hampshire et al. 2004). To date, the ion specificity and function of CtpG remains elusive. In this work, we performed bioinformatics predictions and estimated the tolerance of *M. smegmatis* cells expressing CtpG to sublethal doses of different heavy metal cations, together with the ATPase activity mediated by CtpG of plasma membrane vesicles from recombinant cells. The results obtained evidenced that CtpG preferentially transports Cd^{2+} across the mycobacterial plasma membrane.

Materials and methods

Strains, culture conditions, and genomic DNA isolation

M. smegmatis $mc^{2}155$ (ATCC 700084) cells (Snapper et al. 1990) were grown in Luria–Bertani (LB) broth until an $OD_{600} = 0.3$ for electroporation experiments, 0.1 for cell viability assays and 0.4 for cell membrane isolation. For the viability assays, the cells were harvested and resuspended in LB containing 0.05% Tween 80, separately supplemented with heavy metal cations, and incubated at 37 °C. *E. coli* BL21 (Agilent Technologies, CA, USA) cells were grown at 37 °C in LB broth or plates. *M. tuberculosis* H37Rv (ATCC 27294) genomic DNA was isolated from cells cultured in 7H9-ADC broth (Somerville et al. 2005).

Expression of *M. tuberculosis* H37Rv CtpG in *M. smegmatis* cells

Plasmids and primers used are listed in Table 1. The *ctpG* (Rv1992c) gene was amplified by PCR from genomic DNA of *M. tuberculosis* H37Rv using the primer pairs ctpG-pMV Dir/ctpG-pMV Rev that respectively introduce the restriction sites *EcoR*I and *Sal*I to the 5´ and 3´ ends of the *ctpG* gene. The amplimer obtained was inserted into the mycobacterial/*E. coli* shuttle vector pMV261 (Stover et al. 1991) to produce the pML01 plasmid (Table 1), which was subsequently transformed into *E. coli* BL21 cells (Agilent Technologies, CA, USA). The recombinant cells were screened by colony PCR using the primer pairs pMV comp-up/RT ctpG-Rev. The integrity of the *ctpG* gene was confirmed by sequencing of pML01, using the same primer pairs. Finally, *M. smegmatis* mc^2155 cells were electropored with pML01 or pMV261 (control plasmid), and transformation was confirmed by colony PCR using the primer pairs pMVcomp Up/RT ctpG-Rev and Tm903A/Tm903B, respectively. Finally, the *M. tuberculosis* CtpG protein was expressed on the *M. smegmatis* cell membrane by incubating recombinant cells at 45 °C.

Cell membrane isolation

Differential centrifugation was used to isolate the cell membranes as previously described (Basu et al. 1992; Ayala et al. 2015). Mycobacterial cells were harvested, centrifuged, and resuspended in lysis buffer (10 mM MOPS, 1 mM EDTA, and 0.3 mM phenylmethylsulfonyl fluoride, pH 7.4), and cells were then lysed in a Mini Beadbeater-16 (Biospec, OK, USA). Cell debris was isolated by centrifugation at 25,000*g* for 30 min at 4 °C, and the cell membranes were obtained from the supernatant by centrifugation at 100,000*g* for 90 min at 4 °C. The membrane fraction was resuspended in buffer containing 10 mM MOPS and 250 mM sucrose (pH 7.4), and analyzed by SDS–PAGE.

ATPase activity

ATPase activity was measured for the plasma membrane vesicles from wild-type *M. smegmatis* mc^2155 and cells transformed with pMV261 or pML01. The inorganic phosphate (P_i) release by the ATPase catalytic activity was quantified as previously described (Fiske and Subbarow 1925; Cariani et al. 2004; León-Torres et al. 2015). The enzymatic reactions (50 µL) were performed in incubation buffer (40 mM MOPS, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 5 mM NaN_3, 0.25 mM Na_2MoO_4, and 0.02% Brij-58, pH 7.4) using 8.0 µg membrane vesicles, and individually supplemented with 25 µM (final concentration) of each heavy metal cation: $CoCl_2$, Cu SO_4, $ZnSO_4$, $MnSO_4$, $NiSO_4$, $Pb(CH_3COO)_2$ and $CdCl_2$. Reactions supplemented with Cu^+ were performed in the presence of DTT (2.5 µM) and cysteine (0.2 mM) (León-Torres et al. 2015). The reactions were subsequently initiated by adding 1 mM Na_2ATP, then incubated at 37 °C for 30 min, and terminated by adding 100 µL of stop solution (3% ascorbic acid, 0.5% ammonium molybdate, and 3% SDS in 1.0 M HCl). Finally, 150 µL of 3.5% bismuth citrate and 3.5% sodium citrate in 2.0 M HCl were added, and samples were incubated at 37 °C for 10 min. The difference between the total ATPase activity and the activity obtained with no cations (basal activity) was considered the ATPase activity stimulated by the tested cations. In addition, the ATPase activity attributed to CtpG was estimated by subtracting the

