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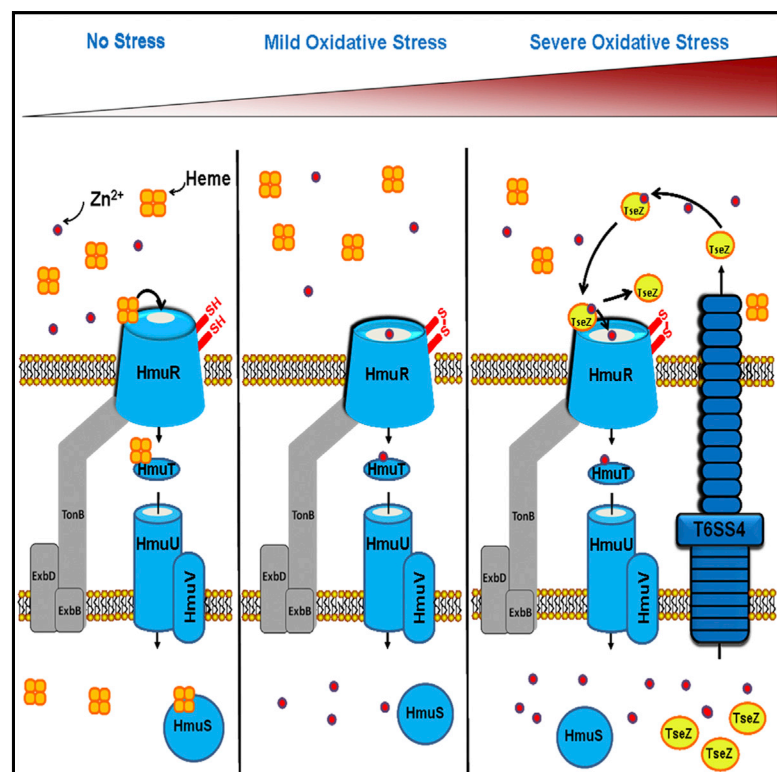
The Type VI Secretion System Engages a Redox-Regulated Dual-Functional Heme Transporter for Zinc Acquisition

Graphical Abstract



Authors

Meiru Si, Yao Wang, Bing Zhang, ..., Lei Zhang, Tao G. Dong, Xihui Shen

Correspondence

xihuishen@nwsuaf.edu.cn

In Brief

Si et al. find that T6SS4 secretes a proteinaceous zincophore, TseZ, that interacts with the outer membrane heme transporter HmuR to acquire zinc under oxidative stress. HmuR is a dual-function transporter, transporting heme under normal conditions but zinc upon sensing extracellular oxidative stress, triggered by forming an intramolecular disulfide bond.

Highlights

- The outer membrane heme transporter HmuR transports zinc during oxidative stress
- HmuR switches substrate specificity by forming an intramolecular disulfide bond
- T6SS4 secretes a proteinaceous zincophore to enhance HmuR zinc acquisition
- HmuR suggests a mechanism for gradually responding to stress

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The Type VI Secretion System Engages a Redox-Regulated Dual-Functional Heme Transporter for Zinc Acquisition

Meiru Si,[1,3,4] Yao Wang,[1,4] Bing Zhang,[1] Chao Zhao,[1] Yiwen Kang,[1] Haonan Bai,[1] Dawei Wei,[1] Lingfang Zhu,[1] Lei Zhang,[1] Tao G. Dong,[2] and Xihui Shen[1,5,*]
[1]State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China
[2]Ecosystem and Public Health, Faculty of Veterinary Medicine, University of Calgary, Calgary, AB T2N 4Z6, Canada
[3]College of Life Sciences, Qufu Normal University, Qufu, Shandong 273165, China
[4]These authors contributed equally
[5]Lead Contact
*Correspondence: xihuishen@nwsuaf.edu.cn
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SUMMARY

The type VI secretion system was recently reported to be involved in zinc acquisition, but the underlying mechanism remains unclear. Here, we report that *Burkholderia thailandensis* T6SS4 is involved in zinc acquisition via secretion of a zinc-scavenging protein, TseZ, that interacts with the outer membrane heme transporter HmuR. We find that HmuR is a redox-regulated dual-functional transporter that transports heme iron under normal conditions but zinc upon sensing extracellular oxidative stress, triggered by formation of an intramolecular disulfide bond. Acting as the first line of defense against oxidative stress, HmuR not only guarantees an immediate response to the changing environment but also provides a fine-tuned mechanism that allows a gradual response to perceived stress. The T6SS/HmuR-mediated active zinc transport system is also involved in bacterial virulence and contact-independent bacterial competition. We describe a sophisticated bacterial zinc acquisition mechanism affording insights into the role of metal ion transport systems.

