Received Date : 20-Jan-2016

Revised Date : 05-Apr-2016

Accepted Date : 11-Apr-2016

Article type : Original Research

Zinc Coordination is Essential for the Function and Activity of the Type II Secretion ATPase EpsE

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Running title: Role of zinc in the type II secretion ATPase EpsE

Key words: type II secretion, ATPase, zinc, tetracysteine

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Summary

The type II secretion system Eps in *Vibrio cholerae* promotes the extracellular transport of cholera toxin and several hydrolytic enzymes, and is a major virulence system in many Gram-negative pathogens that is structurally related to the type IV pilus system. The cytoplasmic ATPase EpsE provides the energy for exoprotein secretion through ATP hydrolysis. EpsE contains a unique metal-binding domain that coordinates zinc through a tetracysteine motif ($CXXCX_{29}CXXC$), which is also present in type IV pilus assembly but not retraction ATPases. Deletion of the entire domain or substitution of any of the cysteine residues that coordinate zinc completely abrogates secretion in an EpsE-deficient strain and has a dominant negative effect on secretion in the presence of WT EpsE. Consistent with the *in vivo* data, chemical depletion of zinc from purified EpsE hexamers results in loss of *in vitro* ATPase activity. In contrast, exchanging the residues between the two dicysteines with those from the homologous ATPase XcpR from *Pseudomonas aeruginosa* does not have a significant impact on EpsE. These results indicate that, although the individual residues in the metal binding domain are generally interchangeable, zinc coordination is essential for the activity and function of EpsE.

Graphical Abstract

Type II secretion ATPases contain a unique zinc-binding domain which is absent from homologous type IV pilus retraction ATPases and type IV secretion ATPases. Removal of the entire zinc-binding domain or disruption of zinc coordination in the type II secretion ATPase EpsE abrogates secretion and prevents ATP hydrolysis, indicating that zinc coordination is essential for the function and activity of type II secretion ATPases.

Introduction

The type II secretion (T2S) system mediates the extracellular transport of virulence factors, such as toxins and hydrolytic enzymes, in many Gram-negative pathogens (Sandkvist, 2001; Cianciotto, 2005; Korotkov *et al.*, 2012). *Vibrio cholerae* uses the T2S system to secrete cholera toxin, which is largely

responsible for the severe diarrhea that characterizes cholera, as well as at least 20 other proteins such as proteases, chitinases, lipases, and biofilm matrix proteins (Overbye *et al.*, 1993; Connell *et al.*, 1998; Davis *et al.*, 2000; Sikora *et al.*, 2011; Johnson *et al.*, 2014). Type II-secreted proteins first cross the inner membrane using the Sec or Tat general export pathways, signal sequences are then removed, and these proteins are subsequently transported across the outer membrane via T2S (Pugsley, 1993; Voulhoux *et al.*, 2001). Type II-secreted factors are important for nutrient acquisition and/or modulating the surroundings to benefit the bacteria in both the aquatic environment as well as in the human small intestine (Sandkvist, 2001; Cianciotto, 2005; Sikora, 2013).

The T2S apparatus in *V. cholerae* spans both the inner and outer membranes and is composed of 12 Eps (*e*xtracellular *p*rotein *s*ecretion) proteins (denoted EpsC through EpsM) and PilD (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997; Marsh & Taylor, 1998; Fullner & Mekalanos, 1999). The cytoplasmic ATPase EpsE is associated with the inner membrane through interactions with the bitopic inner membrane protein EpsL (Sandkvist *et al.*, 1995; Abendroth *et al.*, 2005). EpsE acts as a molecular motor to provide the energy for exoprotein secretion through ATP hydrolysis (Camberg & Sandkvist, 2005). A recent study from our laboratory indicates that EpsL may provide a molecular link between EpsE and the major pseudopilin component EpsG. Protein-protein interactions between EpsG and EpsL were established through chemical crosslinking and co-immunoprecipitation followed by immunoblotting for EpsG or EpsL. EpsG prepilin processing by PilD is required for this EpsL interaction, although no other T2S proteins are necessary. The results suggest that the energy produced during ATP hydrolysis by EpsE may be transduced through EpsL to the major pseudopilin EpsG to power pseudopilus assembly for T2S (Gray *et al.*, 2011).

EpsE belongs to a large family of type II/IV secretion ATPases, including those involved in protein secretion, type IV pilus (T4P) biogenesis, competence, and archaeal flagella (archaellum) assembly (Planet *et al.*, 2001). Family members consist of two distinct domains: the N-terminal domain (NTD) and the C-terminal domain (CTD), which are connected by a short flexible linker (Robien *et al.*, 2003; Satyshur *et al.*, 2007; Misic *et al.*, 2010; Rose *et al.*, 2011; Reindl *et al.*, 2013). The CTD contains the conserved ATP-binding motifs that collectively form the nucleotide-binding pocket, including Walker A and B motifs and Asp and His boxes (Robien *et al.*, 2003; Chiang *et al*., 2008). Within this family, EpsE and other T2S ATPases as well as ATPases required for T4P assembly form the subfamily of GspE/PilB ATPases, alternatively referred to as pilus assembly ATPases (Planet *et al.*, 2001). Members of this

subfamily contain a unique domain called the C-terminal metal binding domain (C_M) that coordinates zinc through a tetracysteine motif ($CXXCX_{21\text{-}40}CXXC$) (Fig. 1; Planet *et al.*, 2001; Robien *et al.*, 2003; Camberg & Sandkvist, 2005). The C_M domain is notably absent among T4P retraction ATPases such as PilT and PilU (Robien *et al.*, 2003; Satyshur *et al.*, 2007; Misic *et al.*, 2010; Whitchurch & Mattick, 1994). The tetracysteine motif is conserved among all T2S ATPases except for *Xylella fastidiosa* XpsE and *Xanthomonas campestris* XpsE (Robien *et al.*, 2003). The EpsE C_M domain spans residues C397-C433 in EpsE, with the amino acids in between the two dicysteines forming a hairpin turn, or loop. The C_M loop residues share low sequence homology (~30%) between homologous T2S ATPases (Robien *et al.*, 2003; Camberg & Sandkvist, 2005). Structural analysis shows that the C_M domain is located on the outside of the EpsE hexamer (Lu *et al.*, 2013).

Zinc-coordinating domains have been implicated in diverse roles such as stability, protein-protein interactions, and regulation of activity (Fekkes *et al.*, 1999; Jakob *et al.*, 2000; Salzer *et al.*, 2014). The importance of cysteine residues to the C_M domain of GspE/PilB ATPases has been previously suggested by other studies (Possot & Pugsley, 1997; Salzer *et al.*, 2014). The T2S ATPase PulE from *Klebsiella oxytoca* contains a tetracysteine motif similar to EpsE, and loses the ability to support secretion of pullulanase when at least two adjacent cysteines are replaced with serines. However, the insolubility of native PulE and lack of protein purification techniques prevented study of PulE *in vitro* (Possot & Pugsley, 1997). *Thermus thermophilus* PilF is an ATPase involved in T4P biogenesis and DNA uptake, and was recently shown to require zinc for stability of PilF hexameric complexes but not for ATPase activity *in vitro*. Additionally, cysteine residues were important for PilF's role in piliation at high temperatures but not for transformation *in vivo* (Salzer *et al.*, 2014).

Understanding the function of EpsE and its individual domains is essential for elucidating the mechanism of T2S and its contribution to pathogenesis. In this study, we investigate the role of the tetracysteine motif and zinc in the EpsE C_M domain, as EpsE is a well-characterized ATPase involved in the secretion of cholera toxin and many hydrolytic enzymes in *V. cholerae*, an important human pathogen and established model organism. We show that zinc coordination by the C_M domain is required for the function of EpsE in T2S, but the C_M residues between the two dicysteines are interchangeable with that of a homologue. Zinc coordination provides stability to the EpsE hexamer, as removal of zinc results in a loss of ATPase activity *in vitro*, an inability to support T2S *in vivo*, and an alteration in the protein's conformation.

Results

The EpsE C_M domain is required for Type II Secretion

The C_M domain is conserved among T2S and T4P assembly ATPases such as EpsE and PilB, while it is absent in homologous T4P retraction ATPases such as PilT and type IV secretion ATPases including HP0525 (Fig. 1), suggesting that it may be required for a process unique to T2S and T4P assembly. In order to examine the importance of the C_M domain in T2S, we designed mutations in EpsE that remove the entire C_M domain and connect the residues at the point in the CTD where the β-strands most closely converge based on structural superposition of EpsE and HP0525, which lacks the C_M domain (Fig. 1). We removed the entire domain including the four cysteines (EpsE ΔC_M) or replaced it with a proline residue (EpsE ΔC_MPro) in order to generate EpsE variants that resemble ATPases lacking the C_M domain. Although wild-type (WT) EpsE can complement the loss of secretion of the serine protease VesB and lipase in an *epsE*::*kan* strain of *V. cholerae*, EpsE ΔC_M and EpsE ΔC_MPro are deficient in their ability to support secretion in this mutant (Fig. 2A, B). While EpsE ΔC_M and EpsE ΔC_MPro are expressed, they are detected at slightly lower levels than WT EpsE, suggesting a small decrease in stability (Fig. 2C). However, both EpsE ΔC_M and EpsE ΔC_MPro exhibit negative dominance, as over-expression of these EpsE variants prevents T2S in WT *V. cholerae* (Fig. 3). This dominant negative phenotype may be explained by competition between WT and mutant forms of EpsE for interaction with other components within the T2S complex, or by the formation of mixed EpsE oligomers with lower overall activity. Together, these results suggest that both deletion constructs are expressed in *V. cholerae*, and that the C_M domain is necessary for EpsE's function in T2S.