Table 1 Bacterial strains, plasmids and primers used in this study

	Description	Reference
Strains		
M. smegmatis mc^2155	Efficiently transformable strain	ATCC 700084
E. coli BL21	pET15b compatible expression host	Novagen
Vectors and plasmids		
pGEM-T easy vector	*E. coli* cloning vector for PCR fragments, Ampr	Promega
pMV261	*E. coli*/mycobacteria shuttle vector, Kmr	Stover, (1991)
pML01	*ctpG* gene cloned into the pMV261 vector, Kmr	This study
Primers (5′–3′)		
ctpG-pMV Dir	ttttgaattcgtgacgactgtagttgacg (EcoRI site)	This study
ctpG-pMV Rev	ttttgtcgacagtgacgctattgctgg (SalI site)	This study
pMV comp Up	cagcgaggacaacttgagc	This study
pMV comp Down	tatttgatgcctggcagtcg	This study
RT ctpG- Dir	ggtccagctgaccgttgtat	This study
RT ctpG-Rev	attgcgcgtgaataccagat	This study
Tm903 A	ctcgtgaagaaggtgttgct	This study
Tm903 B	ccgaccatcaagcattttat	This study

activity of the membrane vesicles from cells transformed with pMV261 to the activity obtained from cells transformed with pML01. The enzymatic activity was reported as nmol of Pi released/mg of protein × min, and assessed from three independent experiments.

Cell viability assay

Mycobacterial cells (*M. smegmatis* mc^2155 transformed with pML01 or pMV261) were harvested and diluted in culture medium until an OD_{595} of 0.05 was reached. Then, 100 µL of bacterial suspension were separately mixed in 96-well plates with 100 µL of serial dilutions of heavy metal cations: Cu^{2+} (4.0 to 0.75 mM), Co^{2+} (1.2 to 0.4 mM), Mn^{2+} (100 to 10 mM), Ni^{2+} (2 to 0.4 mM), Zn^{2+} (4.0 to 1.0 mM), Cd^{2+} (0.3 to 0.035 mM), and Pb^{2+} (3.5 to 1.0 mM, pH = 5.5). Subsequently, the cultures were incubated at 37 °C for 72 h at 80 rpm. Finally, the OD_{595} of the cultures was measured in an iMARKTM Microplate Reader (Bio-Rad, CA, USA). Cultures supplemented with no cation and kanamycin were considered as 100 and 0% growth, respectively (Ayala-Torres et al. 2015; León-Torres et al. 2015).

Kinetic parameters of CtpG

The ATPase activity stimulated by the heavy metal cations in plasma membrane vesicles expressing *M. tuberculosis* CtpG was estimated as previously described (León-Torres et al. 2015). The optimum quantity of the membrane protein was estimated using 2–15 µg of membrane vesicles in the enzymatic reactions. The pH dependence of ion metal transport was evaluated in reactions performed with 8.0 µg of membrane vesicles and 25 µM of each cation, varying the pH from 5.9 to 8.7 using 10 mM MOPS (pH values below 7.9) and 10 mM TRIS (pH values above 8.2). The optimum temperature was evaluated in reactions at pH 7.5, by varying the temperature from 4 to 60 °C. All of the enzymatic reactions were performed for 30 min. The enzymatic activity was reported as nmol of Pi released/mg of protein × min and assessed from three independent experiments.

Bioinformatics analyses

The *ctpG* nucleotide sequence was retrieved from Tuberculist (Lew et al. 2011). The topology of the TMS was predicted with ExPASy (Gasteiger et al. 2005), TMHMM 2.0 (Zankari et al. 2013), TMpred (Hofmann and Stoffel 1993), HMMTOP (Tusnády and Simon 2001), Phobius (Käll et al. 2004), DAS (Cserzo et al. 1997), TMDET (Tusnády et al. 2005), and PPM server (Lomize et al. 2012). The tertiary structure and potential binding sites were predicted using Phyre2 (Kelley and Sternberg 2009). Modeling and validation of the tertiary structure was performed using Swiss Model (Biasini et al. 2014), What If (Vriend 1990), and PROCHECK (Laskowski et al.1993). The quality of the model was evaluated using PROSA (Wiederstein and Sippl 2007). ClustalW2 (Larkin et al. 2007) and BLASTP were used to align the CtpG amino acid sequence with the well characterized bacterial P-type ATPases ZntA from *E. coli* (P37617), ZntA of *Shigella sonnei* (Q3YW59), CopA (O29777) and CopB (O30085) from *Archaeoglobus fulgidus* (Mana-Capelli et al. 2003), and ZosA from *Bacillus subtilis* (O31668).

