



# A Novel Calcium Uptake Transporter of Uncharacterized P-Type ATPase Family Supplies Calcium for Cell Surface Integrity in *Mycobacterium smegmatis*

Hemant Kumar Gupta,<sup>a</sup> Shruti Shrivastava,<sup>a</sup>  Rakesh Sharma<sup>a,b</sup>

CSIR-Institute of Genomics and Integrative Biology, Council of Scientific and Industrial Research (CSIR), New Delhi, India<sup>a</sup>; Academy of Scientific and Industrial Research (AcSIR), New Delhi, India<sup>b</sup>

**ABSTRACT**  $\text{Ca}^{2+}$  plays an important role in the physiology of bacteria. Intracellular  $\text{Ca}^{2+}$  concentrations are tightly maintained in the nanomolar range. Molecular mechanisms of  $\text{Ca}^{2+}$  uptake in bacteria remain elusive. Here we show that CtpE is responsible for  $\text{Ca}^{2+}$  uptake in *Mycobacterium smegmatis*. It represents a previously uncharacterized P-type ATPase family in bacteria. Disruption of *ctpE* in *M. smegmatis* resulted in a mutant with impaired growth under  $\text{Ca}^{2+}$ -deficient conditions. The growth defect of the mutant could be rescued by  $\text{Ca}^{2+}$  or by ectopic expression of *ctpE* from *M. smegmatis* or the orthologous gene (*Rv0908*) from *Mycobacterium tuberculosis* H37Rv. Radioactive transport assays revealed that CtpE is a  $\text{Ca}^{2+}$ -specific transporter.  $\text{Ca}^{2+}$  deficiency increased expression of *ctpE*, resulting in increased  $^{45}\text{Ca}^{2+}$  accumulation in cells. *ctpE* is a gene that is part of an operon, which is negatively regulated by  $\text{Ca}^{2+}$ . The *ctpE* mutant also showed hypersensitivity to polymyxin B, increased biofilm formation, and higher cell aggregation, indicating cell envelope defects. Our work establishes, for the first time, the presence of  $\text{Ca}^{2+}$  uptake pumps of the energy-dependent P-type ATPase superfamily in bacteria and also implicates that intracellular  $\text{Ca}^{2+}$  is essential for growth and cell envelope integrity in *M. smegmatis*.

**IMPORTANCE**  $\text{Ca}^{2+}$  is essential for gene regulation, enzymatic activity, and maintenance of structural integrity of cell walls in bacteria. Bacteria maintain intracellular calcium concentrations in a narrow range, creating a gradient with low cytoplasmic calcium concentration and high extracellular calcium concentration. Due to this steep gradient, active pumps belonging to family 2 of P-type ATPases and antiporters are used for  $\text{Ca}^{2+}$  efflux, whereas  $\text{Ca}^{2+}$  uptake is usually carried out by channels. Molecular mechanisms of  $\text{Ca}^{2+}$  uptake in bacteria are still elusive and are mainly limited to a nonproteinaceous channel in *Escherichia coli* and a pH-dependent channel protein from *Bacillus subtilis*. Energy-dependent active transporters are not reported for  $\text{Ca}^{2+}$  uptake from any organism. Here we show that CtpE belonging to a family of previously uncharacterized bacterial P-type ATPases is involved in specific uptake of  $\text{Ca}^{2+}$  into *Mycobacterium smegmatis*. We also demonstrate that intracellular  $\text{Ca}^{2+}$  obtained through CtpE is essential for growth and maintenance of cell surface properties under  $\text{Ca}^{2+}$ -deficient conditions.

**KEYWORDS** calcium, *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, P-type ATPase, transporter

Calcium ( $\text{Ca}^{2+}$ ) plays an important role in the physiology of living organisms. These functions have been well characterized for eukaryotes and have begun to be characterized in prokaryotes (1). In prokaryotes, calcium has been implicated in chemotaxis (2), heterocyst differentiation (3, 4), twitching (5) and swarming (6) motility,

Received 18 August 2017 Accepted 22 August 2017 Published 26 September 2017

**Citation** Gupta HK, Shrivastava S, Sharma R. 2017. A novel calcium uptake transporter of uncharacterized P-type ATPase family supplies calcium for cell surface integrity in *Mycobacterium smegmatis*. mBio 8:e01388-17. <https://doi.org/10.1128/mBio.01388-17>.

**Invited Editor** Gloria Marcela Rodriguez, PHRI-Rutgers

**Editor** Eric J. Rubin, Harvard School of Public Health

**Copyright** © 2017 Gupta et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Rakesh Sharma, [rsharma@igib.res.in](mailto:rsharma@igib.res.in).

gene regulation (7), virulence (8, 9), and survival in an eukaryotic host (10). Another important function of  $\text{Ca}^{2+}$  is to maintain structural integrity of the bacterial membranes and cell wall (11). These functions are mediated by  $\text{Ca}^{2+}$ , either by acting as a structural or catalytic cofactor for various enzymes (12, 13) or by interaction of various  $\text{Ca}^{2+}$ -binding proteins (3, 5, 14, 15).

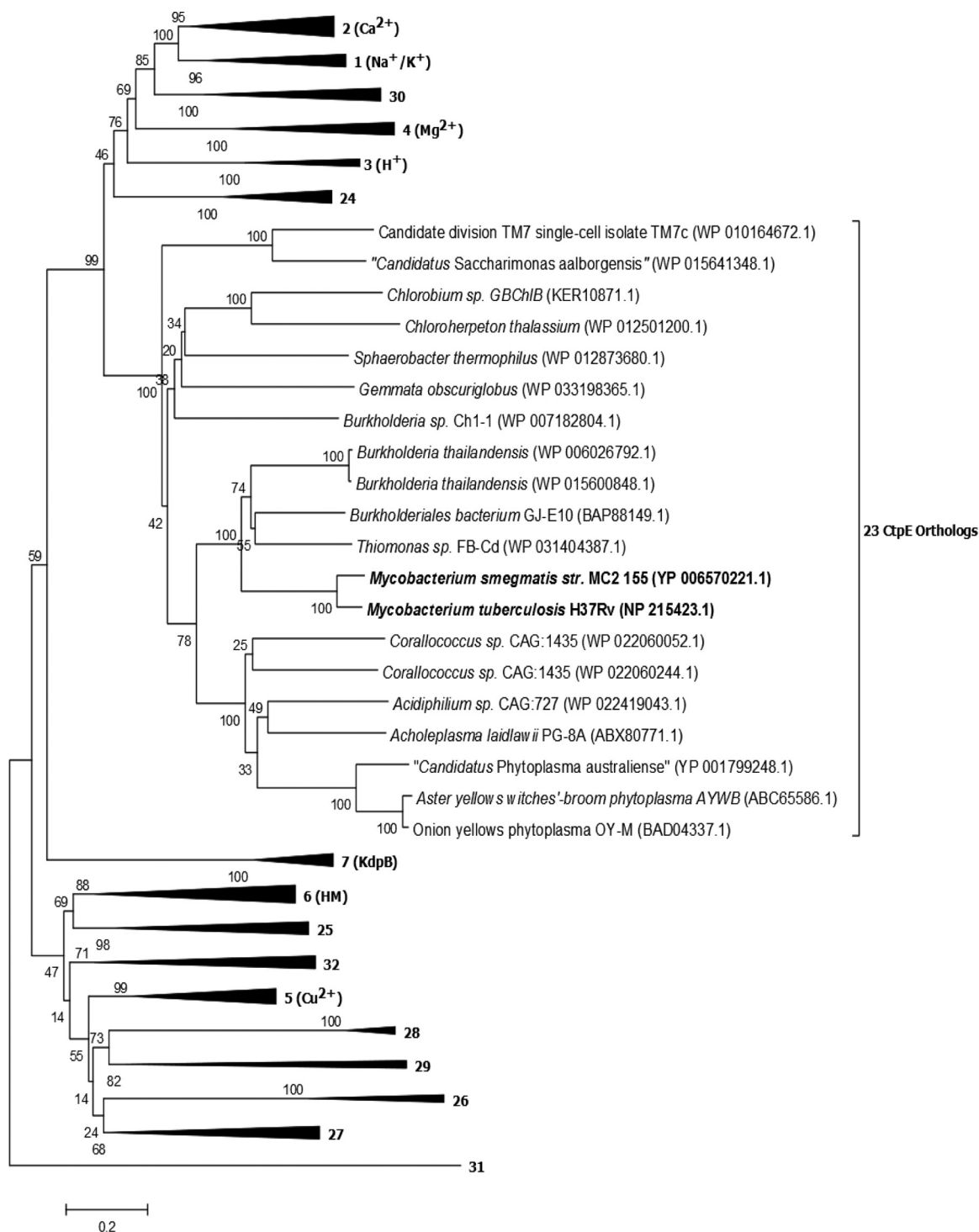
$\text{Ca}^{2+}$  concentration is tightly maintained in bacteria, usually many folds lower than the surrounding environment (1, 16, 17).  $\text{Ca}^{2+}$  homeostasis requires orchestrated activity of efflux and uptake systems along with  $\text{Ca}^{2+}$ -binding proteins.  $\text{Ca}^{2+}$  efflux requires transport against a stiff concentration gradient. Active transporters belonging to P-type ATPases of family 2 and antiporters have been identified from bacteria for their role in  $\text{Ca}^{2+}$  efflux (10, 18–21). Examples of molecular mechanisms of  $\text{Ca}^{2+}$  uptake in bacteria are few and limited to  $\text{Ca}^{2+}$  channels, *viz.*, a nonproteinaceous  $\text{Ca}^{2+}$ -selective channel from *Escherichia coli* (22) and a recently identified pH-sensitive  $\text{Ca}^{2+}$  channel from *Bacillus subtilis* (23). Though energy-dependent  $\text{Ca}^{2+}$  uptake is expected to be essential for survival in  $\text{Ca}^{2+}$ -deficient situations, to the best of our knowledge, to date, no active transporter has been identified and characterized from any organism.