We have previously reported that *V. cholerae* T2S mutants leak periplasmic content likely due to outer membrane damage (Sikora *et al*., 2007). Therefore, we also evaluated the ability of EpsE ΔC_M and EpsE ΔC_MPro to restore outer membrane integrity by determining their effect on non-specific extracellular release of periplasmic β-lactamase. We observed higher percentages of β-lactamase activity in the supernatant of *epsE*::*kan* strains containing empty vector or expressing the C_M deletion variants of EpsE (35-45%) compared to WT *V. cholerae* and the *epsE*::*kan* strain expressing WT EpsE (15-20%) further indicating that these EpsE variants are non-functional (Fig. S1).

Residues in the C_M loop are interchangeable for EpsE's function in vivo *and* in vitro

After establishing the importance of the EpsE C_M domain, we then investigated the role of the 29 amino acids in between the two dicysteines. In order to understand the contribution of these residues to EpsE's function, we decided to swap the loop from EpsE with that of a homologue. This technique was selected over mutation of individual residues as it allowed us to simultaneously substitute multiple residues and to evaluate whether the C_M loop residues participate in species-specific protein-protein interactions (Sandkvist *et al.*, 1995; Sandkvist *et al.*, 2000). We compared the C_M loop residues among several EpsE homologues and found that both the length of the loop and the specific residues vary significantly (Fig. 4). The EpsE C_M loop contains 29 amino acids, and in order to alter the specific residues of the C_M loop without changing the length, we chose to genetically engineer a chimeric construct, EpsE-XcpR C_M, wherein the 29 C_M loop residues between the two dicysteines from EpsE were exchanged with those of XcpR from *P. aeruginosa*. The cysteines remain intact, but the exchanged region from EpsE differs from that of XcpR by 17 out of 29 residues, mostly in the central portion of the loop (Fig. 4). The EpsE-XcpR C_M chimera was found to complement the loss of VesB and lipase secretion in the *epsE*::*kan* strain of *V. cholerae* (Fig. 5A, B) and is expressed at nearly WT levels (Fig. 5C). Although restoration of protease secretion was much more variable compared to WT EpsE, it was consistently more substantial than the C_M deletion mutant phenotypes (Fig. 2A, B). Consistent with its ability to complement the secretion defect in the *epsE*::*kan* mutant, the EpsE-XcpR C_M chimera had no negative effect on VesB secretion when overexpressed in WT *V. cholerae* (Fig. 5A). It is possible that either the presence of WT EpsE can overcome a slight defect in the interaction of the EpsE-XcpR C_M chimera with the T2S system or that this chimera causes a deficiency in oligomer formation or stability.

EpsE hexamers have a greatly increased ATPase activity over monomeric EpsE, suggesting that EpsE likely functions as a hexamer *in vivo* (Sandkvist and Camberg, 2005). The crystal structure of EpsE was first solved as a helical filament, but modeled as a hexamer based on the structure of *H. pylori* HP0525 (Yeo *et al*., 2000; Robien et al., 2003). In a later study, a refined hexameric model was proposed based on *P. aeruginosa* PilT and tested by mutagenesis of residues predicted to participate in subunit-subunit interactions (Patrick *et al*., 2011). We recently constructed a fusion protein consisting of Hcp1, a hexameric protein from *P. aeruginosa* (Mougous *et al.*, 2006), fused to the C-terminus of EpsE (Lu *et al*., 2013). This resulted in a stable EpsE hexamer with increased ATPase activity that was amenable to purification, crystallization and structure determination. Similarly, the C-terminal Hcp1 fusion strategy has also been recently used to purify the hexameric form of the homologous T4P assembly ATPase PilB (Bischof *et al*., 2016). To determine whether stabilization of the EpsE-XcpR C_M chimera by the "assistant

hexamer" Hcp1 can overcome a possible oligomerization defect, we fused Hcp1 to EpsE-XcpR C_M and compared its ability to support secretion with EpsE-Hcp1 in the *epsE*::*kan* mutant. We found that the EpsE-XcpR C_M–Hcp1 chimera fusion with the loop swap is able to support T2S to the same extent as EpsE-Hcp1 (Fig. 6), suggesting that most of the residues in the EpsE C_M domain can be interchanged with those of a homologue without having a negative impact on EpsE's ability to support T2S. In order to verify that the fusions are stable and do not break down into native EpsE, we analyzed cell extracts by SDS-PAGE and immunoblotting for EpsE and Hcp1 (Fig. 7). The results show that both WT and chimeric EpsE-Hcp1 fusions remain intact *in vivo* and migrate according to their predicted molecular weights similarly to EpsE-Hcp1-His_6 purified by metal affinity and size-exclusion chromatography from *E. coli* cell extracts (Fig. S2, S3). Thus, the complementation of the T2S-defect in the *epsE*::*kan* mutant by EpsE-Hcp1 and EpsE-XcpR C_M-Hcp1 is likely due to the intact fusions and not break-down products, indicating that the fusions are functional. In addition, these results show that the variability in complementation of T2S by the EpsE-XcpR C_M chimera is mitigated when the assistant hexamer Hcp1 is present, suggesting that there is no apparent species-specific role of the C_M loop residues between the dicysteines. As expected, when we overexpressed and purified hexameric His_6-tagged forms of EpsE-Hcp1 and EpsE-XcpR C_M-Hcp1 from *E. coli* by metal affinity chromatography and gel filtration (Fig. S2, S3) and determined their ability to hydrolyze ATP, there is no decrease in ATPase activity when the EpsE C_M loop residues are exchanged with those of the homologue XcpR (Fig. 8).

Zinc binding to the C_M domain is required for T2S and EpsE ATPase activity

Given the importance of having an intact C_M domain, and yet the relative flexibility in the requirement for precise C_M loop residues for the function of EpsE, we next sought to understand the contribution of the dicysteines to EpsE and T2S. We constructed a series of EpsE C_M cysteine to serine substitution mutants and tested their ability to complement the loss of T2-secreted protease and lipase activities and restoration of outer membrane integrity in the *epsE::kan* mutant as described above. We analyzed single (C400S, C430S), double (C397SC400S, C430SC433S), triple (C400SC430SC433S), and quadruple (C397SC400SC430SC433S) cysteine to serine substitution mutants. Similarly to the C_M domain deletion variants EpsE ΔC_M and EpsE ΔC_MPro, all EpsE C_M cysteine mutants are unable to complement the T2S defects in the *epsE::kan* strain (Fig. 2, Fig. S1). While expression of the C400S and C3xS variants results in what appear to be EpsE dimers, perhaps due to the exposure of single free cysteines, these bands are not detected in the double or quadruple cysteine mutants (Fig. 2C). In order to test whether the cysteine mutant variants of EpsE are nonfunctional simply due to misfolding, we tested for negative dominance

in vivo. All cysteine mutants exhibited negative dominance and inhibited the ability of WT EpsE to support protease secretion, suggesting that they are not completely misfolded and are able to interact with WT EpsE and/or other components of the T2S complex such as EpsL (Fig. 3A). We also confirmed the negative dominant phenotype of the EpsE C4xS variant by analyzing cholera toxin secretion, and show that expression of EpsE C4xS inhibits the secretion of the cholera toxin B subunit in WT *V. cholerae* to approximately the same level as in a T2S mutant (Fig. 3B). Collectively, these results indicate the importance of cysteines for EpsE's function in T2S and suggest that all four cysteine residues are essential.

In order to analyze the metal content of the cysteine mutant proteins and the effect of these mutations on activity, we attempted to purify hexahistidine-tagged proteins; however, these variant proteins were not amenable to purification, as they aggregated when overexpressed in *E. coli*. We were also unable to purify cysteine mutant variants of the EpsE-Hcp1 fusion (data not shown). This suggests that the folding and/or stability of the mutant proteins is severely compromised when any of the cysteine residues are exchanged for serines, and lends support to the notion that zinc binding to this domain may be crucial for the overall conformation/stability of EpsE.