Results

Different divalent heavy metal cations are possibly transported by CtpG

The *ctpG* gene (Rv1992c) is annotated in the *M. tuberculosis* H37Rv genome as a 771 amino acid (2316 bp) transmembrane protein that possibly catalyzes the transport of an undetermined metal cation with hydrolysis of ATP, and part of the P-type ATPases classified in the group of acid anhydride hydrolases (Lew et al. 2011). The predicted isoelectric point and molecular weight of CtpG are 5.55 and 79.3 kDa, respectively. In a previous study, we classified CtpG as a P_{1B}-type ATPase, due to the presence of the WI (YE) (RG) motif located between positions 406 and 409 (TMS6), and the LS motif in TMS7 that it is associated to Zn^{2+} P-type ATPases (Futai et al. 2004; Lewinson et al. 2009). The hydrophobicity profile of *M. tuberculosis* CtpG is similar to that of ATPase pumps experimentally associated with Cd^{2+}, Zn^{2+}, and Co^{2+} transport (Novoa-Aponte et al. 2012). According to the PPM server, CtpG displays a topology typical of heavy metal transporting P_{1B}-type ATPases, which includes eight TMS, whose function is to transport heavy-metal cations. In addition, the InterProScan server suggests that CtpG contains its phosphorylation site (DKTGTLT) within TMS6 and TMS7 (Fig. 1). Interestingly, an uncommon heavy metal binding motif APCAL was found in TMS6, suggesting that CtpG is an atypical P_{1B}-type ATPases. On the other hand, the modelled 3D structure demonstrated that the transport domain of CtpG is typical of type I ATPases.

The *M. tuberculosis* CtpG amino acid sequence shows 38% identity with the *M. smegmatis* CtpD protein, a Co^{2+} transporting P_{1B}-type ATPase (Raimunda et al. 2012). *M. smegmatis* CtpD is orthologous of *M. tuberculosis* CtpD, which is also a Co^{2+} transporting P_{1B}-type ATPase in *M. tuberculosis* together with the paralogous heavy-metal transporter CtpJ. Although the amino acid sequences of all of the above-mentioned $P\text{-}_{1B}$ type ATPases contain the catalytic domains of heavy metal $P_{1B\text{-}4}$ type ATPases, specifically CtpG does not show the SCP motif in the TMS6 (Novoa-Aponte et al. 2012). Therefore, CtpG could display cation