P-type ATPases form a large superfamily of integral membrane proteins involved mainly in ATP-dependent transport of cations and phospholipids (24). Nine functionally characterized P-type ATPase families have been identified and are further classified into three groups; topological type I with 8 transmembrane  $\alpha$ -helical segments (TMS) represents heavy metal ATPases, topological type II with 10 TMS represents the  $\text{Na}^+/\text{K}^+$ ,  $\text{Ca}^{2+}$ ,  $\text{H}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{Na}^+$ ,  $\text{K}^+$ , or phospholipid ATPases, and topological type III represents 4 component  $\text{K}^+$  transporters (25). Genome sequencing has revealed previously uncharacterized P-type ATPases from eukaryotes as well as prokaryotes (25, 26). A comparative study on bacterial P-type ATPases has identified 10 new P-type ATPase families of unknown physiological functions (26). Out of these 10 new families, two families (families 23 and 24) clustered loosely together with family 2  $\text{Ca}^{2+}$ -ATPases (26) and belong to topology type II, indicating their possible involvement in  $\text{Ca}^{2+}$  transport. In this study, we selected CtpE, a member of family 23, from *Mycobacterium smegmatis* for further characterization. Here we present evidence that CtpE is a  $\text{Ca}^{2+}$ -specific uptake transporter in mycobacteria. It is expressed as part of an operon, and expression of this operon was found to be negatively regulated by  $\text{Ca}^{2+}$ . We further demonstrate that under conditions of  $\text{Ca}^{2+}$  deficiency, CtpE supplies  $\text{Ca}^{2+}$  for growth and maintenance of cell surface integrity.

## RESULTS

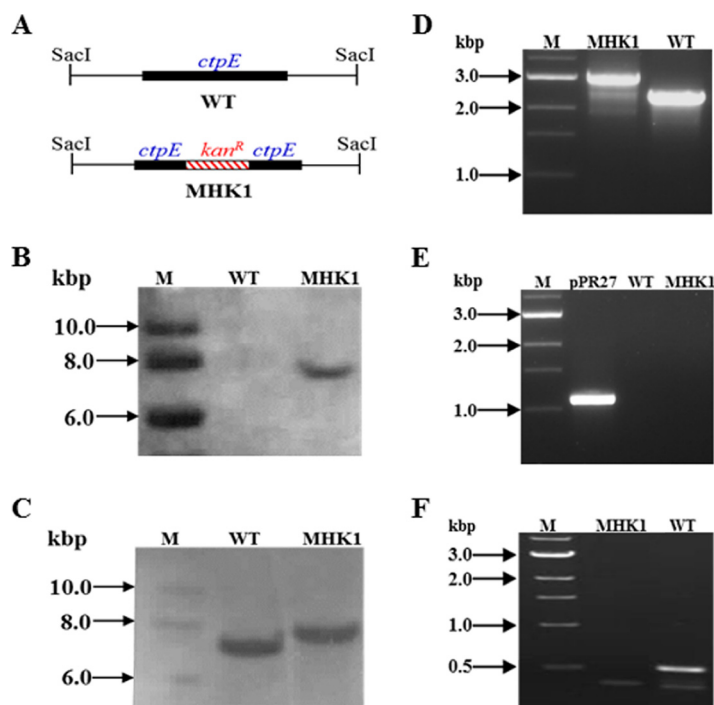
**Disruption of *ctpE* results in a mutant sensitive to  $\text{Ca}^{2+}$  chelators.** *Mycobacterium smegmatis* CtpE belongs to family 23 of functionally uncharacterized P-type ATPases of bacteria (26). We observed that in addition to the reported members from *Actinobacteria*, *Firmicutes*, and *Cyanobacteria* for this uncharacterized family, orthologs were also present in "*Candidatus* Saccharibacteria," *Chlorobi*, *Chloroflexi*, *Planctomycetes*, *Proteobacteria*, and *Tenericutes* (Fig. 1 and see Table S1 in the supplemental material). *M. smegmatis* *ctpE* encodes a putative P-type ATPase of 791 amino acids. It has 10 predicted transmembrane helices (TM) and 2 cytoplasmic loops after TM2 and TM4 (Fig. S1). It has all the conserved motifs for functional activity that P-type ATPase has (Fig. S1). A conserved motif VPEGL was observed in TM4, which is similar to the IPEG(A)L motif observed in  $\text{Ca}^{2+}$ -transporting P-type ATPases of family 2, indicating that  $\text{Ca}^{2+}$  may be a probable substrate for family 23.

To ascertain the functional role of *ctpE*, the *ctpE* gene was disrupted by homologous recombination, and disruption of *ctpE* was confirmed by Southern hybridization and PCR amplifications (Fig. 2). The *ctpE*-disrupted strain was designated MHK1. The mutant showed normal growth in standard Luria-Bertani (LB) broth and Middlebrook 7H9 medium, but growth of the mutant was slow in Sauton's medium, and culturing the mutant in Sauton's medium could not achieve a final cell density similar to that of the wild type (WT) (routine observation). Most P-type ATPases are cation transporters; we screened strain MHK1 against increasing concentrations of



**FIG 1** Phylogenetic tree depicting P-type ATPases families across bacterial divisions. The boldface numbers after the black triangles or line give the P-type ATPase family numbers; substrates for the characterized families are shown after the family number in parentheses. CtpE orthologs from "Candidatus Saccharibacteria," Chlorobi, Chloroflexi, Planctomycetes, Proteobacteria, and Tenericutes that were not previously reported are shown along with CtpE from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*. The accession numbers for CtpE orthologs are shown in parentheses. The accession numbers of the sequences representing other families are provided in Text S1 in the supplemental material. The bar indicates 0.2 nucleotide substitutions per position.

various cations and chelators. The MHK1 strain was sensitive to Ca<sup>2+</sup>-specific chelators EGTA and 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) compared to the WT (Fig. 3A and B) but not to other chelators such as EDTA, *N,N,N',N'*-tetrakis(2-pyridylmethyl)ethane-1,2-diamine (TPEN), or phenanth-