Because we were unable to analyze the effect of the cysteine-to-serine substitutions on purified proteins, we instead took a chemical approach to determine the contribution of zinc binding to the C_M domain. We used p-chloromercuribenzoic acid (PCMB) to release zinc and measured free zinc using the zinc-complexing agent 4-(2-pyridylazo)resorcinol (PAR). As these experiments required large amounts of purified proteins, we used the previously described hexameric N-terminally truncated ΔN1-EpsE-Hcp1 fusion (Lu *et al.*, 2013), which can be purified in much greater quantities and has about 3-fold higher *in vitro* ATPase activity than the full-length fusion (Fig. S4, Fig. 9B). This form of EpsE is unable to function *in vivo* because it lacks the first 90 NTD residues known to interact with EpsL, which are necessary for EpsE to support T2S (Sandkvist *et al.*, 1995; Sandkvist *et al.*, 2000; Abendroth *et al.*, 2005) (data not shown). Figure 9A shows that increasing amounts of PCMB cause an increase in the amount of zinc released by ΔN1-EpsE-Hcp1 hexamers, which coordinate metal at a 1:1 ratio of zinc:EpsE, consistent with our previous findings with monomeric EpsE (Camberg & Sandkvist, 2005). Additionally, when the EpsE-Hcp1 fusion is pre-treated with a 4-fold molar excess of PCMB for 10 minutes at room temperature, it has nearly abolished ATPase activity *in vitro* compared to untreated and mock-treated controls (Fig. 9B). Thus, zinc binding to the EpsE C_M domain is necessary for *in vitro* ATPase activity.

Zinc stabilizes the conformation of EpsE

Based on the aggregation of EpsE C_M cysteine mutants when overexpressed in *E. coli* and the loss of ΔN1-EpsE-Hcp1's *in vitro* ATPase activity upon zinc release, we hypothesized that zinc contributes to the overall protein conformation of hexameric EpsE. In order to test this, we analyzed purified protein migration profiles using native polyacrylamide gel electrophoresis. Purified ΔN1-EpsE-Hcp1 protein was either untreated or incubated with a 4-fold molar excess of PCMB or mock-treated for 10 minutes at room temperature. As seen in Fig. 9C, zinc removal results in a change in the native migration pattern of the ΔN1-EpsE-Hcp1 hexamer, indicating that conformational changes occur following zinc release.

Discussion

This study demonstrates that the EpsE C_M domain is required for type II secretion in *V. cholerae*. Although many of the loop residues that form the elbow of the C_M domain in between the dicysteines are interchangeable, the tetracysteine motif ($CXXCX_{29}CXXC$) must be intact in order for EpsE to function as the molecular motor for T2S. Substitution of any of the cysteine residues resulted in EpsE variants that are unable to support secretion. These EpsE variants are produced in *V. cholerae*; however, none of them were amenable to purification due to aggregation when overexpressed in *E. coli*. It is possible that these variant proteins are misfolded when they are overproduced in isolation from the rest of the T2S complex. However, the negative dominance by the EpsE cysteine mutants when expressed in WT *V. cholerae* demonstrates that they are still able to interact with other components of the T2S complex and/or WT EpsE, suggesting that they retain some native properties and are not completely misfolded. For several of our experiments, we utilized a form of EpsE fused to the assistant hexamer Hcp1, as this construct forms hexamers in the absence of other Eps proteins, has high ATPase activity *in vitro* (Fig. S2-S4, Fig. 8) (Lu *et al.*, 2013) and is functional *in vivo* (Fig. 6). When zinc was chemically removed from purified ΔN1-EpsE-Hcp1 fusion protein using PCMB, we observed a loss of ATPase activity and a change in the migration pattern using native-PAGE (Fig. 9). Collectively, these data indicate that zinc binding to the C_M domain is necessary to support hexameric complex stability.

We have previously characterized many aspects of the function and activity of EpsE. Among others, we have shown that while monomeric EpsE is capable of hydrolyzing ATP, hexamerization results in greatly increased ATPase activity (Camberg & Sandkvist, 2005; Camberg *et al.*, 2007; Patrick *et al.*, 2011; Lu *et*

al., 2013). The role of zinc was first examined using purified GST-tagged EpsE that primarily yields monomers with low ATPase activity and only a small fraction of highly active hexamers (Camberg & Sandkvist, 2005). Titration of this purified material with *p*-hydroxymercuriphenylsulfonic acid (PMPS) in the presence of PAR revealed that EpsE binds 1 mol of zinc per mol of EpsE, which corresponds with our results in the present study showing that equimolar amounts of zinc are released by ΔN1-hexameric EpsE-Hcp1 using PCMB. When purified EpsE was treated with a four-fold molar excess of PMPS, there was only a 50% decrease in EpsE's ATPase activity, suggesting that zinc does not significantly contribute to the stability of monomeric EpsE (Camberg & Sandkvist, 2005). In contrast, our current study shows that there is a nearly complete reduction in ATPase activity of EpsE hexamers. As the ability to hydrolyze ATP is sensitive to the conformational state of EpsE, the removal of zinc has a greater impact on the ATPase activity of the hexamer than the monomer.

Previous studies examining the tetracysteine motifs of similar Type II/IV secretion ATPases have also indicated the importance of this motif, although some key differences also exist between those reports and the current study. Possot and Pugsley (1997) showed that secretion of the T2S substrate pullulanase was decreased upon substitution of single or double cysteine to serine substitutions to the ATPase PulE, and was abrogated following a triple cysteine to serine substitution. The two different single cysteine substitutions also exhibited varying amounts of negative dominance, with C391S exhibiting 31% dominance and C419S only 3% (Possot & Pugsley, 1997). We have found that even removing a single cysteine abolishes the ability of EpsE to support T2S, and all variants display very similar levels of negative dominance. Unlike WT PulE, which cannot be purified due to the aggregation of the protein when produced in the absence of other T2S components, WT EpsE is soluble and readily amenable to purification. This allowed us to determine the difference in protein solubility upon substituting cysteine residues, which showed that all cysteines are required for EpsE solubility when overexpressed in *E. coli*.

An investigation of the tetracysteine motif in *T. thermophilus* PilF by Salzer *et al* (2014) showed these cysteines are required in order to support piliation at high temperatures. PilF requires at least three cysteine residues to coordinate zinc, and may be able to use H_2O to substitute for the fourth cysteine. These authors used cysteine to alanine substitutions, whereas we chose to substitute cysteines with serines in order to maintain more closely-related amino acid side chains. At lower growth temperatures, however, cysteine substitutions do not affect PilF function, suggesting that zinc binding is not essential for pilus assembly, but rather provides the protein stability necessary for proper function at elevated

temperatures. Our data indicates that EpsE's stability is also compromised when zinc is removed; however, EpsE is non-functional upon replacement of even one of its cysteines. Additionally, zinc is not necessary for PilF ATPase activity, whereas our data show that zinc is required for ATPase activity of EpsE. PilF requires neither ATP nor zinc-coordinating cysteine residues for hexameric complex assembly and hexamerization does not appear to be a prerequisite for ATP hydrolysis, perhaps due to its extended N-terminus, which the authors suggest may provide additional stability compared to homologues lacking these additional residues. As this study focused on an extremophile, our results are more likely to be widely applicable to other mesophilic organisms, including important pathogens that express T2S and/or T4P systems.

Zinc frequently plays an important role in protein conformation and stabilization, with zinc-coordinating domains most commonly supporting overall or domain-specific protein folding and/or stability or participating in interactions with DNA, RNA, or proteins (Krishna *et al.*, 2003; Maret & Li, 2009). Zinc may stabilize a particular conformation that is important for activity, such as a redox sensor, or to position a domain for protein-protein interactions. For example, the chaperone Hsp33 acts as a molecular "redox switch," by remaining inactive in its zinc-coordinating reduced state and becoming activated upon cellular oxidation, resulting in disulfide bonding and dimerization (Jakob *et al.*, 1999; Jakob *et al.*, 2000; Graumann *et al.*, 2001). SecA, on the other hand, contains a zinc-binding domain that stabilizes the position of basic residues involved in SecB interactions. (Fekkes *et al.*, 1999; Zhou & Xu, 2003). Similarly, the ATP-dependent chaperone ClpX interacts with the cofactor $SspB_2$ via a hydrophobic patch of residues located in a zinc-binding domain (Thibault *et al.*, 2006).

The work presented here demonstrates that the zinc-coordinating C_M domain is necessary for the activity and function of EpsE, the motor protein that energizes T2S. One lingering question is whether the EpsE C_M domain shares any functions found in similar zinc-coordinating domains, such as Hsp33 and SecA. We have not ruled out the possibility that zinc binding to the C_M domain may offer a means for bacteria to modulate energy production for T2S in addition to, or as a consequence of, providing protein stability. EpsE is unlikely to act as a redox switch similar to Hsp33, as removal of zinc causes a conformational change resulting in a complete loss of activity. It is feasible that the C_M domain is necessary for correctly positioning residues involved in protein-protein interactions in a similar manner to SecA and ClpX; however, if the loop residues participate in protein-protein interactions, this interaction is largely insensitive to the amino acid substitutions in the EpsE-XcpR C_M loop chimera (Fig.