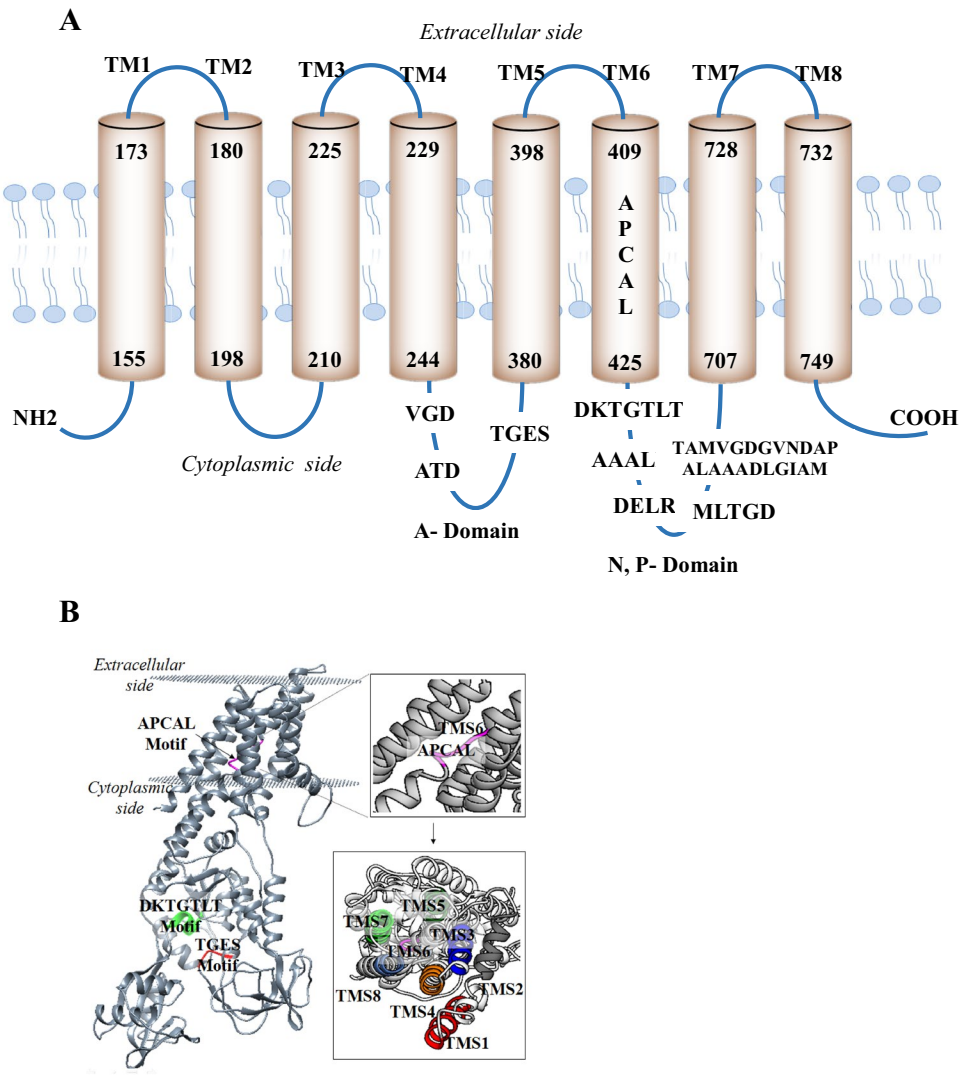

Fig. 1 Prediction of metal-binding sites of *M. tuberculosis* CtpG. **a** Membrane type I topology of CtpG. **b** Model of the tertiary structure of CtpG (Ramachandran *Z* score −0.581), constructed using the 4 templates: 4umv (35.3% identity). The model was validated using PROSA (Wiederstein and Sippl 2007) obtaining a *Z* score −7.66, PROCHECK (Laskowski et al. 1993), and WHAT-IF (Vriend 1990) obtaining a Ramachandran *Z* score −0.581

transporting characteristics different to *M. tuberculosis* CtpJ, and CtpD from *M. tuberculosis* and *M. smegmatis*. According to the alignment with other heavy metal P_{1B}-type ATPases, CtpG showed a similarity between 34 and 39% (query cover from 78 to 80%) with ZntA from *E. coli*, CopA and CopB of *A. fulgidus*, and ZosA from *B. subtilis* that are putative Cu^{+} and Cu^{2+} P-type ATPases, respectively. In conclusion, the bioinformatics analyses suggest that different heavy metal cations, such as Cd^{2+}, Zn^{2+}, Cu^{2+}, and Co^{2+}, are potentially transported by *M. tuberculosis* CtpG.

M. tuberculosis CtpG was heterologously expressed in the *M. smegmatis* $mc^{2}155$ plasma membrane

The heterologous expression of CtpG in the cell membrane of the non-pathogenic and environmental *M. smegmatis* allows estimation of the activity and biological effect of CtpG embedded in the natural environment of the mycobacterial cell membrane. The *EcoR*I and *Sal*I restriction sites introduced by PCR in the 5`and 3′-ends of the *M. tuberculosis ctpG* gene, respectively, allowed *ctpG* to be directionally ligated into the pMV261shuttle vector, which contains replication origins for *E. coli* (*oriE*) and one for mycobacteria (*oriM*). PCR amplification, enzyme digestion mapping, and DNA sequencing verified the integrity of *ctpG* and its insertion into the correct open-reading frame with the *hsp60* promoter of pMV261 in the pML01 recombinant plasmid (Supplementary Fig. 1).

The ATPase activity associated with *M. tuberculosis* CtpG is stimulated by different heavy metal cations in the mycobacterial plasma membrane

Based on the bioinformatics predictions and previous works (Botella et al. 2011), we assessed the ATPase activity in plasma membrane vesicles enriched with CtpG and separately stimulated by different heavy metal cations (Co^{2+}, Cu^{2+}, $Cu^{+,}$ Zn^{2+}, Mn^{2+}, Ni^{2+}, $Pb^{2+}$and Cd^{2+}), as a first