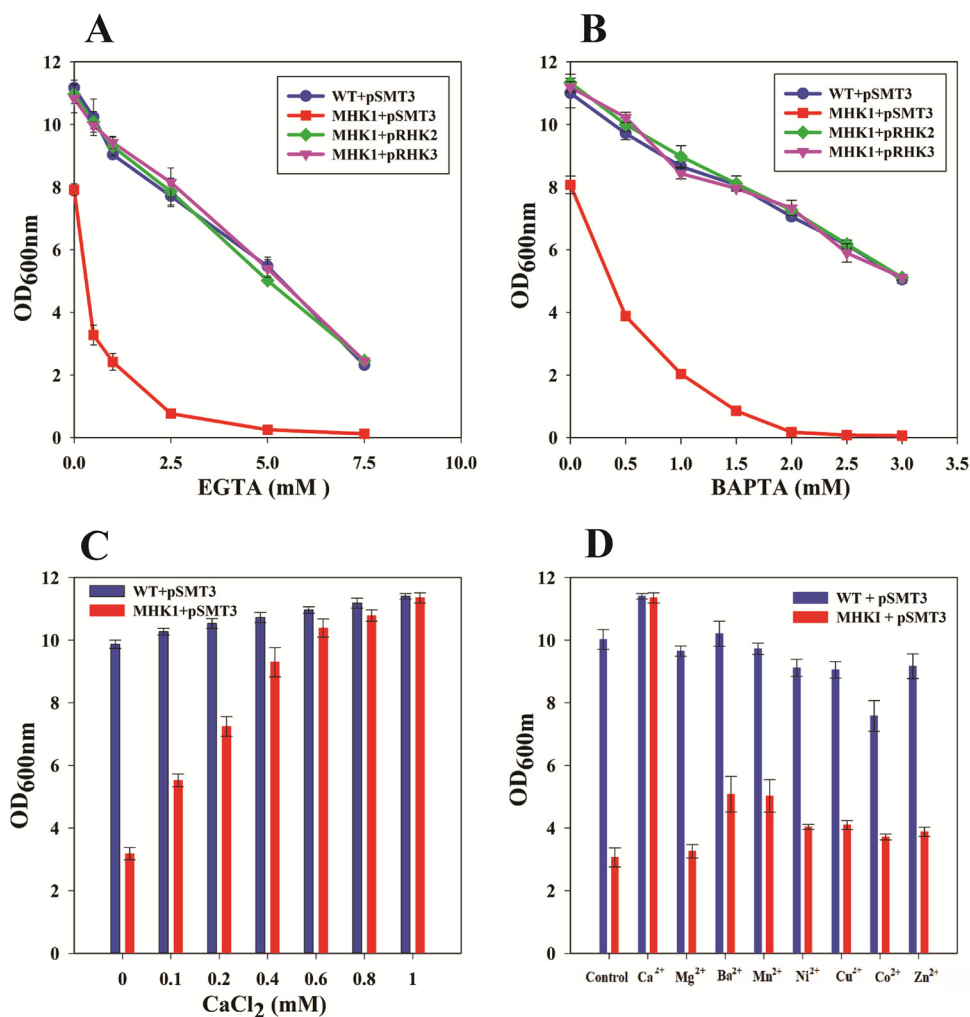


**FIG 2** Confirmation of *ctpE* disruption in mutant MHK1. (A) Schematic representation of *ctpE* flanking region after *SacI* digestion from wild-type *Mycobacterium smegmatis* mc<sup>2</sup>155 (WT) and the mutant (MHK1), and the fragment sizes were 7.2 kb and 7.8 kb, respectively. (B and C) Genomic DNA from the WT and MHK1 strains was digested with *SacI*, and the Southern blot was developed by using probe for kanamycin cassette (B) or *ctpE* (C). Lanes M, DNA ladder. Amplification was carried out from genomic DNA isolated from the WT or MHK1 strain or plasmid pPR27. (D) The *ctpE* gene was amplified using primers HK11F and HK11R. The expected band was 2,996 bp from MHK1 and 2,367 bp from the WT. (E) The *sacB* gene was amplified using primers SACBF and SACBR, and the expected band was 1,127 bp from the plasmid control (pPR27) or single crossover mutant. The absence of amplification in strain MHK1 confirmed a double crossover mutant. (F) Amplification against the portion of *ctpE* deleted after digestion with *SphI* and *NcoI* in disruption construct using primers LOSSF and LOSSR. The expected band size was 611 bp, and the presence of this band was expected only in the WT.

roline (Fig. S2). To determine whether the EGTA- and BAPTA-sensitive phenotype was due to specific scavenging of Ca<sup>2+</sup> ions from the medium and not due to nonspecific chelation of other cations, we supplemented Sauton's medium with different cations in the presence of 1.0 mM EGTA. Ca<sup>2+</sup> was able to rescue the growth (Fig. 3C) of strain MHK1, and the growth defect was reversed in a dose-dependent manner (Fig. 3C). Equimolar concentrations of other cations such as Mg<sup>2+</sup>, Ba<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, and Cu<sup>2+</sup> or 0.5 mM Co<sup>2+</sup> or Zn<sup>2+</sup> were not able to restore the growth of MHK1 (Fig. 3D).

To confirm that the EGTA- or BAPTA-sensitive phenotype of strain MHK1 was due to disruption of *ctpE*, we complemented MHK1 with ectopic expression of *ctpE* under the control of the *hsp60* promoter. Complemented strain MHK1 carrying pRHK2 was completely restored for the EGTA- or BAPTA-sensitive phenotype (Fig. 3A and B). CtpE ortholog Rv0908 from pathogenic *Mycobacterium tuberculosis* has 79% identity at the protein level with CtpE from *M. smegmatis* and was expected to have a similar physiological function. Complementation of strain MHK1 with heterologous expression of Rv0908 also restored the growth of MHK1 in the presence of EGTA or BAPTA similar to *ctpE* from *M. smegmatis* (Fig. 3A and B).

**CtpE is responsible for uptake of Ca<sup>2+</sup> in *M. smegmatis*.** Sensitivity of MHK1 to Ca<sup>2+</sup> chelators indicated that CtpE may be responsible for transport of Ca<sup>2+</sup> into the cells. *M. smegmatis* strains were grown in Ca<sup>2+</sup>-depleted Sauton's medium, and Ca<sup>2+</sup> uptake was determined by using <sup>45</sup>Ca<sup>2+</sup> as a tracer (Fig. 4A). The accumulation of <sup>45</sup>Ca<sup>2+</sup> increased with time in the wild type, but there was no significant accumulation of <sup>45</sup>Ca<sup>2+</sup> in the MHK1 mutant strain. Expression of *ctpE* from *M. smegmatis* or its

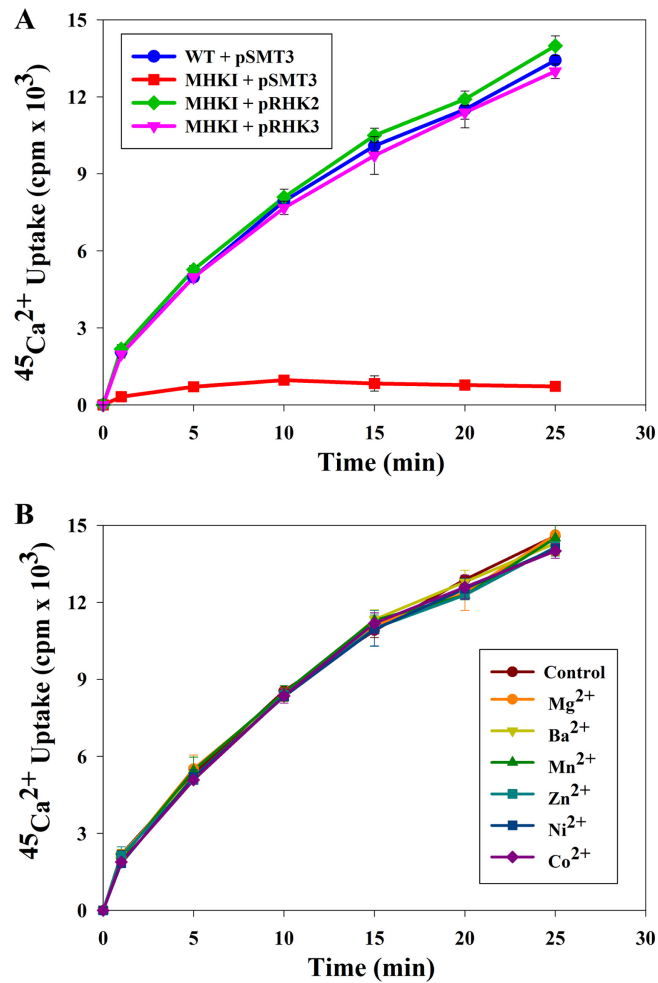


**FIG 3** Growth defects of the MHK1 mutant and restoration by complementation or CaCl<sub>2</sub>. Wild-type *Mycobacterium smegmatis* mc<sup>2</sup>155 carrying pSMT3 (WT+pSMT3), the MHK1 mutant carrying pSMT3 (MHK1+pSMT3), mutant complemented with *M. smegmatis* *ctpE* (MHK1 carrying pRHK2 [MHK1+pRHK2]), and mutant complemented with *M. tuberculosis* Rv0908 (MHK1+pRHK3) were grown in Sauton's medium containing various concentrations of EGTA (A) and BAPTA (B). (C) Ca<sup>2+</sup> is able to restore the growth of MHK1 in a dose-dependent manner. Strains were inoculated in Sauton's medium containing 1.0 mM EGTA and 0.1 to 1.0 mM of Ca<sup>2+</sup>. (D) Growth of the MHK1 mutant was not restored by cations other than Ca<sup>2+</sup>. Strains were inoculated in Sauton's medium containing 1.0 mM EGTA and 1.0 mM concentrations of different cations except Zn<sup>2+</sup> and Co<sup>2+</sup>, which were used at 0.5 mM. Cell growth in these experiments was monitored after 48 h of incubation at 37°C by measuring absorbance or optical density at 600 nm. Experiments were performed three times; values are averages, and standard deviations are shown as error bars.

ortholog from *M. tuberculosis*, Rv0908, both restored the <sup>45</sup>Ca<sup>2+</sup> uptake in the mutant (Fig. 4A).

Cation selectivity of CtpE was examined by evaluating other cations as competitive inhibitors of Ca<sup>2+</sup> uptake in the cells (Fig. 4B). Equimolar concentrations of MgCl<sub>2</sub>, MnCl<sub>2</sub>, ZnCl<sub>2</sub>, CoCl<sub>2</sub>, and NiCl<sub>2</sub>, or 1.0 mM BaCl<sub>2</sub> (which precipitates at higher concentrations under assay conditions) did not compete for <sup>45</sup>Ca<sup>2+</sup> uptake, indicating that CtpE is a Ca<sup>2+</sup>-specific P-type ATPase.