4). Potentially, modulation of the C_M domain through C_M zinc coordination/abrogation or protein-protein interaction could affect EpsE activity by altering the positioning of important residues in the β-strands that enter and exit the C_M domain (Figs. 1, 10). Structural analysis indicates that R441 in the strand leaving the C_M domain contacts the adenyl and ribose moieties of the nucleotide (Fig. 10) (Robien *et al.*, 2003). On the opposite strand, entering the C_M domain, R394 contacts a leucine residue in a neighboring subunit in 2 out of the 6 subunits in the elongated hexameric structure of ΔN1-EpsE-8aa-Hcp1 (Fig. 10) (Lu *et al.*, 2013). Changes in the contacts between subunits in the EpsE hexamer might prevent the adoption of important transient conformations that are essential for the functioning of EpsE in T2S. Repositioning of R394 and R441 in Zn-depleted EpsE variants could explain the loss of ATPase activity *in vitro* and/or T2S function *in vivo*.

The T2S system shares many structural similarities not only to the closely related T4P system, but also to the archaeal flagellar system (archaellum) and competence systems of Gram-positive bacteria (Korotkov *et al.*, 2012). The assembly ATPases supporting each of these systems share many structural features, including C_M domains; therefore, results of this study should inform further research not only on T2S, but also among many different molecular motor systems across bacterial and archaeal domains (Planet *et al.*, 2001; Robien *et al.*, 2003; Korotkov *et al.*, 2012). Knowledge about EpsE structure and function relationships may also provide insight into mechanisms of the antagonistic functions of the T4P assembly and retraction ATPases.

Experimental Procedures

Bacterial Strains and Growth Conditions

Vibrio cholerae TRH7000 (El Tor, *thy* Hg^R (*ctxA-ctxB*)), *V. cholerae* 3083 (El Tor, serotype Ogawa), and *Escherichia coli* BL21(DE3) were used in this study. *V. cholerae* strains were grown at 37°C in LB broth supplemented with 100 mg ml^{-1} thymine for TRH7000. Those strains containing plasmids were grown in the presence of 200 μg ml^{-1} carbenicillin and induced with isopropyl-D-thiogalactopyranoside (IPTG) as described in the figure legends.

Cloning and Expression

The EpsE C_M domain deletion mutants were constructed using PCR with the following primers: ΔC_M: 5'-TAAGGTGCGCACCAAGCGCTGAG-3' and 5'-TACCGTGGCCGAACCGGTAT-3'; ΔC_MPro: 5'-CCATACCGTGGCCGAACCGGTAT-3'. The EpsE ΔC_M construct is missing residues 396-437, while ΔC_MPro replaces those residues with a proline. The *epsE* fragments containing mutations were cloned into pMMB384 (wild-type *epsE* in pMMB67EH) (Sandkvist *et al.*, 1995) by exchange of an MfeI/BamHI fragment to create the pMMB EpsE variant plasmids. The pMMB plasmids were then introduced to *epsE*::*kan* and wild type (WT) *V. cholerae* strains through conjugation.

The pMMB*epsE-xcpR* C_M chimera plasmid was constructed by first amplifying the beginning of *epsE* and creating a 3' region of overlap with the beginning of the *xcpR* C_M domain (5'-GAGGGATCCTGAGCAGATGGAAGCCAAGCAATGACCGAA-3' (BamHI) and 5'-GCGCGGTAGGGCTCCTTGCAATCTGGGCATAAGG-3'.) The *xcpR* C_M fragment was amplified from pMMB-*xcpR* (Turner, 1993) using the primers 5'-CCTTATGCCCAGATTGCAAGGAGCCCTACCGCGC-3' and 5'-TGGTTACATTTAGGGCAGCCGCGGGCGCGATGCA-3'. The downstream part of *epsE* was then amplified with a 5' overlap of the end of the *xcpR* C_M domain using the primers 5'-TGCATCGCGCCCGCGGCTGCCCTAAATGTAACCA-3' and 5'-CTCCTGCAGCAAACGCGGCCATTAGGACTCCTTAGTC-3' (PstI). The three amplified fragments were used as a template for the amplification of the entire region using the first and last primers listed (to yield the *epsE-xcpR* C_M PCR product) and cloned into pMMB67EH using the BamHI and PstI restriction sites and introduced into to *epsE::kan* and WT *V. cholerae* strains through conjugation. The protein purification expression vector pET21(d)*epsE*(2-503)-*xcpR*C_M-*epsL*(1-253)-his_6 was constructed by exchange of a NotI/BsmI fragment containing the C_M domain from the *epsE-xcpR* C_M PCR product.

The full-length EpsE-Hcp1 fusion is encoded by the expression vector pET21(d)*epsE*(1-503)-8aa-*hcp1*-his_6, and the truncated ΔN1-EpsE-Hcp1 fusion from pET21(d)*epsE*(100-503)-8aa-*hcp1*-his_6. pET21(d)*epsE*(100-503)-*xcpR*C_M-8aa-*hcp1*-his_6 was created by the exchange of a NotI/BsmI fragment from pET21(d)*epsE*(2-503)-*xcpR*C_M-*epsL*(1-253)-his_6. The pMMB*epsE-hcp1* and *epsE-xcpR*C_M-*hcp1* constructs were made by PCR amplification of the region of interest from pET21(d)*epsE*(100-503)-8aa-*hcp1*-his_6 or pET21(d)*epsE*(100-503)-*xcpR*C_M-8aa-*hcp1*-his_6, respectively, using the primers 5'-GAGGGATCCGAAGGAGATATACATGGACTTCTTC-3' (BamHI) and 5'-GAGCTGCAGATATCAGGCCTGCACGTTCTG-3' (PstI) and cloning into pMMB67EH using the BamHI and PstI restriction sites. The plasmids were conjugated into *epsE::kan* and WT *V. cholerae* as before.

EpsE point mutations were constructed using the QuikChange Site-Directed Mutagenesis kit (Stratagene) as directed. The following primers were used to construct the cysteine mutations: C400S (5'-CCTTATGCCCAGATTCCAAAGAGCCTTACGAGGC-3' and 5'-GCCTCGTAAGGCTCTTTGGAATCTGGGCATAAGG-3'), C430S (5'-CGTGCAACGGGCTCCCCTAAATGTAACC-3' and 5'-GGTTACATTTAGGGGAGCCCGTTGCACG-3'), C397SC400S (5'-GGTGCGCACCTTATCCCCAGATTCCAAAGAGCCTTAC-3' and 5'-GTAAGGCTCTTTGGAATCTGGGGATAAGGTGCGCACC-3'), C430SC433S (5'-CGTGCAACGGGCTCCCCTAAATCTAACCACAAAGG-3' and 5'-CCTTTGTGGTTAGATTTAGGGGAGCCCGTTGCACG-3'). *epsE*C400SC430SC433S (C3xS) was constructed using site-directed mutagenesis with *epsE*C430SC433S template DNA and the primers for C400S listed above, and *epsE*C397SC400SC430SC433S (C4xS) was made similarly using *epsE*C397SC400S as a template and the primers for C430SC433S. Regions containing mutations were cloned into pMMB384 by exchange of an MfeI/BamHI fragment to create the individual pMMB EpsE variant plasmids. Plasmids were then introduced to *epsE::kan* and WT *V. cholerae* strains through conjugation.

Protease and Lipase Secretion Assays

Measurements of extracellular protease activity were performed as described (Sikora *et al.*, 2007). Briefly, the fluorogenic probe, *N*-tert-butoxy-carbonyl-Gln-Ala-Arg-7-amido-4-methylcoumarin (Sigma-Aldrich) was added to overnight culture supernatants and 10-minute kinetic protease activity was measured using fluorescence at excitation and emission wavelengths 385 nm and 440 nm, respectively. Lipase activity was quantified as described previously by incubating *V. cholerae* overnight culture supernatants with 4-nitrophenyl myristate and measuring 4-nitrophenol release as the change in absorbance at 415 nm over a 30-minute period (Johnson *et al*., 2015). All assays were performed in triplicate, normalized to the density of the culture at 600 nm, and mean and SEM are displayed.

SDS-PAGE and Immunoblotting

Cell lysates were boiled in SDS sample buffer and analyzed by SDS-PAGE using 4-12% Bis-Tris gels (NuPAGE, Invitrogen) and MES or MOPS running buffer. Proteins were transferred to nitrocellulose membranes (Protran, GE Healthcare) using NuPage transfer buffer (Invitrogen) and probed with either 1:10,000 α-EpsE antibodies or 1:5000 α-cholera toxin antibodies followed by 1:20,000 horseradish peroxidase-conjugated goat α-rabbit immunoglobulin G (BioRad) or 1:1,000 α-Hcp1 antibodies followed

by 1:10,000 goat α-rabbit IgG-HRP. Blots were developed using Ecl2 (Pierce) and imaged using a Typhoon FLA 9500 (GE Healthcare).