INTRODUCTION

As the second most abundant transition metal ion in living organisms after iron, zinc is essential for life by serving both catalytic and structural roles within a large number of proteins. To acquire sufficient zinc for survival and pathogenesis, bacteria have evolved a number of efficient zinc transporters. Transport of Zn^{2+} across the outer membrane is mediated by either nonspecific porins or TonB-dependent receptors, such as *Neisseria meningitidis* ZnuD and CbpA (Kumar et al., 2012; Stork et al., 2010). When in the periplasm, zinc can cross the cytoplasmic membrane through either the high-affinity ATP-binding cassette family transporter ZnuACB (Claverys, 2001) or the low-affinity ZRT/IRT-related protein (ZIP) family transporter ZupT (Cerasi et al., 2014). Additionally, some pathogenic bacteria were reported to secrete Zn^{2+}-chelating compounds analogous to siderophores as a mechanism of acquiring this nutrient during infection (Bobrov et al., 2014). Zur, a Zn^{2+}-sensing metalloregulatory protein belonging to the ferric uptake regulator family, is used by many bacteria to repress the transcription of Zn^{2+} uptake systems to maintain zinc homeostasis (Mazzon et al., 2014; Sein-Echaluce et al., 2015).

Type VI secretion systems (T6SSs) are used by many Gram-negative bacteria to translocate effector proteins into eukaryotic or prokaryotic cells. Many genomes harbor multiple evolutionarily distinct T6SS clusters that presumably play different roles in bacterial life cycles. T6SSs are known to mediate competition among rival bacteria in polymicrobial environments by translocating antibacterial toxins (e.g., cell wall-degrading enzymes, nucleases, and membrane-targeting enzymes) into competitor bacterial cells (Durand et al., 2014; Cianfanelli et al., 2016). Moreover, some T6SSs associated with pathogens are involved in bacterial pathogenesis by translocating antieukaryotic effectors into eukaryotic cells, thus modulating host immunity and inflammation (Aubert et al., 2016; Hachani et al., 2016).

Recently, we reported that T6SS was involved in the acquisition of metal ions such as iron, manganese, and zinc, highlighting an unexpected role for T6SS in enhancing bacterial fitness via competition for essential nutrients. In *P. aeruginosa*, the T6SS effector TseF facilitates the delivery of outer membrane vesicle-associated iron to bacterial cells by engaging the Fe(III)-pyochelin receptor FptA and the porin OprF (Lin et al., 2017). In *Burkholderia thailandensis*, a manganese-binding T6SS effector, TseM, scavenges extracellular Mn^{2+} and delivers that Mn^{2+} via direct interaction with the TonB-dependent outer membrane transporter MnoT to fulfill the increased cellular demand for Mn^{2+} under conditions of oxidative stress (Si et al., 2017). Additionally, T6SS4 of *Yersinia pseudotuberculosis* acquires zinc via secretion of the zinc-chelating effector

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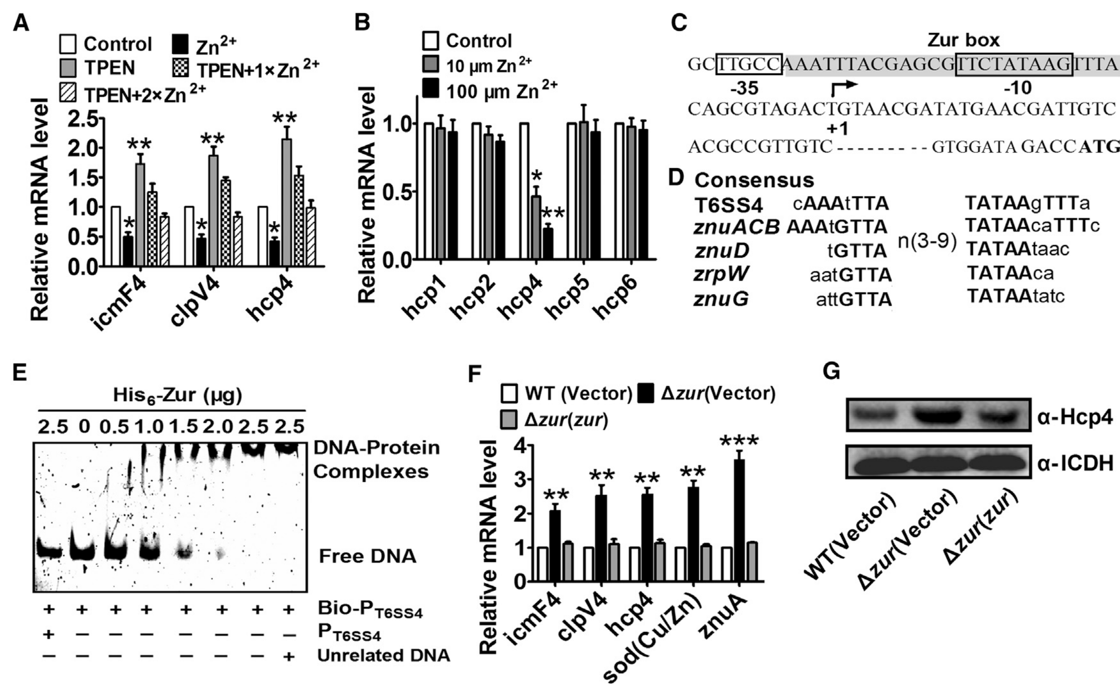