approach to identify the possible ion specificity of CtpG. Since the ATPase activity was assessed on membrane vesicles containing ion transporters other than P-type ATPases, the enzymatic activity associated with CtpG was calculated by subtracting the ATPase activity value of membranes from *M. smegmatis* cells transformed with the expression vector pMV261 from the activity obtained from cell membranes expressing CtpG. First, the optimal quantity of protein in the enzymatic reactions was estimated. Therefore, the reaction samples were supplemented with at least 8.0 µg of membrane vesicles to produce values of ATPase activity independent of the amount of protein. The enzymatic reactions were also separately supplemented with 25 µM of each heavy metal cation, which ensured high enzymatic activity in every case (data not shown). Statistically, significant ATPase activities associated with *M. tuberculosis* CtpG were observed by stimulation with Cu^{2+} (9.42 ± 0.44 U/mg of protein × min), Co^{2+} (4.08 ± 0.22 U/mg of protein × min), Ni^{2+} (2.67 ± 0.69 U/mg of protein × min), Zn^{2+} (2.22 ± 0.44 U/mg of protein × min), Cd^{2+} (5.03 ± 1.69 U/mg of protein × min), Pb^{2+} (4.28 ± 0.70 U/mg of protein × min), and Mn^{2+} (1.83 ± 0.27 U/mg of protein × min). Only the presence of Cu^{+} ions did not stimulate the ATPase activity of CtpG. The obtained values of ATPase activity suggested that Cu^{2+}, Co^{2+}, Pb^{2+}, and Cd^{2+} are the most likely substrates of CtpG; however, this transporter is able to transport other divalent heavy metal cations at a lower level across the mycobacterial cell membrane (Fig. 2).

CtpG is associated with Cu^{2+} and Cd^{2+} pumping across the mycobacterial plasma membrane

Cell viability assays were performed to evaluate the tolerance of CtpG-expressing *M. smegmatis* cells to toxic levels of the potentially transported cations, to gain insight of the actual heavy metal ions pumped by this cell membrane transporter. Since cultures supplemented with high doses of cations were sometimes colored, the percentage of growth was taken as the difference between the OD_{595} of culture growth in the presence and absence of heavy metal cations. As shown in Fig. 3, the cell viability of *M. smegmatis* transformed with pML01 or the control vector pMV261 did not tolerate toxic concentrations of Ni^{+}, Zn^{2+}, Mn^{2+}, Co^{2+}, and Pb^{2+}. Conversely, the viability of *M. smegmatis* cells expressing CtpG was up to 4.4-fold higher under toxic levels of Cu^{2+} (1.5 mM) compared to the control cells. Similarly, the viability of recombinant cells was between 11 and 36-fold higher than the viability of control cells under toxic levels of Cd^{2+} (0.05 to 0.1 mM). Therefore, the cell viability assays strongly suggest that CtpG is associated with the tolerance of toxic concentrations of copper and/or cadmium.

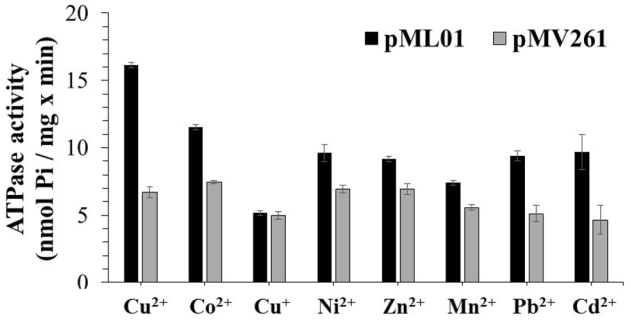

Fig. 2 ATPase activity of the mycobacterial cell membrane stimulated by heavy metal cations. The ATPase activity associated with *M. tuberculosis* CtpG was estimated as the difference between the activity stimulated by the heavy metal cations and the basal ATPase activity of cells expressing the recombinant plasmid pML01 and control cells. The ATPase activity corresponds to nmol of Pi released/mg of protein × min. The SD was calculated from triplicates of two independent experiments

CtpG preferentially transports Cd^{2+} across the mycobacterial plasma membrane

The enzymatic reactions were always supplemented with excess ATP to estimate the actual dependence of enzymatic activity on other experimental variables. Based on the results obtained in the cell viability assays, we estimated the dependence of the ATPase activity on Cu^{2+} and Cd^{2+} concentrations. We observed that the ATPase activity increased with the amount of membrane vesicles, up to a maximum of 6 µg of protein (Fig. 4). Regarding pH dependence, the optimal ATPase activity stimulated by the heavy metal cations ranged from pH 6.8 to 7.4. Additionally, the optimal temperature was always 37 °C (Fig. 4). The enzyme kinetics showed an apparent K_m of 0.108 ± 0.007 µM and a V_{max} of 0.856 ± 0.011 (nmol of Pi/mg of protein × min) for Cd^{2+}, and K_m of 0.981 ± 0.0422 µM and V_{max} of 1.051 ± 0.155 (nmol of Pi/mg of protein × min) for Cu^{2+} (Fig. 5). Thus, CtpG displayed a V_{max}/K_m ratio 7.4-fold higher for Cd^{2+} compared with Cu^{2+} ions, suggesting that CtpG preferentially transports Cd^{2+} across the mycobacterial cell membrane.