Protein Purification

Constructs for purification were introduced into pET21(d)*epsE*(100-503)-8aa-*hcp1*-his_6 (ΔN1-EpsE-Hcp1) (Lu *et al.*, 2013) or pET21(d)*epsE*(1-503)-8aa-*hcp1*-his_6 (full-EpsE-Hcp1) and expressed in *E. coli* BL21(DE3) under IPTG-inducing conditions. Proteins were purified using metal affinity chromatography on cobalt resin (Talon, Clontech). Subsequently, size-exclusion chromatography was performed using a Superose 6 column (GE Healthcare) as described (Robien *et al.*, 2003; Lu *et al.*, 2013) and compared to known protein standards. Gel filtration fractions containing protein peaks were analyzed using SDS-PAGE and visualized by staining the gel with Gel Code Blue (Thermo Scientific).

ATPase Activity Assays

Purified EpsE-Hcp1 and ΔN1-EpsE-Hcp1 fusion proteins were assayed for *in vitro* ATPase activity according to (Lu *et al.*, 2013) using BIOMOL Green reagent (Enzo Life Sciences) to detect free Pi.

PAR/PCMB Assay

Zinc release was measured using a modified PAR/PCMB assay (Camberg & Sandkvist, 2005; Ilbert *et al.*, 2007). Briefly, zinc was removed from ΔN1-EpsE-Hcp1 purified protein using a titration of p-chloromercuribenzoic acid (PCMB; Sigma) in the presence of the zinc-complexing agent 4-(2-pyridylazo)resorcinol (PAR; Sigma). Zinc release was measured at 500 nm and compared to a $ZnCl_2$ standard curve. Assays were performed in duplicate and SEM is shown.

Native-PAGE

Proteins were treated with a 4-fold molar excess of PCMB or mock-treated for 10 minutes at room temperature and analyzed on a 4-20% Tris-Glycine native gel (NuPAGE, Invitrogen) with Tris-Glycine running buffer at 125V for 5 hours on ice. Proteins were visualized by staining the gel with Gel Code Blue (Thermo Scientific).

β-Lactamase activity assay

Periplasmic contents were isolated by incubating cells from overnight cultures with 2,000 U/ml polymyxin B sulfate in PBS on ice for 30 minutes, centrifuging at 8,000 rpm for 10 minutes, and isolating

the supernatant (periplasmic extract) from spheroplasts. β-Lactamase activity was measured in overnight culture supernatants and periplasmic extracts as previously described with some modifications (Sikora *et al*., 2007). Nitrocefin (EMD Chemicals) was added to supernatants and periplasmic extracts in PBS buffer and the absorbance at 482 nm was measured over the course of 5 minutes at 37 °C.

Acknowledgments

We acknowledge Dr. Connie Lu for plasmids pET21(d)*epsE*(1-503)-8aa-*hcp1*-his$_6$ and pET21(d)*epsE*(100-503)-8aa-*hcp1*-his$_6$, Dr. Stephen Lory for the pMMB-*xcpR* plasmid, Dr. John Mekalanos for the α-Hcp1 Pa antibody, and Wilhelm Voth and Dr. Ursula Jakob for assistance with the PCMB/PAR assay. This work was supported by National Institutes of Health grants R01AI049294 (to M. S.) and R01AI34501 (to W. G. J. H.).

Figure 1. Structural comparison of type II/IV secretion ATPases. The structures of the *V. cholerae* T2S ATPase EpsE (left, PDB 1P9W), the *P. aeruginosa* type IV pilus retraction ATPase PilT (center, PDB 3JVV), and the *H. pylori* type IV secretion ATPase HP0525 (right, PDB 1G6O) are shown. N-terminal domains are colored green, C-terminal domains in cyan, and the C_M domain in EpsE is displayed in red with zinc represented as a blue sphere. Nucleotide is shown in orange.

Figure 2. The EpsE C_M domain is required for secretion. A. WT and *epsE::kan* strains of *V. cholerae* TRH7000 containing empty vector (-) or pMMB plasmids encoding WT or mutant EpsE variants described on the x-axis were grown with 200μg ml^{-1} carbenicillin and 10μM IPTG. Culture supernatants were analyzed for VesB protease activity using a cleavable fluorogenic probe as described in *Experimental Procedures*. All EpsE variants showed statistical significance compared to WT EpsE ($p<0.0001$). **B.** The same overnight culture supernatants as in A were analyzed for lipase activity as described in *Experimental Procedures.* All EpsE variants showed statistical significance compared to WT EpsE ($p<0.03$) **C.** Expression of EpsE in WT *V. cholerae* TRH7000, followed by *epsE::kan V. cholerae* containing empty vector and *epsE::kan V. cholerae* expressing either WT EpsE, or variants of EpsE. Samples were analyzed by SDS-PAGE and immunoblotting for EpsE. The size of EpsE and EpsE ΔC_M are indicated by black arrows, and EpsE dimers by a gray arrow.

Figure 3. A. WT *V. cholerae* TRH7000 containing pMMB plasmids encoding WT or mutant EpsE variants described on the x-axis were grown with 200μg ml^{-1} carbenicillin and 100μM IPTG. Samples were prepared and assayed for protease activity as in Fig. 2. Assays were performed in triplicate and standard error is shown. All EpsE variants showed statistical significance compared to WT EpsE ($p<0.0001$). **B.** WT *V. cholerae* 3083 and isogenic ΔepsC containing pMMB plasmids (empty vector (-), WT EpsE, or EpsE C4xS) were grown with 200μg ml^{-1} carbenicillin and 100μM IPTG. Cells (C) and supernatants (S) were analyzed by SDS-PAGE and immunoblotting for cholera toxin.

Figure 4. Alignment of C_M domains in T2S ATPase homologues. Clustal Omega alignment of $CXXCX_{27\text{-}30}CXXC$ motifs of T2S ATPase homologues. Asterisks below indicate residue conservation identity, and colons and periods indicate high and low levels of residue homology, respectively.

Figure 5. The EpsE-XcpR C_M chimera partially complements the T2S defect in *epsE::kan* mutants of *V. cholerae*. A. *V. cholerae* TRH7000 WT, followed by *epsE::kan* strains containing empty vector (-) or pMMB encoding EpsE or EpsE-XcpR C_M were grown with 200μg ml^{-1} carbenicillin and 10μM IPTG. The last bar represents WT *V. cholerae* expressing EpsE-XcpR C_M induced with 100μM IPTG to test for negative dominance. Culture supernatants were analyzed for VesB activity using a cleavable fluorogenic probe as described in *Experimental Procedures*. Assays were performed in triplicate and SEM is shown. No statistically significant difference between WT EpsE and EpsE-XcpR C_M was detected using a T-test ($p = 0.37$). **B.** Overnight culture supernatants were assayed for lipase activity as in Fig. 2B. No statistically significant difference between WT EpsE and EpsE-XcpR C_M was detected using a T-test ($P = 0.064$). **C.** Expression of EpsE in WT *V. cholerae* TRH7000 (lane 1), followed by empty vector (lane 2), WT EpsE (lane 3), or EpsE-XcpR C_M expressed in *epsE::kan V. cholerae* (lane 4) and induced with 10μM IPTG. Samples were analyzed by SDS-PAGE and immunoblotting for EpsE.

Figure 6. EpsE-Hcp1 and EpsE-XcpR C_M–Hcp1 fusions support secretion in *V. cholerae*. A. WT and *epsE::kan* strains of *V. cholerae* TRH7000 containing empty vector (-), pMMB encoding EpsE, EpsE-Hcp1, or EpsE-XcpR C_M–Hcp1 were grown with 200μg ml^{-1} carbenicillin and 10μM IPTG. Supernatants were analyzed for VesB activity using a cleavable fluorogenic probe as described in *Experimental Procedures*. Assays were performed in triplicate and standard error is shown. **B.** Lipase assays were performed on overnight culture supernatants as in Fig. 2B. Assays were performed in triplicate with standard errors displayed.

Figure 7. Detection of full-length EpsE-Hcp1 fusions in *V. cholerae*. Overnight cultures of *V. cholerae* TRH7000 WT or *epsE*::kan strains containing empty vector (-) or pMMB plasmids encoding EpsE, EpsE-Hcp1, or EpsE-XcpR C_M–Hcp1 were grown with 200μg ml^{-1} carbenicillin and 10μM IPTG. Cell lysates were analyzed by SDS-PAGE and immunoblotting using α-EpsE antibodies (left) or α-Hcp1 antibodies (right). Molecular mass markers are indicated and the positions of EpsE and EpsE-Hcp1 fusion are shown with arrows. Purified EpsE-Hcp1 protein was included as a positive control.

Figure 8. The EpsE-XcpR C_M–Hcp1 chimera fusion maintains *in vitro* ATPase activity. Purified EpsE-Hcp1 and EpsE-XcpR C_M–Hcp1 fusions were assayed for *in vitro* ATPase activity as described in *Experimental Procedures*.

Figure 9. Removal of zinc results in a loss of *in vitro* ATPase activity and changes the migration pattern of ΔN1-EpsE-Hcp1. A. Zinc release titration curve. Increasing amounts of p-chloromercuribenzoic acid (PCMB) result in increased zinc release. **B.** Treatment of ΔN1-EpsE-Hcp1 protein abolishes *in vitro* ATPase activity. Proteins were either untreated or treated with a 4-fold molar excess of PCMB or mock-treated for 10 minutes at room temperature and assayed for ATPase activity as described in *Experimental Procedures*. **C**. Purified ΔN1-EpsE-Hcp1 was untreated or incubated with a 4-fold molar excess of PCMB or mock-treated for 10 minutes at room temperature. Samples were then analyzed using native-PAGE and stained with Coomassie.