Figure 1. Regulation of T6SS4 Expression by Zur

(A) Zinc-responsive expression of T6SS4. *B. thailandensis* strains were grown in Luria-Bertani (LB) containing 60 μM Zn^{2+}, 60 μM TPEN, 60 μM TPEN together with 60 μM Zn^{2+} (TPEN + 1 × Zn^{2+}), or 60 μM TPEN together with 120 μM Zn^{2+} (TPEN + 2 × Zn^{2+}), respectively; expression was measured by qRT-PCR.

(B) Zinc-responsive expression of different *hcp* genes was determined by qRT-PCR. Relative mRNA levels were calculated using the $\Delta\Delta C_T$ method and represent fold changes in comparison with growth in LB medium.

(C) Identification of a Zur binding site in the T6SS4 promoter region, indicated by gray shading.

(D) Comparison of the putative Zur binding site in the T6SS4 promoter with consensus Zur binding sites.

(E) Binding of Zur to the T6SS4 promoter examined by EMSA. Interaction of Zur with a biotin-labeled probe was detected using streptavidin-conjugated horseradish peroxidase (HRP) and a chemiluminescent substrate. The unlabeled promoter was added to determine the binding specificity of Zur. Bio-P_{T6SS4}, biotin-labeled T6SS4 promoter.

(F) Zur represses T6SS4 expression. Relative expression, measured by qRT-PCR, in the indicated strains was determined. The expression levels of representative T6SS4 genes in Δ*zur*(Vector) and Δ*zur*(*zur*) are shown as fold changes relative to their expression levels in WT(Vector).

(G) The protein level of Hcp4 expressed in relevant *B. thailandensis* strains. Lysates from bacteria grown in LB medium were resolved by SDS-PAGE, and Hcp4 was detected by a specific anti-Hcp4 antibody. Isocitrate dehydrogenase (ICDH) was used as a loading control.

The data shown are the averages and SDs from three independent experiments. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. See also Figures S1 and S2.

YezP (Wang et al., 2015). Although the mechanisms of T6SS-mediated iron and manganese acquisition have thus been clarified, the zinc uptake mechanism involving T6SS remains less described.

Here we describe a sophisticated bacterial Zn^{2+} acquisition pathway consisting of a Zur-regulated T6SS and a TonB-dependent outer membrane heme transporter, HmuR, in *B. thailandensis*. T6SS-mediated zinc acquisition was achieved by secretion of a zinc-binding effector, TseZ, that functions in conjunction with the heme transporter HmuR for active transport of zinc across the outer membrane. Notably, HmuR is a redox-regulated dual-function transporter, transporting heme iron under normal conditions (in the absence of oxidative stress) and, triggered by the formation of an intramolecular disulfide bond, transporting zinc when oxidative stress is in play. HmuR thus serves to sense extracellular oxidative stress, not only allowing the development of an immediate response to a changing environment but also serving as a fine-tuned mechanism allowing a gradual response to stressful conditions.

RESULTS

Zur Represses T6SS4 Expression in *B. thailandensis*

Previously, Burtnick and Brett (2013) reported that the T6SS1s in *B. pseudomallei* and *B. mallei* were negatively regulated by zinc. Although the T6SS1 in *B. thailandensis* was not subject to zinc regulation, the expression of T6SS4 genes (*icmF4*, *clpV4*, and *hcp4*) was found to be repressed by the presence of Zn^{2+}. This repression is specific because exogenous Zn^{2+} had no effect on other *hcp* genes encoded in the T6SS gene clusters of *B. thailandensis* (*hcp1* [*bth_I2962*], *hcp2* [*bth_II0123*], *hcp5* [*bth_II0868*], and *hcp6* [*bth_II0260*]) (Figures 1A and 1B). Moreover, the expression of T6SS4 genes was markedly induced by chelation of Zn^{2+} from the medium with the specific zinc chelator N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) (Stork et al., 2010), and such induction was repressed by exogenous Zn^{2+} in a dose-dependent manner (Figure 1A). The T6SS4 promoter region contains a putative Zur binding site highly similar to the *zur* box identified in *E. coli* (Patzer and Hantke,