Discussion

The ion specificity assigned to bacterial P-type ATPases has been open to interpretation depending on the experimental approaches used to investigate it. The methods used in this work have been previously useful to ascertain the transport preferences of some mycobacterial P-type ATPases (Andreu et al. 2004; Ayala-Torres et al. 2015; León-Torres et al. 2015). The *in silico* hydrophobicity profile of the CtpG is similar to those of ATPases experimentally associated with Cu^{2+}, Cd^{2+}, Co^{2+}, and Zn^{2+} efflux, and typical of P_{1B}-type ATPases. Interestingly, the [WI (YE) (RG)] motif between

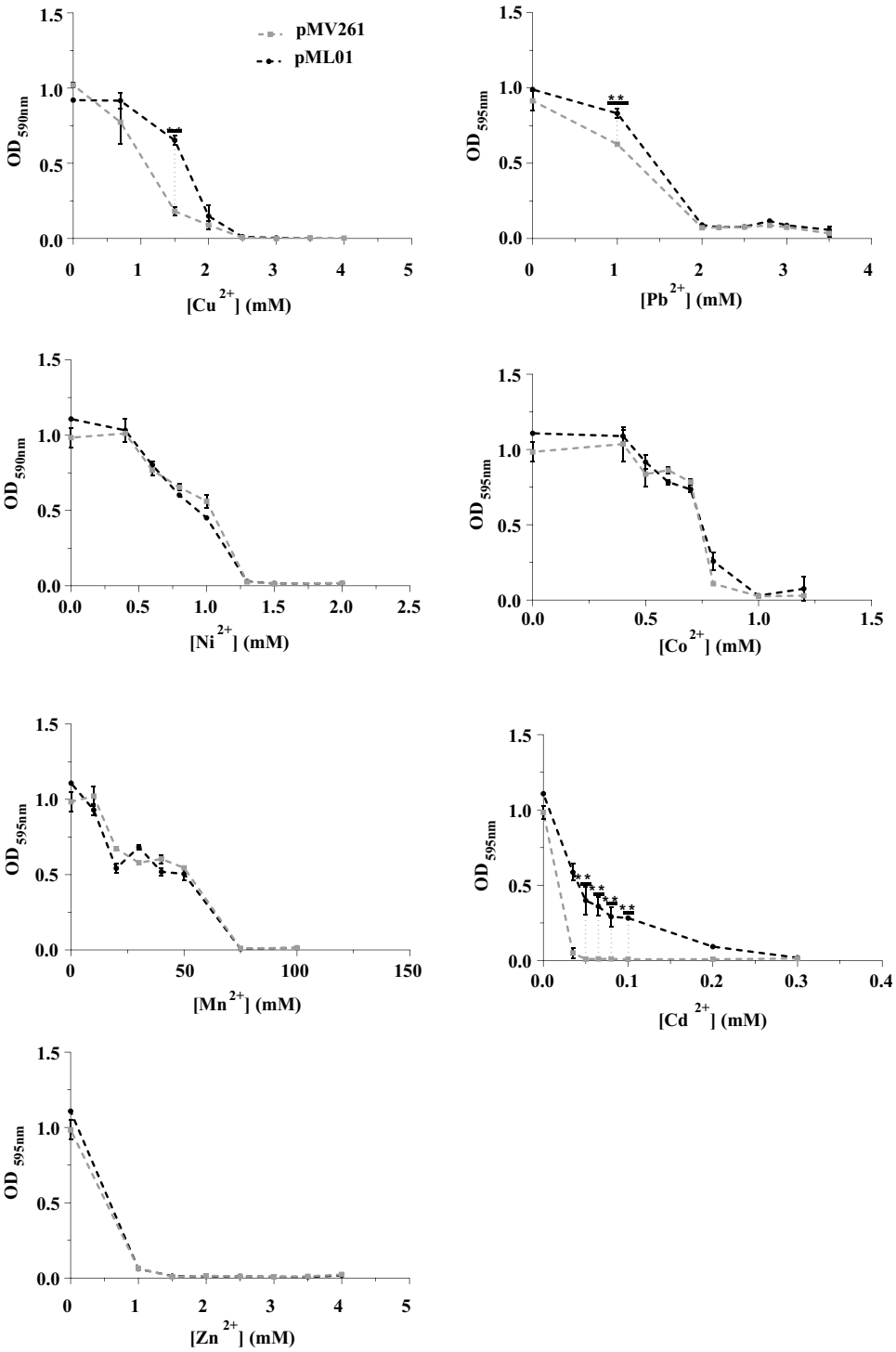

Fig. 3 Metal sensitivity of mycobacterial cells. The OD_{595} of *M. smegmatis* mc^2155 expressing *M. tuberculosis* CtpG was compared with control cells transformed with the vector pMV261. Each data represent triplicates in three independent experiments. The dotted lines (**) show significant differences in cell growth between *M. smegmatis* mc^2155 expressing *M. tuberculosis* CtpG and control cells ($P < 0.05$) cultured under toxic levels of Cu^{2+} (1.5 mM), Pb^{2+} (1.0 mM) and Cd^{2+} (0.05 to 0.1 mM)