***ctpE* is transcribed as part of an operon.** Gene neighborhood analysis of *ctpE* revealed a putative operon with five upstream genes (*pbp* [penicillin-binding protein], *bla* [metallo-β-lactamase], *echa* [enoyl-coenzyme A {CoA} hydratase], *agl* [amylo-α-1,6-glucosidase], and *gtf* [glycosyltransferase]) and two downstream genes (*chp* [conserved hypothetical proteins]) in *M. smegmatis* (Fig. 5A). A similar gene organization was observed in other mycobacterial species (Fig. S3) with the exception that *agl* and *gtf*

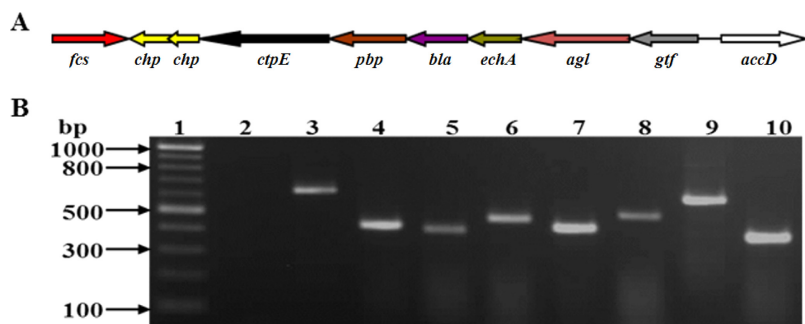


**FIG 4**  $^{45}\text{Ca}^{2+}$  uptake by *Mycobacterium smegmatis* strains. (A) *Mycobacterium smegmatis* mc<sup>2</sup>155 (WT strain carrying pSMT3 [WT+pSMT3]), the mutant (MHK1+pSMT3), mutant complemented with *M. smegmatis* *ctpE* (MHK1+pRHK2), and mutant complemented with *M. tuberculosis* *Rv0908* (MHK1+pRHK3) were grown to mid-log phase in Sauton's medium supplemented with 1.0 mM EGTA, washed, and resuspended in assay buffer.  $^{45}\text{Ca}^{2+}$  uptake was initiated by adding 1  $\mu\text{Ci}/\text{ml}$  of  $^{45}\text{Ca}^{2+}$  and 10 mM  $\text{CaCl}_2$ . Every 5 min, 1.0 ml of culture was taken out and subsequently passed through a 0.45- $\mu\text{m}$  filter membrane. The membrane was washed with 1.0 mM EGTA containing HBSS buffer and dried in an oven. Radioactive counts were determined with a liquid scintillation counter. (B) Uptake of  $^{45}\text{Ca}^{2+}$  in *M. smegmatis* mc<sup>2</sup>155 was not inhibited by the indicated cations at 10 mM concentrations of  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Co}^{2+}$  or by 1.0 mM  $\text{Ba}^{2+}$  in the assay. Experiments were performed in triplicate; values are averages, and standard deviations are shown as error bars.

were absent at the same locus in other species. To confirm whether these eight genes existed in an operon in *M. smegmatis*, the boundaries of the putative operon intergenic regions were amplified by reverse transcriptase PCR (RT-PCR). Amplification of each junction between the genes confirmed that expression of these genes is transcriptionally coupled (Fig. 5B) and that they were transcribed as a polycistronic operon. This operon was designated the *ctpE* operon.

***ctpE* expression is regulated by  $\text{Ca}^{2+}$ .** To check whether  $\text{Ca}^{2+}$  regulates expression of *ctpE*, we employed two approaches,  $^{45}\text{Ca}^{2+}$  uptake assays and semiquantitative reverse transcriptase PCR assay from cells grown in  $\text{Ca}^{2+}$ -depleted or  $\text{Ca}^{2+}$ -rich medium. First,  $\text{Ca}^{2+}$  uptake was monitored in WT cells grown in  $\text{Ca}^{2+}$ -depleted or  $\text{Ca}^{2+}$ -rich medium.  $^{45}\text{Ca}^{2+}$  accumulation increased fourfold in cells grown in  $\text{Ca}^{2+}$ -depleted medium and decreased by nearly twofold in cells grown in  $\text{Ca}^{2+}$ -rich medium compared to the control (cells grown in Sauton's medium without any additives) (Fig. 6A). No such effects were observed on  $^{45}\text{Ca}^{2+}$  uptake in strain MHK1 expressing





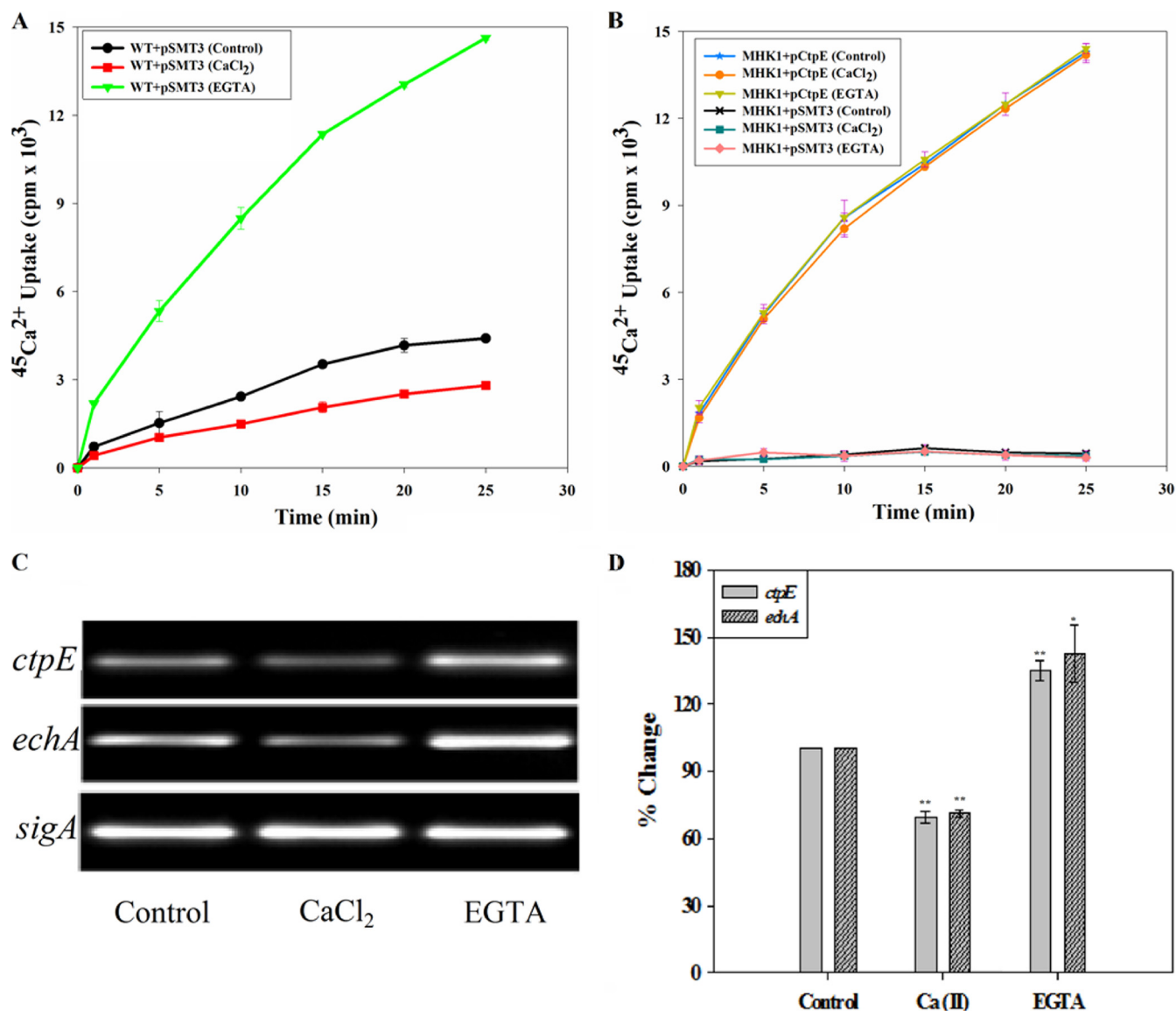
**FIG 5** *ctpE* is expressed as a part of an operon. (A) Schematic representation of the genetic organization of the *M. smegmatis* mc<sup>2155</sup> *ctpE* operon. The genes and the proteins they encode follow: *fcs*, feruloyl-CoA-synthetase; *chp*, conserved hypothetical protein; *ctpE*, cation-transporting P-type ATPase E; *pbp*, penicillin-binding protein 4; *bla*, metallo- $\beta$ -lactamase; *echA*, enoyl-CoA-hydratase; *agl*, amylo- $\alpha$ -1,6-glucosidase; *gtf*, glycosyltransferase; *accD*, acyl-CoA-carboxylase. (B) RT-PCR analysis of the *Mycobacterium smegmatis* mc<sup>2155</sup> *ctpE* operon. The intergenic regions between two genes were amplified. Lane 1, 100-bp DNA ladder; lane 2, RT negative control; lane 3, amplified product of region between *gtf* and *agl*; lane 4, region between *agl* and *echA*; lane 5, region between *echA* and *bla*; lane 6, region between *bla* and *pbp*; lane 7, region between *pbp* and *ctpE*; lane 8, region between *chp* and *chp*; lane 9, region between *ctpE* and *chp*; lane 10, *sigA* RT positive control.

*ctpE* under the *hsp60* promoter (Fig. 6B), indicating that the influence of Ca<sup>2+</sup> observed on <sup>45</sup>Ca<sup>2+</sup> uptake in WT cells is not due to posttranslational modifications of the CtpE protein. These results confirmed that Ca<sup>2+</sup> deficiency induced the transcriptional expression of *ctpE*, resulting in increased uptake. Next, we checked the expression of *ctpE* and another gene in the operon, *echA*, in Ca<sup>2+</sup>-deficient or Ca<sup>2+</sup>-replenished growth conditions by semiquantitative RT-PCR. We observed significant increases in amplification of *ctpE* and *echA* from the RNA isolated from cells induced with Ca<sup>2+</sup>-deficient conditions and significant decreases in amplification of these two genes from RNA isolated from cells grown in Ca<sup>2+</sup>-replenished conditions in comparison to amplification from RNA isolated from cells grown in medium with no additives (Fig. 6C and D), indicating that the expression of *ctpE* was negatively regulated by the Ca<sup>2+</sup> concentration in the medium. A similar level of expression and the Ca<sup>2+</sup> influence on expression of *ctpE* and *echA* further confirmed the presence of both genes in a polycistronic mRNA and regulation of the operon by Ca<sup>2+</sup>.