Figure 10. Close-up view of residues at the base of the C_M domain and potential interactions with adjacent subunits or nucleotide. A. View along the twofold axis of the *V. cholerae* EpsE-8aa-Hcp1 hexamer with C2 symmetry (Lu *et al*., 2013), (PDB code 4KSR). The three independent chains are related by a twofold axis and are displayed as green (chains A, A'), cyan (B, B'), and magenta (C, C') with C_M domains colored red. Arg 394 residues from each subunit are displayed as red spheres, and Leu 349 residues are displayed as spheres according to the color of the corresponding subunit. **B.** Arg 394 from chain C is shown in proximity to Leu 349 of chain A'. The proximity of Arg 441 to AMPPNP is also shown, with an alpha-helix from the EpsE C-terminal domain in purple. Distances between possible contacts are indicated by dotted lines and labeled. Zinc is superimposed from the structure of monomeric EpsE (Robien *et al*., 2003) (PDB code 1P9W) and is displayed as a blue sphere.

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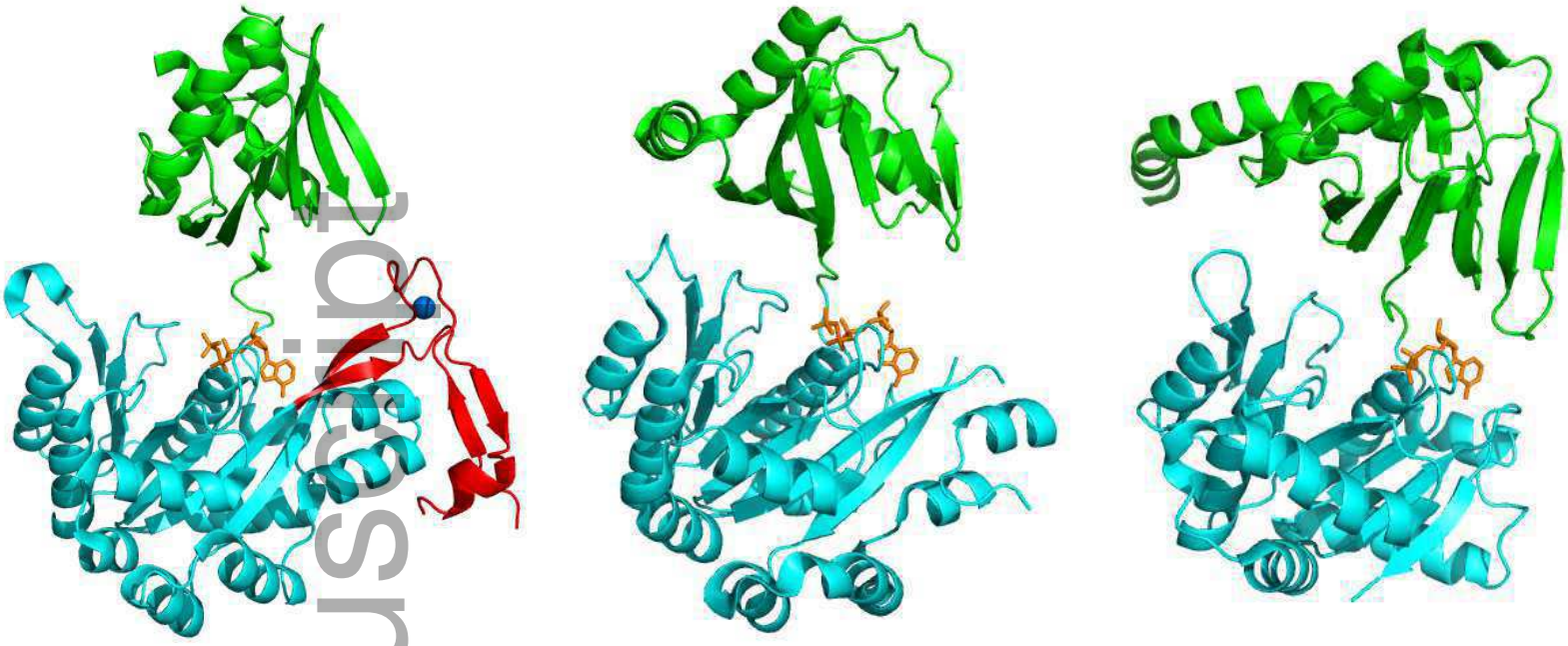

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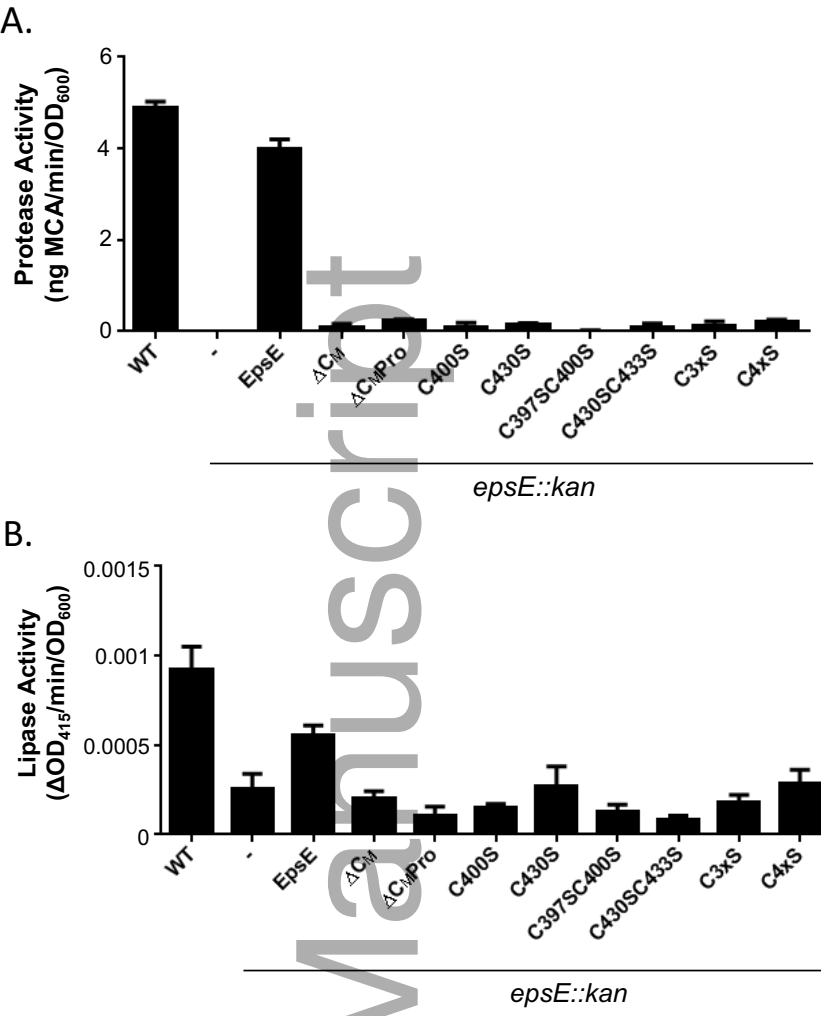