positions 406 and 409 and the [LS] motif associates CtpG with Zn^{2+} transporters. Furthermore, most of the transport residues of CtpG are also found in the previously characterized P-type ATPase ZntA of *S. sonnei* (32.6% identity) suggesting that CtpG could be a Zn^{2+} transporting P-type ATPase. In agreement with this proposal are previous works that indicated the *ctpG* gene is upregulated as a consequence of Zn^{2+} poisoning in macrophages (Botella et al. 2011; Ward et al. 2008). The Zn^{2+} and Cu^{2+} intraphagosomal concentration increases 1 h after macrophages infection with *M. tuberculosis* (up to 37.8 and 426 µM respectively), but considerably diminish after 24 h of infection (Wagner et al. 2005). Therefore, tubercle bacilli need to activate detoxification systems to avoid heavy metal cation promotion of reactive

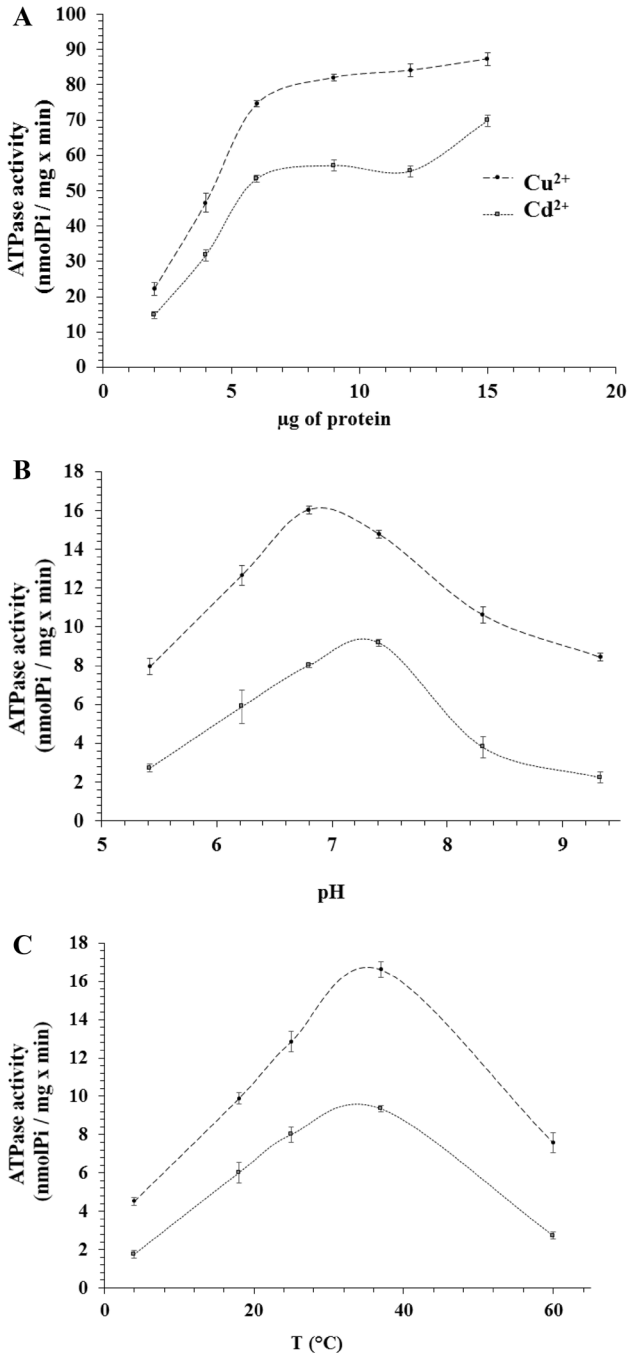

Fig. 4 Kinetic parameters of CtpG expressed in the *M. smegmatis* cell membrane. The kinetic parameters that were evaluated for CtpG-enriched membranes were the dependence in Cu^{2+} and Cd^{2+} ATPase activity of: **a** amount of membrane protein, **b** pH, and **c** Temperature. Bars represent the SD calculated from two independent experiments, each performed in triplicate