**MHK1 shows cell envelope defects.** We routinely observed that the MHK1 colonies were dry and rough in comparison to relatively oily and smooth colonies of the wild type on Ca<sup>2+</sup>-deficient medium. CtpE was found to transport Ca<sup>2+</sup> in *M. smegmatis* and was part of an operon carrying genes with putative function in cell envelope biosynthesis or modification. This demanded an evaluation of the cell envelope defects in the MHK1 strain. We checked sensitivity and survival of MHK1 under various stress conditions to evaluate cell envelope defects and observed that the mutant did not differ significantly from the wild type in the presence of SDS, lysozyme, or cell wall-inhibiting antibiotics such as ethambutol or isoniazid (Fig. S4). Interestingly, MHK1 showed hypersensitivity to polymyxin B compared to the wild type (Fig. 7A) and also showed increased cellular aggregation (Fig. 7B) and increased reticulation in biofilm formation (Fig. 7C). These defects were enhanced by Ca<sup>2+</sup> deficiency (1.0 mM EGTA) and were alleviated in the presence of excess Ca<sup>2+</sup> (500  $\mu$ M to 1.0 mM) in the growth medium. The phenotype of the complemented strain was similar to that of the wild type, indicating that CtpE-mediated Ca<sup>2+</sup> uptake is important for the integrity of the cell envelope of *M. smegmatis* under Ca<sup>2+</sup>-deprived conditions.

## DISCUSSION

In this work, we demonstrate that CtpE is a Ca<sup>2+</sup> uptake transporter in *M. smegmatis*. To the best of our knowledge, this is the first Ca<sup>2+</sup> uptake transporter belonging to the energy-dependent P-type ATPase superfamily reported from any organism. Distribution of CtpE orthologs in several bacterial divisions further indicates the importance and

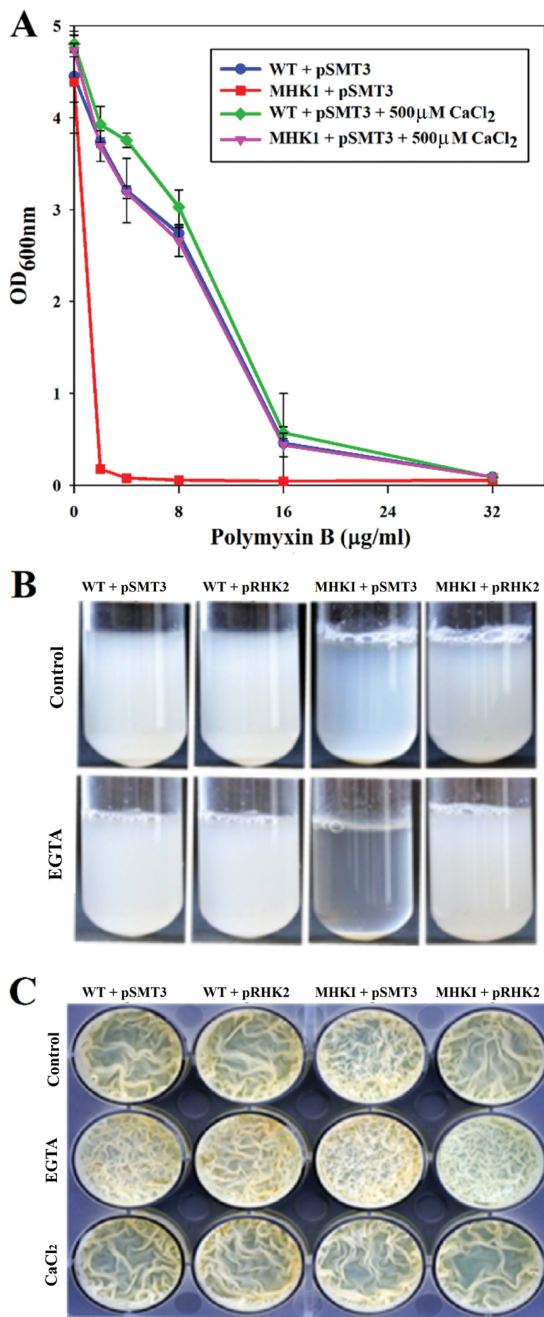


**FIG 6** Ca<sup>2+</sup> negatively regulates expression of *ctpE*. Role of Ca<sup>2+</sup> on expression of *ctpE* in *Mycobacterium smegmatis* mc<sup>2</sup>155 strains. Strains were grown to mid-log phase in Sauton's medium without any supplementation or supplemented with 1.0 mM EGTA or CaCl<sub>2</sub> and used for uptake assays. (A and B) Uptake of <sup>45</sup>Ca<sup>2+</sup> in *M. smegmatis* mc<sup>2</sup>155 (WT) (A) and in the mutant (MHK1+pSMT3) and mutant complemented with *M. smegmatis* *ctpE* (MHK1+pRHK2) (B). (C) Semiquantitative reverse transcriptase PCR analysis of the *M. smegmatis* mc<sup>2</sup>155 *ctpE* and *echA*. *M. smegmatis* mc<sup>2</sup>155 was grown to mid-log phase in Sauton's medium and treated with EGTA or CaCl<sub>2</sub> for 2 h. RNA was isolated, and RT-PCR was carried out for *ctpE*, *echA*, and *sigA*. (D) Transcription profile of *ctpE* and *echA*. Quantification of amplified PCR product (Fig. 6C) was done by densitometry. *sigA* was taken as an endogenous control. The experiment was performed three times independently; values are averages, and standard deviations are shown as error bars. Values that are significantly different are indicated by asterisks as follows: \*, *P* < 0.05; \*\*, *P* < 0.01.

essential role of Ca<sup>2+</sup> in the growth and physiology of prokaryotes. Orthologs of CtpE were identified from 290 strains and were absent in archaea and many other divisions in bacteria, indicating that this family of P-type ATPases has recently evolved for specific Ca<sup>2+</sup> uptake required for essential physiological functions or survival of these species under low-Ca<sup>2+</sup> growth conditions. We observed that strain MHK1 showed significant growth defect only in the presence of high concentrations of EGTA or BAPTA, which indicates that CtpE is required for acquisition of Ca<sup>2+</sup> under Ca<sup>2+</sup>-deficient conditions and that *M. smegmatis* possesses yet uncharacterized alternate Ca<sup>2+</sup> uptake mechanism(s) operating at higher Ca<sup>2+</sup> concentrations.

We observed that *ctpE* is negatively regulated at the transcription level by Ca<sup>2+</sup>. This Ca<sup>2+</sup>-mediated regulation of *ctpE* may be due to a Ca<sup>2+</sup>-sensing/regulatory system in





**FIG 7** The MHK1 mutant shows defects in cell surface integrity. (A) The MHK1 mutant strain shows hypersensitivity to polymyxin B. The cultures were grown in Middlebrook 7H9 medium containing the indicated concentrations of polymyxin B and CaCl<sub>2</sub>. Cell growth was monitored after 72 h of incubation at 37°C by measuring absorbance at 600 nm. (B) MHK1 shows increased cell aggregation. All strains, *Mycobacterium smegmatis* mc<sup>2</sup>155 (WT+pSMT3), the mutant (MHK1+pSMT3), and mutant complemented with expression of *M. smegmatis* *ctpE* (MHK1+pRHK2), were grown in Middlebrook 7H9 medium up to mid-log phase and diluted in fresh medium with 1.0 mM EGTA or without EGTA. The cells were grown for up to 48 h at 37°C with shaking and left standing for 1.0 h. (C) MHK1 shows enhanced reticulation in biofilm formation. All the strains were grown in Sauton's medium supplemented with 2.0% glucose in microtiter plates for 5 days at 37°C without any additive or with 1.0 mM EGTA or CaCl<sub>2</sub>.

*M. smegmatis*. Eukaryotic calcium channels, plasma membrane calcium transporter (PMCA) and sarcoendoplasmic reticulum calcium transporter (SERCA) are known to be regulated at the transcription level by Ca<sup>2+</sup>-dependent signaling involving calmodulin and protein kinases (27). There are few examples of gene regulation by Ca<sup>2+</sup>-binding

proteins in prokaryotes (7), but mechanisms regulating expression of  $\text{Ca}^{2+}$  channels or transporters are not known in prokaryotes. Bacteria are known to possess calmodulin-like and EF-hand proteins, which are speculated to play a role in  $\text{Ca}^{2+}$  storage and signaling. *M. smegmatis* has a calmodulin-like protein (CAML) with functional similarity to eukaryotic calmodulin (28). This protein was implicated to have a role in phospholipid metabolism (29), and CAMLP from *Mycobacterium tuberculosis* has recently been shown to be important during infection (30). A  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (Cam-kinase) was also purified and characterized from *M. smegmatis* (31), but direct roles of these proteins in gene regulation in *M. smegmatis* remain unexplored. The presence of components of  $\text{Ca}^{2+}$ -sensing (CAML) and regulatory (Cam-kinase) machinery and our observation of  $\text{Ca}^{2+}$ -dependent regulation of *ctpE* in *M. smegmatis* tempt us to speculate on possible roles of CAMLP and Cam-kinase in *ctpE* regulation.