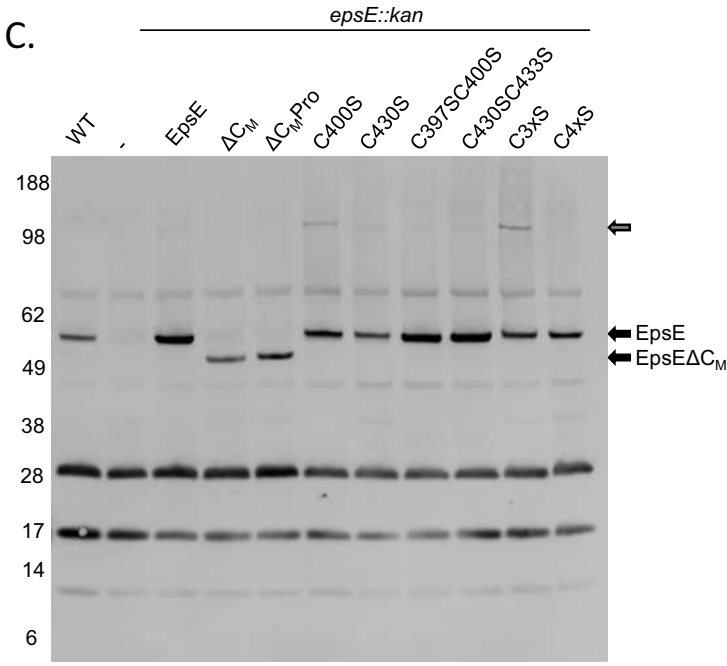

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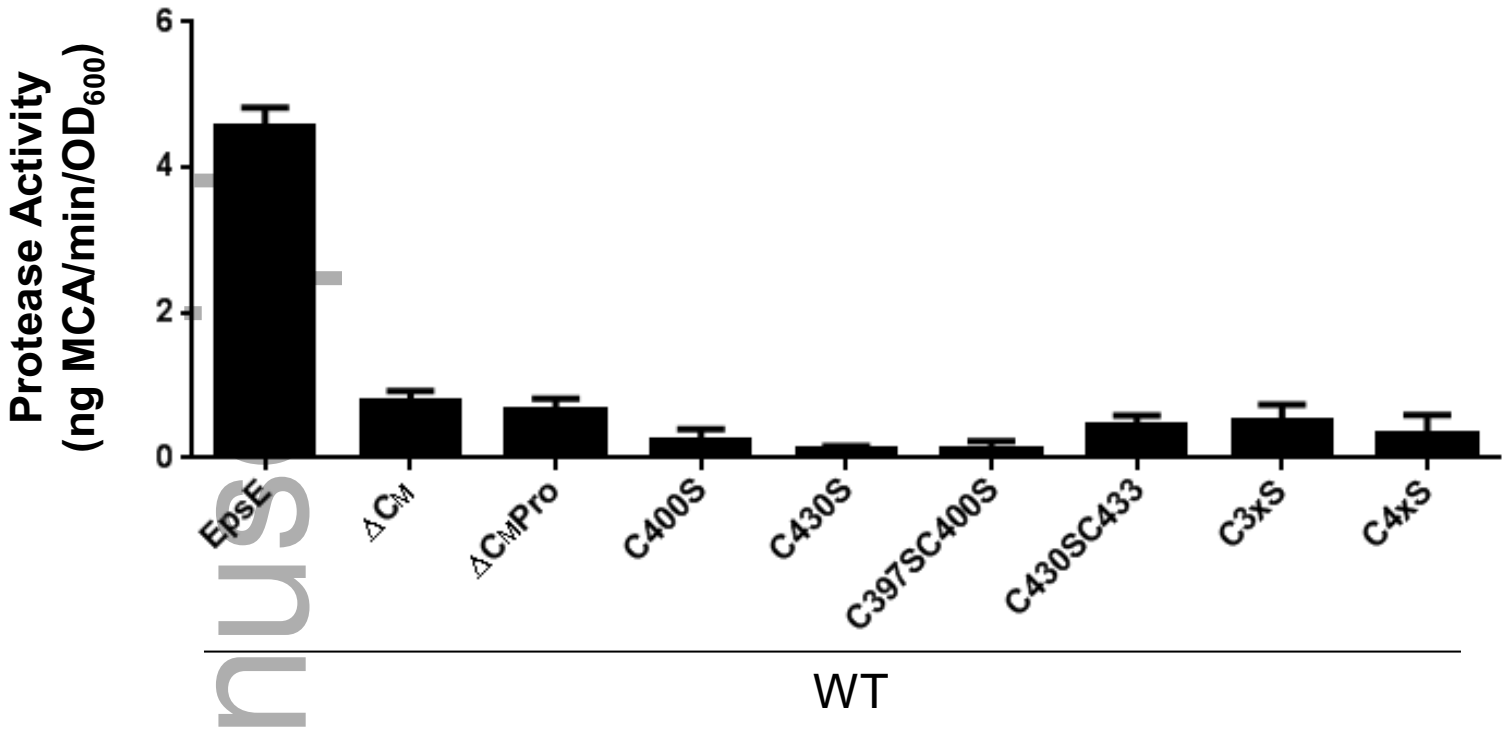
A.



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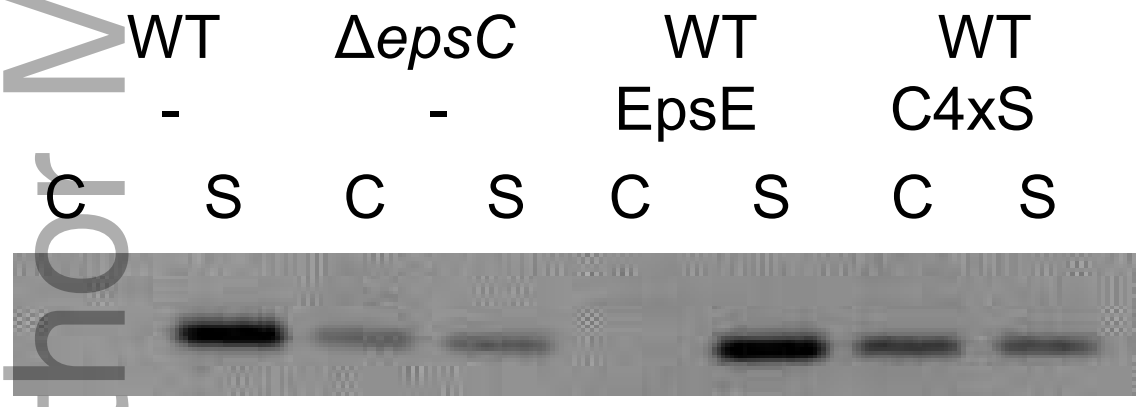

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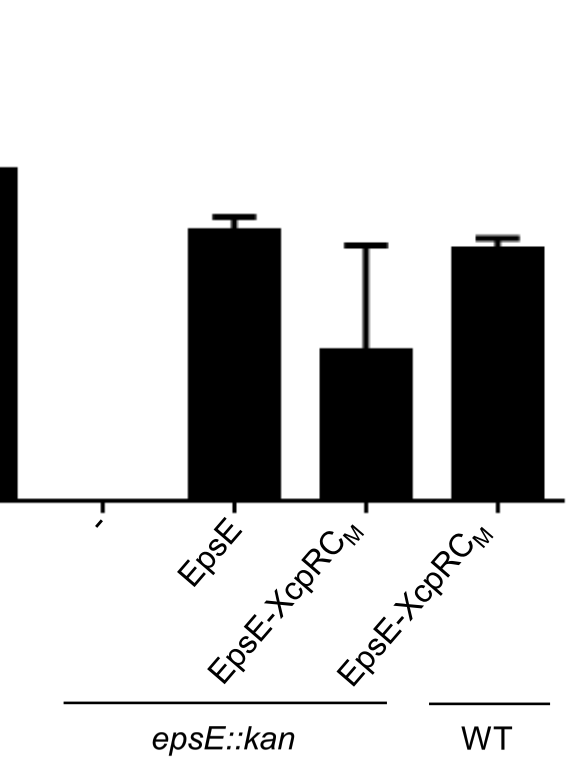
V. cholerae EpsE cpdckepyeadkeqrklfdskkkep-lilyratgcpkc
P. aeruginosa XcpR cpackepyradeaecallgvdpaap-ptlhrargcgec
A. hydrophila ExeE cpdcraprpiteqerlamgmelapd-qqvwrpvgceqc
K. oxytoca PulE cphcrqqepanadt--ahqmeiapg-talwqprgcaec
D. dadantii OutE cpscrqpyvindeq--aqqtglaag-ttlyhpggcekc
L. pneumophila GspE cshcktphelrqeekelmglkpdadvsqvfepkgcdyc
* *: : . ** *



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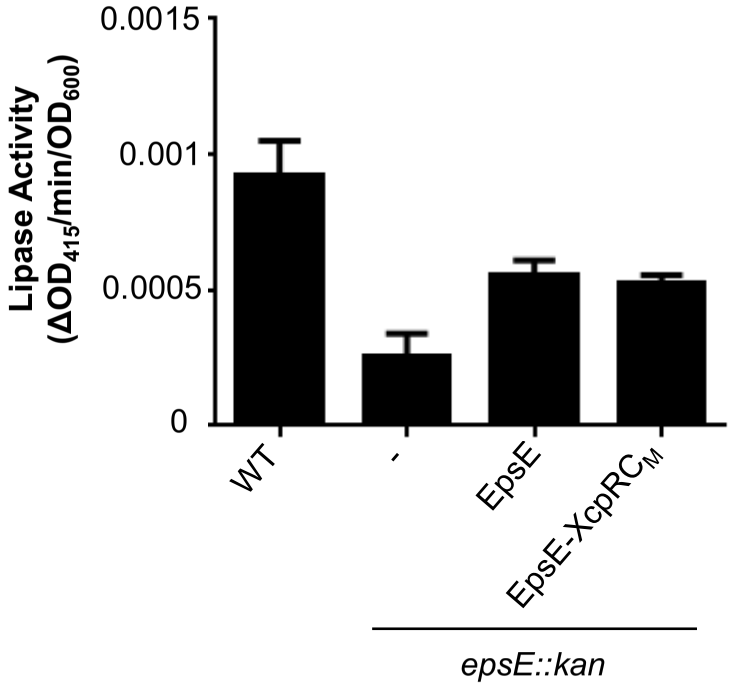