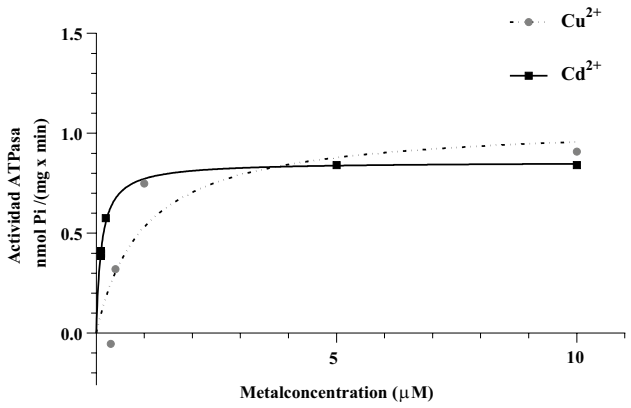

Fig. 5 Michaelis–Menten kinetics of recombinant *M. tuberculosis* CtpG expressed in the *M. smegmatis* cell membrane stimulated with Cu^{2+} and Cd^{2+} ions. Bars represent the SD calculated from two independent experiments, each performed in triplicate

oxygen species (ROS) and membrane destabilization during early infection (Ward et al. 2010). We observed that *M. smegmatis* cells expressing *M. tuberculosis* CtpG do not tolerate toxic levels of Zn^{2+}. In contrast, the recombinant cells tolerate high doses of other divalent heavy metal cations, such as Cu^{2+} and Cd^{2+}. This behavior suggests that CtpG contains the structural elements to bind different cations, which compete to be transported by this transporter. P-type ATPases recognizing different substrates are relatively common in bacteria; for example, the *E. coli* Zn^{2+} transporting P-type ATPase ZntA is able to bind other divalent cations such as Ni^{2+}, Co^{2+}, and Cu^{2+} with similar stoichiometric affinities (Liu et al. 2006). Regarding the *Mycobacterium* genus, the *M. smegmatis* Co^{2+} transporting P_{1B4}-type ATPase CtpD is also activated by different divalent cations, such as Ni^{2+} and Zn^{2+} to a lesser extent (Raimunda et al. 2012).

If CtpG potentially recognizes different divalent heavy metal cations, it raises the question as to which is the preferred substrate for this P-type ATPase. Mycobacterial viability assays showed that although CtpG is associated with Cd^{2+} and Cu^{2+} pumping across the *M. tuberculosis* cell membrane, the Michaelis–Menten kinetics (V_{max}/K_m) indicate that Cd^{2+} is preferentially transported by CtpG. It is therefore not surprising that toxic levels of Cd^{2+} activate CtpG *in vivo*. On the other hand, CmtR is a DNA-binding repressor that senses Cd^{2+} and regulates genes involved in reducing the intracellular levels of this heavy metal ion in human alveolar macrophages infected with tubercle bacilli (Grasseschi et al. 2003). Cd^{2+} de-represses the regulator CmtR, allowing the transcription of the *cmtR-Rv1993c-ctpG* operon of *M. tuberculosis* (Chauhan et al. 2009) and the CtpG expression.

It is known that cadmium from pollution and cigarette smoke accumulates in the pulmonary alveoli (Grasseschi et al. 2003) and *M. tuberculosis* subsequently exposes to high concentrations of this heavy metal inside macrophages. Thus, *M. tuberculosis* must activate cadmium detoxification and efflux systems, as could be for as the P-type ATPase, CtpG. In consequence, CtpG may be relevant during the

first hours of the *M. tuberculosis* infection (early infection) when the intrafagosomal concentration of Cd^{2+} and Cu^{2+} is elevated. In this context, the results obtained in this work could be significant because demonstrate that although Cd^{2+} and Cu^{2+} are potentially transported by CtpG, cadmium is the heavy metal more efficiently pumped outside mycobacterial cells; therefore, CtpG is a possible cadmium detoxification system belonging to the P-type ATPase family. If there was not an efficient cadmium detoxification system in mycobacterial cells, this toxic heavy metal could displace Ca^{2+} and Zn^{2+} from proteins and makes bacteria susceptible to oxidative stress (Paulsen et al. 1996; Haney et al. 2005).

On the other hand, regarding CtpG as a possible Cu^{2+} transporting P-type ATPase of *M. tuberculosis*, there are more relevant Cu^{2+} detoxification systems in *M. tuberculosis* (Rowland and Niederweis 2012) than CtpG; however, alternative copper transporters could be activated to preserve tubercle bacilli virulence. For instance, CtpV, which is associated to *M. tuberculosis* tolerance to toxic levels of copper ions, (Ward et al. 2010) displays the function of an alternative copper mycobacterial transporter. Therefore, it is not possible to exclude CtpG as an alternative copper transporter in *M. tuberculosis*.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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