We observed that *ctpE* is part of an operon with genes predicted to be involved in membrane/cell wall biosynthesis or integrity in *M. smegmatis* and other mycobacterial species (see Fig. S3 in the supplemental material), indicating the requirement of  $\text{Ca}^{2+}$  in processes involving these proteins.  $\text{Ca}^{2+}$  is known to influence phospholipid metabolism and membrane structure (11).  $\text{Ca}^{2+}$  concentration changes and  $\text{Ca}^{2+}$ /calmodulin-mediated regulation have been implicated in phospholipid biosynthesis and translocation of phospholipids between the monolayers of bilayer membranes (11). In *Pseudomonas aeruginosa*, a lytic transglycosylase (SltB1) and penicillin-binding protein (PBP2) form a complex in the presence of  $\text{Ca}^{2+}$  through an EF-hand motif of PBP2. This complex is required for peptidoglycan polymerization and integrity of the cell envelope (15). Sequence analysis using Prosite (32) did not reveal an EF-hand pattern in any of the proteins encoded by the *ctpE* operon, but we observed that amylo- $\alpha$ -1,6-glucosidase (AGL) possessed conserved DXDXDG motif known for  $\text{Ca}^{2+}$  binding (33). It will be interesting to validate possible  $\text{Ca}^{2+}$  binding to AGL and interactions of this protein with other proteins encoded by the CtpE operon. We observed several morphological changes, namely, colony surface, increased cell aggregation, denser biofilm, and polymyxin B sensitivity in the MHK1 strain, reflecting the influence of intracellular  $\text{Ca}^{2+}$  on the cell envelope composition and integrity in *M. smegmatis*. It is not difficult to speculate that other proteins encoded by the *ctpE* operon play a role in maintaining the cell envelope integrity through a  $\text{Ca}^{2+}$ -influenced process, but the role of other  $\text{Ca}^{2+}$ -regulated systems cannot be ruled out at this stage.

In conclusion, we demonstrate that CtpE of *M. smegmatis* and *M. tuberculosis* is a  $\text{Ca}^{2+}$  uptake transporter, which is essential for growth under  $\text{Ca}^{2+}$ -deficient conditions. CtpE is expressed as a part of an operon and  $\text{Ca}^{2+}$  negatively regulates expression of this operon. Intracellular  $\text{Ca}^{2+}$  acquired by CtpE is important for cell wall integrity in *M. smegmatis*. Structural and functional comparison of CtpE with  $\text{Ca}^{2+}$  efflux pumps of family 2 will help in identifying molecular determinants responsible for directionality in the  $\text{Ca}^{2+}$ -transporting P-type ATPases.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture media.** Bacterial strains and plasmids used in this study are listed in Table 1. For oligonucleotides, see Table S2 in the supplemental material. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth and LB agar supplemented with appropriate antibiotics. *Mycobacterium smegmatis* mc<sup>2</sup>155 (wild-type [WT]) and mutant strains were grown on LB medium supplemented with 0.05% Tween 80 or Middlebrook 7H9 medium supplemented with albumin-dextrose-catalase (ADC) or oleic acid-albumin-dextrose-catalase (OADC) or Sauton's medium (pH 7.5) supplemented with 0.05% Tween 80 as described below for the particular experimental procedure.

**Bioinformatic analysis.** Orthologs were predicted with OrthoLogoDB (34) or by reciprocal best-hits method (35). Multiple sequence alignments of the CtpE sequence from *M. smegmatis* and orthologous sequences from other species was done by ClustalW, and a phylogenetic tree was constructed by neighbor-joining method using MEGA version 6.0 (36). Topology predictions were carried out by TMHMM (37), and the operon was predicted with the pattern/Markov chain-based bacterial operon and gene prediction program, fgenesB (38).

**Molecular biology methods and cloning.** DNA manipulations, such as PCR, restriction digestions, and ligations, were conducted using standard procedures (39). To generate expression clones under the control of the *hsp60* promoter, *ctpE* from *M. smegmatis* mc<sup>2</sup>155 (using primers MSEF and MSER [F stands for forward, and R stands for reverse]) and its homologue *Rv0908* from *M. tuberculosis* (using primers RVEF

**TABLE 1** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<i>E. coli</i> DH10B	F <sup>-</sup> <i>mcrA</i> Δ( <i>mrr-hsdRMS-mcrBC</i> ) φ80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>ara</i> Δ139 ( <i>ara leu</i> )7697 <i>galU</i> <i>galk</i> λ <sup>-</sup> <i>rpsL</i> <i>endA1</i> <i>nupG</i>	Gibco-BRL
<i>M. smegmatis</i> mc <sup>2</sup> 155	High-frequency transformation strain derived from wild-type <i>M. smegmatis</i> mc <sup>2</sup> -6	41
MHK1	<i>M. smegmatis</i> mc <sup>2</sup> 155; <i>ctpE</i> ::Km <sup>r</sup>	This work
Plasmids		
pET23a	<i>E. coli</i> expression vector; T7 promoter; His tag	Novagen
pPR27	<i>Mycobacterium-E. coli</i> shuttle vector; temperature-sensitive; <i>oriM</i> <i>sacB</i> Gent <sup>r</sup>	40
pUC4K	Kanamycin resistance cassette containing vector; Km <sup>r</sup> Amp <sup>r</sup>	GE Healthcare
pSMT3	<i>E. coli-Mycobacterium</i> shuttle vector; <i>hsp60</i> promoter; Hyg <sup>r</sup>	44
pRHK1	<i>M. smegmatis</i> <i>ctpE</i> with insertion of kanamycin resistance cassette and cloned in pPR27; Km <sup>r</sup> Gent <sup>r</sup>	This work
pRHK2	<i>M. smegmatis</i> <i>ctpE</i> cloned in pSMT3; Hyg <sup>r</sup>	This work
pRHK3	<i>M. tuberculosis</i> <i>Rv0908</i> cloned in pSMT3; Hyg <sup>r</sup>	This work

and RVER) were amplified, appropriately digested, and ligated into pSMT3 at the EcoRV or HindIII site to generate pRHK2 and pRHK3, respectively.

**Construction of *ctpE*-disrupted mutant.** The disruption mutant for *ctpE* in *M. smegmatis* mc<sup>2</sup>155 was generated by homologous recombination with sucrose counterselection (40). *ctpE* was amplified from *M. smegmatis* mc<sup>2</sup>155 genomic DNA by PCR using primers HK11F and HK11R. The amplified product was cloned into pET23a at the XbaI site, and the resultant plasmid was further digested with SphI and NcoI, which resulted in loss of the 611-bp fragment, causing disruption of the *ctpE*. The ends were blunt ended using T<sub>4</sub> DNA polymerase. The kanamycin resistance cassette was excised as a PstI fragment from pUC4K and was made blunt ended. It was then ligated with pET23a containing disrupted *ctpE*. This kanamycin cassette-inserted *ctpE* was excised with XbaI and cloned into suicidal vector pPR27 at the XbaI site to generate plasmid pRHK1. The pRHK1 vector was electroporated into *M. smegmatis* mc<sup>2</sup>155 (41), and the transformants were allowed to grow at 30°C on LB agar plates containing kanamycin. A single colony of the transformant was inoculated into LB broth with kanamycin at 30°C for 24 h to allow recombination. The mutants were then selected at 42°C on LB agar plates supplemented with 10% sucrose and kanamycin. The loss of suicidal plasmid was confirmed by the absence of growth of the mutant on gentamicin-containing LB agar medium at 30°C.

**Southern hybridization.** Five micrograms of the genomic DNA was restriction digested with SacI, and Southern hybridization was performed as described previously (39). The kanamycin resistance cassette from pUC4K or a 771-bp fragment of *ctpE* (amplified using primers CESF and CESR) was used as a probe for confirmation of *ctpE* disruption in the MHK1 mutant. The labeling of the probe and hybridization and detection of hybridizing fragment were carried out according to the manufacturer's instructions (NEBlot Phototope kit and Phototope-Star Detection kit; New England Biolabs Inc., USA).

**Restoration of MHK1 growth with cations.** To check the growth of strain MHK1 with different cations, starter cultures of *M. smegmatis* mc<sup>2</sup>155 and MHK1 were grown in Sauton's medium supplemented with 0.05% Tween 80 for 24 h, diluted 1:100, and added to medium containing 1.0 mM EGTA. Restoration of growth with different cations was monitored with the addition of 0.1 to 1.0 mM concentrations of CaCl<sub>2</sub>, 1.0 mM MgCl<sub>2</sub>, BaCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub>, or CuSO<sub>4</sub>, or 0.5 mM CoCl<sub>2</sub> or ZnCl<sub>2</sub>. Cells were cultured for 48 h with shaking at 37°C, and growth was monitored at 600 nm.

**Complementation analysis.** *M. smegmatis* MHK1 and mc<sup>2</sup>155 were transformed with pSMT3, pRHK2, or pRHK3 by electroporation. Complementation was checked by growing all strains in Sauton's medium supplemented with 0.05% Tween 80 and 50 μg ml<sup>-1</sup> hygromycin with different concentrations of EGTA or 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA) for 48 h, and the growth was monitored at 600 nm.