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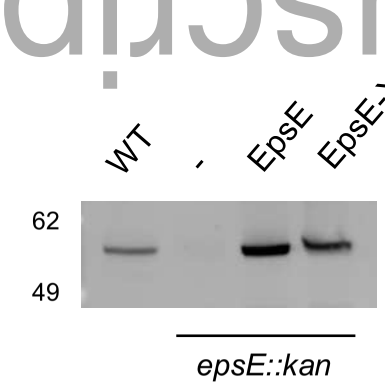

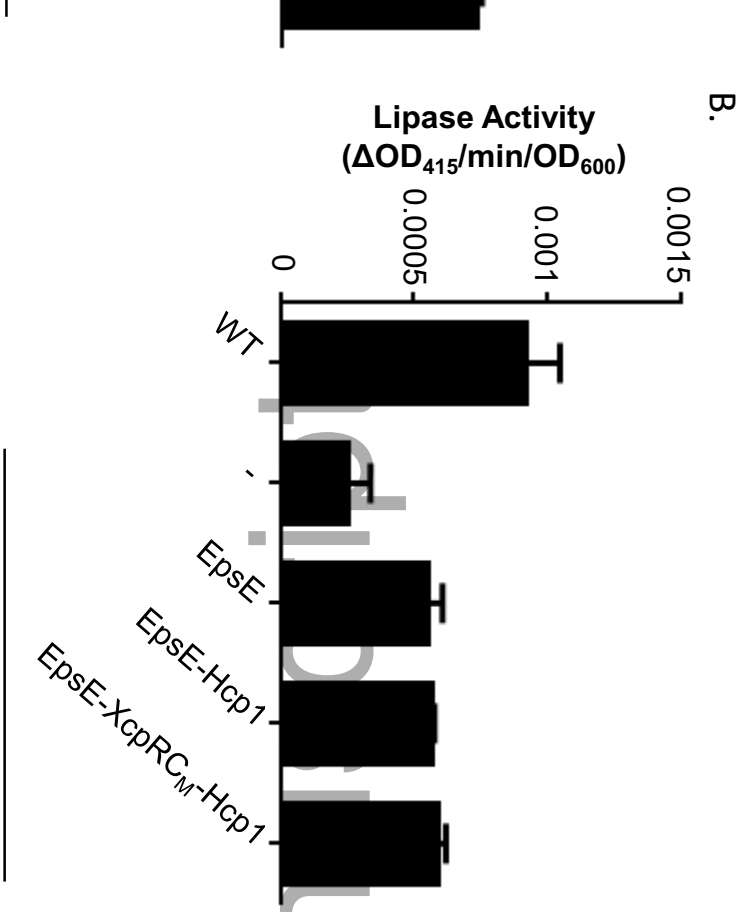

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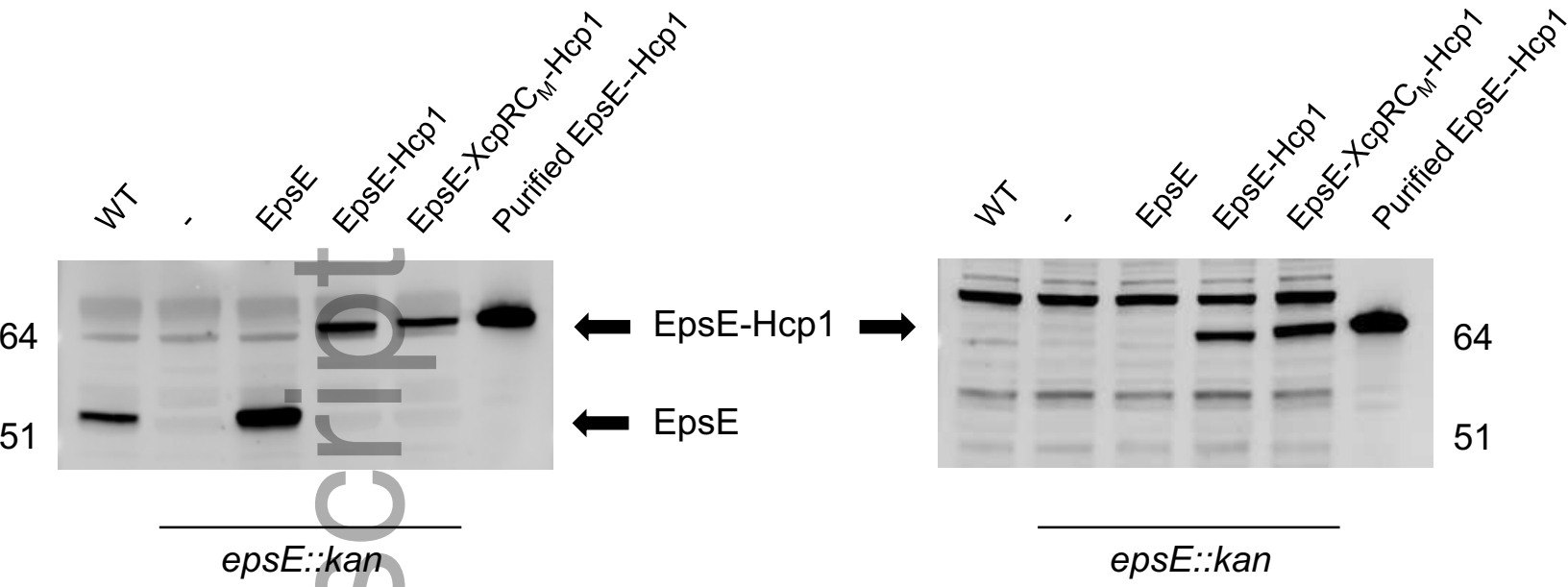

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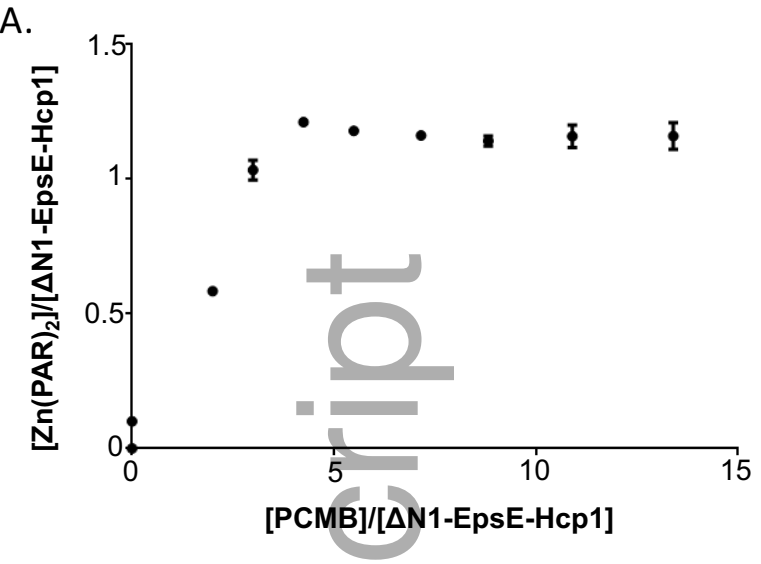

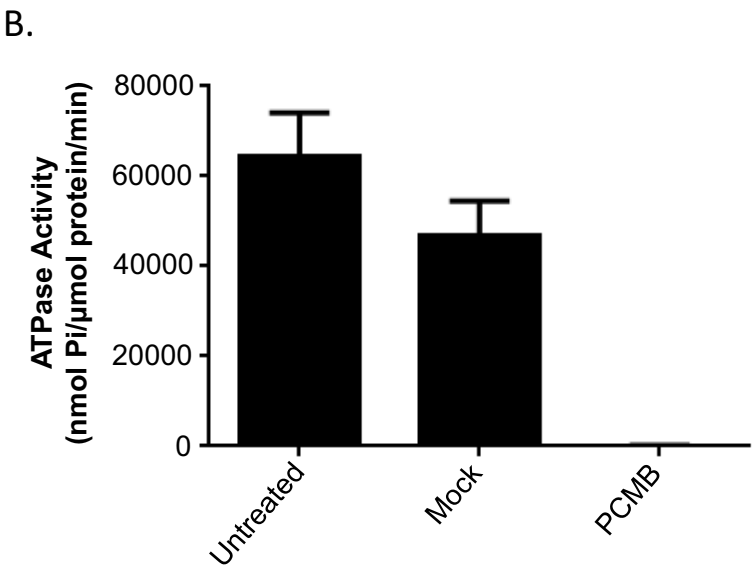

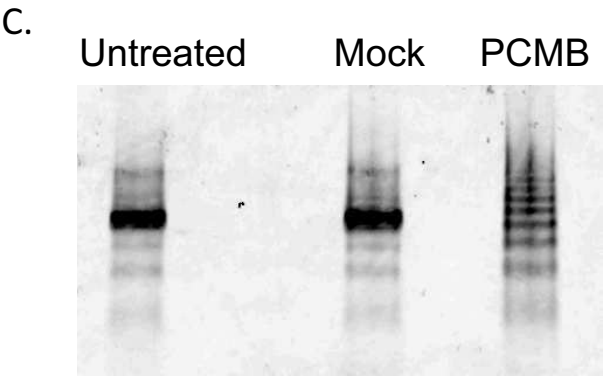

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