**Ca<sup>2+</sup> uptake assays.** *M. smegmatis* strains were grown in Sauton's medium without any additive or with 1.0 mM EGTA or 1.0 mM Ca<sup>2+</sup>. The cells were harvested, washed twice, and resuspended at a cell density of 1.0 OD<sub>600</sub> (optical density at 600 nm) unit/ml in Sauton's medium without any additive. The cells were incubated at 37°C for 15 min, and then <sup>45</sup>Ca<sup>2+</sup> uptake was initiated by the addition of <sup>45</sup>Ca<sup>2+</sup> to a final concentration of 1.0 μCi/ml and 10 mM cold carrier CaCl<sub>2</sub>. At the indicated time, 1.0-ml aliquots were filtered through 0.45-μm nitrocellulose filters, which were prewashed with 1.0 mM EGTA containing Hanks' balanced salt solution (HBSS). The cells were washed twice with 1.0 mM EGTA containing HBSS, dried in an oven, and immersed in 1.0 ml of toluene-based scintillation fluid, and the amount of cell-accumulated <sup>45</sup>Ca<sup>2+</sup> was determined by liquid scintillation counting using a Wallac MicroBeta TriLux instrument (Perkin Elmer). Identical assay reaction mixtures were incubating on ice as controls. The counts from the ice controls were deducted from the counts of the test reaction mixtures incubated at 37°C.

**RNA isolation and reverse transcriptase PCR.** Total RNA from *M. smegmatis* mc<sup>2</sup>155 was isolated using an RNA isolation kit (Qiagen). The RNA was treated with DNase I and purified using an RNeasy kit (Qiagen). The absence of DNA contamination in RNA samples was confirmed by the absence of PCR amplification of *sigA* using SIGAF and SIGAR primers set by *Taq* polymerase.

To characterize the *ctpE* operon, approximately 400- to 600-bp junction regions between two genes were amplified with the help of junction primers listed in Table S2. Reverse transcription PCR was carried out using 1.0  $\mu$ g total RNA by One-Step RT-PCR (reverse transcriptase PCR) kit (Qiagen). Reaction mixtures were incubated at 50°C for 30 min, followed by denaturation at 95°C for 15 min. This was followed by 30 cycles, with 1 cycle consisting of denaturation at 94°C for 30 s, annealing at (55°C to 65°C) for 1 min, and extension at 72°C for 30 to 40 s for different amplifications. The final extension was done at 72°C for 10 min. Amplification products were detected in 1.0% agarose gels along with the 100-bp ladder. For each sample, a reverse transcriptase-negative reaction was also performed to rule out DNA contamination. *sigA* was amplified as a positive control of RT-PCR using SIGAF and SIGAR primers.

To perform semiquantitative RT-PCR, the *M. smegmatis* mc<sup>2</sup>155 cultures were grown in Sauton's medium until they reached an OD<sub>600</sub> of 1.0. The cultures were then treated with 7.5 mM EGTA or 2.5 mM CaCl<sub>2</sub> and allowed to grow for 2 h, and the total RNA was isolated using RNA isolation kit (Qiagen). Total RNA was quantified with a NanoDrop spectrophotometer (NanoDrop 1000; Thermo Scientific). Semiquantitative RT-PCR is carried out using 1.0  $\mu$ g total RNA of each sample with a One-Step RT-PCR kit (Qiagen). The reaction mixture was incubated at 50°C for 30 min, followed by denaturation at 95°C for 15 min. The products were then amplified by 30 cycles, with 1 cycle consisting of denaturation at 94°C for 30 s, annealing (with JRT4F and JRT4R primers or ECHF and ECHR primers for *ctpE* and *echA*, respectively) at 60°C for 1 min, and extension at 72°C for 30 s. The final extension was carried out at 72°C for 10 min. Amplification products were detected in 1.5% agarose gels along with a 100-bp ladder. *sigA* was used as an internal control and was amplified using sigsA2F and -A2R primers. Quantitation of amplified PCR product was done by densitometry using AlphaEaseFC 4.0. Densitometry values of *ctpE* and *echA* were normalized with internal control *sigA*. *P* values were calculated using GraphPad and indicated as follows: \*, *P* < 0.05; \*\*, *P* < 0.01. Experiments were performed three times independently.

**Antibiotic sensitivity assays.** The antibiotic sensitivity assays were performed by growing *M. smegmatis* strains in Middlebrook 7H9 medium supplemented with ADC for 24 h and then inoculating the cultures as 1:200 dilutions in fresh medium containing a range of antibiotic concentrations, followed by incubation at 37°C and 200 rpm for 72 h. Growth was monitored at 600 nm.

**Biofilm formation assays.** Biofilm formation was analyzed by the method of Ojha et al. (42) with small modifications. Briefly, 12-well cell culture plates containing Sauton's medium supplemented with 4.0 ml of 2.0% glucose without any other additive or with 1.0 mM CaCl<sub>2</sub> or 1.0 mM EGTA were inoculated with 40  $\mu$ l of mid-log-phase culture of each strain. Plates were sealed with Parafilm and incubated undisturbed at 37°C, and biofilm formation was observed after 5 days.

**Aggregation assays.** Aggregation assays were performed by the method of Klepp et al. (43) with slight modifications. Briefly, the strains were grown in Middlebrook 7H9 medium up to mid-log phase, diluted 1:100, and added to fresh medium containing 1.0 mM EGTA or lacking EGTA. The cells were grown for 48 h at 37°C and 200 rpm and then left undisturbed for 1.0 h at room temperature and observed for cell aggregation.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.01388-17>.

**TEXT S1**, DOCX file, 0.02 MB.

**FIG S1**, TIF file, 8.2 MB.

**FIG S2**, TIF file, 4.6 MB.

**FIG S3**, TIF file, 15.8 MB.

**FIG S4**, TIF file, 8.4 MB.

**TABLE S1**, DOCX file, 0.1 MB.

**TABLE S2**, DOCX file, 0.02 MB.

## ACKNOWLEDGMENTS

We are thankful to the Director of the CSIR-Institute of Genomics and Integrative Biology for encouragement and financial support.

This work was supported by exploratory grant EXP0003 from the CSIR-Institute of Genomics and Integrative Biology of India and by grant EMR/2016/007780 from the Science and Engineering Research Board of the Department of Science and Technology (DST) to R.S. H.K.G. was supported by a junior research fellowship from the Indian Council of Medical Research (ICMR).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.



## REFERENCES

- Dominguez DC, Guragain M, Patrauchan M. 2015. Calcium binding proteins and calcium signaling in prokaryotes. *Cell Calcium* 57:151–165. <https://doi.org/10.1016/j.ceca.2014.12.006>.
- Tisa LS, Adler J. 1992. Calcium ions are involved in *Escherichia coli* chemotaxis. *Proc Natl Acad Sci U S A* 89:11804–11808. <https://doi.org/10.1073/pnas.89.24.11804>.
- Zhao Y, Shi Y, Zhao W, Huang X, Wang D, Brown N, Brand J, Zhao J. 2005. CcbP, a calcium-binding protein from *Anabaena* sp. PCC 7120, provides evidence that calcium ions regulate heterocyst differentiation. *Proc Natl Acad Sci U S A* 102:5744–5748. <https://doi.org/10.1073/pnas.0501782102>.
- Shi Y, Zhao W, Zhang W, Ye Z, Zhao J. 2006. Regulation of intracellular free calcium concentration during heterocyst differentiation by HetR and NtcA in *Anabaena* sp. PCC 7120. *Proc Natl Acad Sci U S A* 103:11334–11339. <https://doi.org/10.1073/pnas.0602839103>.
- Orans J, Johnson MDL, Coggan KA, Sperlazza JR, Heiniger RW, Wolfgang MC, Redinbo MR. 2010. Crystal structure analysis reveals *Pseudomonas* PilY1 as an essential calcium-dependent regulator of bacterial surface motility. *Proc Natl Acad Sci U S A* 107:1065–1070. <https://doi.org/10.1073/pnas.0911616107>.
- Guragain M, Lenaburg DL, Moore FS, Reutlinger I, Patrauchan MA. 2013. Calcium homeostasis in *Pseudomonas aeruginosa* requires multiple transporters and modulates swarming motility. *Cell Calcium* 54:350–361. <https://doi.org/10.1016/j.ceca.2013.08.004>.
- Wolfgang MC, Lee VT, Gilmore ME, Lory S. 2003. Coordinate regulation of bacterial virulence genes by a novel adenylate cyclase-dependent signaling pathway. *Dev Cell* 4:253–263. [https://doi.org/10.1016/S1534-5807\(03\)00019-4](https://doi.org/10.1016/S1534-5807(03)00019-4).
- Sarkisova SA, Lotlikar SR, Guragain M, Kubat R, Cloud J, Franklin MJ, Patrauchan MA. 2014. A *Pseudomonas aeruginosa* EF-hand protein, EfhP (PA4107), modulates stress responses and virulence at high calcium concentration. *PLoS One* 9:e98985. <https://doi.org/10.1371/journal.pone.0098985>.
- DeBord KL, Galanopoulos NS, Schneewind O. 2003. The *ttsA* gene is required for low-calcium-induced type III secretion of Yop proteins and virulence of *Yersinia enterocolitica* W22703. *J Bacteriol* 185:3499–3507. <https://doi.org/10.1128/JB.185.12.3499-3507.2003>.
- Rosch JW, Sublett J, Gao G, Wang Y-D, Tuomanen EI. 2008. Calcium efflux is essential for bacterial survival in the eukaryotic host. *Mol Microbiol* 70:435–444. <https://doi.org/10.1111/j.1365-2958.2008.06425.x>.
- Smith RJ. 1995. Calcium and bacteria. *Adv Microb Physiol* 37:83–133. [https://doi.org/10.1016/S0065-2911\(08\)60144-7](https://doi.org/10.1016/S0065-2911(08)60144-7).
- Karkare S, Yousafzai F, Mitchenall LA, Maxwell A. 2012. The role of Ca<sup>2+</sup> in the activity of *Mycobacterium tuberculosis* DNA gyrase. *Nucleic Acids Res* 40:9774–9787. <https://doi.org/10.1093/nar/gks704>.
- Ubarretxena-Belandia I, Boots JW, Verheij HM, Dekker N. 1998. Role of the cofactor calcium in the activation of outer membrane phospholipase A. *Biochemistry* 37:16011–16018. <https://doi.org/10.1021/bi9814181>.
- Xi C, Schoeters E, Vanderleyden J, Michiels J. 2000. Symbiosis-specific expression of *Rhizobium etli* *casA* encoding a secreted calmodulin-related protein. *Proc Natl Acad Sci U S A* 97:11114–11119. <https://doi.org/10.1073/pnas.210181097>.
- Nikolaidis I, Izoré T, Job V, Thielens N, Breukink E, Dessen A. 2012. Calcium-dependent complex formation between PBP2 and lytic transglycosylase SltB1 of *Pseudomonas aeruginosa*. *Microb Drug Resist* 18:298–305. <https://doi.org/10.1089/mdr.2012.0006>.
- Gangola P, Rosen BP. 1987. Maintenance of intracellular calcium in *Escherichia coli*. *J Biol Chem* 262:12570–12574.
- Shemarova IV, Nesterov VP. 2014. Ca<sup>2+</sup> signaling in prokaryotes. *Mikrobiologiya* 83:511–518. (In Russian.) <https://doi.org/10.1134/S0026261714050233>.
- Peiffer WE, Desrosiers MG, Menick DR. 1996. Cloning and expression of the unique Ca<sup>2+</sup>-ATPase from *Flavobacterium odoratum*. *J Biol Chem* 271:5095–5100.
- Faxén KJ, Andersen JL, Gourdon P, Fedosova N, Morth JP, Nissen P, Møller JV. 2011. Characterization of a *Listeria monocytogenes* Ca<sup>2+</sup> pump: a SERCA-type ATPase with only one Ca<sup>2+</sup>-binding site. *J Biol Chem* 286:1609–1617. <https://doi.org/10.1074/jbc.M110.176784>.
- Ivey DM, Guffanti AA, Zemsky J, Pinner E, Karpel R, Padan E, Schuldiner S, Krulwich TA. 1993. Cloning and characterization of a putative Ca<sup>2+</sup>/H<sup>+</sup> antiporter gene from *Escherichia coli* upon functional complementation of Na<sup>+</sup>/H<sup>+</sup> antiporter-deficient strains by the overexpressed gene. *J Biol Chem* 268:11296–11303.
- Fujisawa M, Wada Y, Tsuchiya T, Ito M. 2009. Characterization of *Bacillus subtilis* YfkE (ChaA): a calcium-specific Ca<sup>2+</sup>/H<sup>+</sup> antiporter of the CaCA family. *Arch Microbiol* 191:649–657. <https://doi.org/10.1007/s00203-009-0494-7>.
- Reusch RN, Huang R, Bramble LL. 1995. Poly-3-hydroxybutyrate/polyphosphate complexes form voltage-activated Ca<sup>2+</sup> channels in the plasma membranes of *Escherichia coli*. *Biophys J* 69:754–766. [https://doi.org/10.1016/S0006-3495\(95\)79958-1](https://doi.org/10.1016/S0006-3495(95)79958-1).
- Chang Y, Bruni R, Kloss B, Assur Z, Kloppmann E, Rost B, Hendrickson WA, Liu Q. 2014. Structural basis for a pH-sensitive calcium leak across membranes. *Science* 344:1131–1135. <https://doi.org/10.1126/science.1252043>.
- Palmgren MG, Nissen P. 2011. P-type ATPases. *Annu Rev Biophys* 40:243–266. <https://doi.org/10.1146/annurev.biophys.093008.131331>.
- Thever MD, Saier MH. 2009. Bioinformatic characterization of p-type ATPases encoded within the fully sequenced genomes of 26 eukaryotes. *J Membr Biol* 229:115–130. <https://doi.org/10.1007/s00232-009-9176-2>.
- Chan H, Babayan V, Blyumin E, Gandhi C, Hak K, Harake D, Kumar K, Lee P, Li TT, Liu HY, Lo TCT, Meyer CJ, Stanford S, Zamora KS, Saier MH. 2010. The p-type ATPase superfamily. *J Mol Microbiol Biotechnol* 19:5–104. <https://doi.org/10.1159/000319588>.
- Naranjo JR, Mellström B. 2012. Ca<sup>2+</sup>-dependent transcriptional control of Ca<sup>2+</sup> homeostasis. *J Biol Chem* 287:31674–31680. <https://doi.org/10.1074/jbc.R112.384982>.
- Reddy PT, Prasad CR, Reddy PH, Reeder D, McKenney K, Jaffe H, Dimitrova MN, Ginsburg A, Peterkofsky A, Murthy PS. 2003. Cloning and expression of the gene for a novel protein from *Mycobacterium smegmatis* with functional similarity to eukaryotic calmodulin. *J Bacteriol* 185:5263–5268. <https://doi.org/10.1128/JB.185.17.5263-5268.2003>.
- Burra SS, Reddy PH, Falah SM, Venkatasubramanian TA, Murthy PS. 1991. Calmodulin-like protein and the phospholipids of *Mycobacterium smegmatis*. *FEMS Microbiol Lett* 64:189–194.
- Advani MJ, Rajagopalan M, Reddy PH. 2014. Calmodulin-like protein from *M. tuberculosis* H37Rv is required during infection. *Sci Rep* 4:6861. <https://doi.org/10.1038/srep06861>.
- Sharma S, Giri S, Khuller GK. 1998. Ca<sup>2+</sup>/calmodulin dependent protein kinase from *Mycobacterium smegmatis* ATCC 607. *Mol Cell Biochem* 183:183–191. <https://doi.org/10.1023/A:1006883006402>.
- Sigrist CJA, de Castro E, Cerutti L, Cuche BA, Hulo N, Bridge A, Bougueleret L, Xenarios I. 2013. New and continuing developments at PROSITE. *Nucleic Acids Res* 41:D344–D347. <https://doi.org/10.1093/nar/gks1067>.
- Rigden DJ, Galperin MY. 2004. The Dx/Dx motif for calcium binding: multiple structural contexts and implications for evolution. *J Mol Biol* 343:971–984. <https://doi.org/10.1016/j.jmb.2004.08.077>.
- Whiteside MD, Winsor GL, Laird MR, Brinkman FSL. 2013. OrtholugeDB: a bacterial and archaeal orthology resource for improved comparative genomic analysis. *Nucleic Acids Res* 41:D366–D376. <https://doi.org/10.1093/nar/gks1241>.
- Moreno-Hagelsieb G, Latimer K. 2008. Choosing BLAST options for better detection of orthologs as reciprocal best hits. *Bioinformatics* 24:319–324. <https://doi.org/10.1093/bioinformatics/btm585>.
- Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30:2725–2729. <https://doi.org/10.1093/molbev/mst197>.
- Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305:567–580. <https://doi.org/10.1006/jmbi.2000.4315>.
- Li RW. 2011. Metagenomics and its applications in agriculture, biomedicine, and environmental studies. Nova Science Publishers, Hauppauge, NY.
- Sambrook J, Russell DW. 2001. *Molecular cloning: a laboratory manual*, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Pellic V, Jackson M, Reytrat JM, Jacobs WR, Gicquel B, Guilhot C. 1997. Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* 94:10955–10960. <https://doi.org/10.1073/pnas.94.20.10955>.
- Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR. 1990. Isolation

- and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* 4:1911–1919. <https://doi.org/10.1111/j.1365-2958.1990.tb02040.x>.
42. Ojha AK, Baughn AD, Sambandan D, Hsu T, Trivelli X, Guerardel Y, Alahari A, Kremer L, Jacobs WR, Hatfull GF. 2008. Growth of *Mycobacterium tuberculosis* biofilms containing free mycolic acids and harbouring drug-tolerant bacteria. *Mol Microbiol* 69:164–174. <https://doi.org/10.1111/j.1365-2958.2008.06274.x>.
43. Klepp LI, Forrellad MA, Osella AV, Blanco FC, Stella EJ, Bianco MV, Santangelo MDLP, Sassetti C, Jackson M, Cataldi AA, Bigi F, Morbidoni HR. 2012. Impact of the deletion of the six mce operons in *Mycobacterium smegmatis*. *Microbes Infect Inst Pasteur* 14:590–599.
44. Ó Gaora P, Barnini S, Hayward C, Filley E, Rook G, Young D, Thole J. 1997. Mycobacteria as immunogens: development of expression vectors for use in multiple mycobacterial species. *Med Princ Pract* 6:91–96. <https://doi.org/10.1159/000157432>.