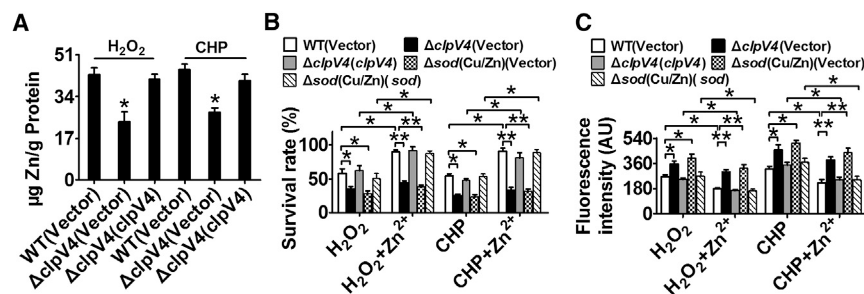

Figure 2. T6SS4 Combats Oxidative Stress by Importing Zn^{2+}

(A) Zn^{2+} uptake requires T6SS4 under conditions of oxidative stress. Stationary phase *B. thailandensis* strains were exposed to 1 mM H_2O_2 and 0.25 mM CHP for 20 min in PBS containing 1 μM $ZnSO_4$. Zn^{2+} associated with bacterial cells was measured by inductively coupled plasmon resonance atomic absorption spectrometry (ICP-MS).

(B) Alleviation of the sensitivity of *B. thailandensis* strains to H_2O_2 and CHP by exogenous Zn^{2+} (1 μM) requires T6SS4. Relevant stationary phase bacterial strains were exposed to 1.0 mM H_2O_2 or 0.25 mM CHP in M9 medium with or without exogenously provided Zn^{2+} (1 μM), and the viability of the cells was determined.

(C) Reduction of intracellular ROS in H_2O_2- and CHP-treated *B. thailandensis* strains by exogenous Zn^{2+} (1 μM) requires T6SS4.

The data shown are the averages of three independent experiments, and error bars indicate the SDs from three independent experiments. **$p < 0.01$, *$p < 0.05$. See also Figure S1.

2000), *Neisseria meningitidis* (Kumar et al., 2012), and *Caulobacter crescentus* (Mazzon et al., 2014; Figures 1C and 1D).

Sequence comparisons allowed identification of the gene encoding Zur (*bth_I0691*); this gene exhibited 36% and 34% amino acid sequence identities with the Zur-encoding genes of *N. meningitidis* and *E. coli*, respectively (Figure S1A). Consistent with the role played by Zur under conditions of oxidative stress (Sein-Echaluce et al., 2015), a Δ*zur* mutant was more resistant to hydrogen peroxide (H_2O_2)-mediated and cumene hydroperoxide (CHP)-mediated challenge than wild-type and complemented strains (Figure S1B). Additionally, *zur* expression was specifically regulated by zinc but not iron, manganese, or cobalt (Figure S1C).

Incubation of the T6SS4 promoter probe (P_{T6SS4}) with His_6-Zur triggered formation of DNA-protein complexes, evident in an electrophoretic mobility shift assay (EMSA) (Figure 1E). These DNA-protein complexes were completely disrupted upon addition of excess unlabeled probe (Figure 1E, leftmost lane) but not by addition of unrelated DNA (Figure 1E, rightmost lane), indicating that the T6SS4 promoter and Zur engaged in a specific interaction, in turn suggesting that T6SS4 expression was directly regulated by Zur.

As expected, deletion of *zur* markedly enhanced the expression of T6SS4 genes (*icmF4*, *clpV4*, and *hcp4*) as well as *znuA* and *sod*(Cu/Zn), two established target genes of Zur (Sein-Echaluce et al., 2015). Moreover, the increased expression could be fully restored to the wild-type level by complementation (Figure 1F). Similar to *icmF4* and *hcp4*, the expression levels of *znuA* and *sod*(Cu/Zn) were regulated specifically by zinc but not iron or cobalt, and such zinc-regulated expression was mediated principally by Zur (Figure S1D). Recently, we showed that *B. thailandensis* T6SS4 mediated oxidative stress resistance by importing manganese; T6SS4 expression was inhibited by high-level Mn^{2+} (Si et al., 2017). Consistent with this, expression of *icmF4* and *hcp4*, but not *znuA* or *sod*(Cu/Zn), was also repressed by high Mn^{2+} concentrations, but these effects were not mediated by Zur (Figure S1D). The negative regulatory effect of Zur on T6SS4 was further confirmed at the protein level with the observation that Hcp4 production was significantly elevated in the Δ*zur* mutant compared with the wild-type and complemented strains (Figure 1G; Figure S2A). These observations indicate that Zur directly represses T6SS4 expression.

T6SS4 Combats Oxidative Stress by Importing Zinc

Both manganese and zinc are crucial for protection against oxidative damage, either by acting as a cofactor for antioxidant enzymes or by competitively inhibiting the damaging Fenton reaction (Gaballa and Helmann, 2002; Faulkner and Helmann, 2011). The observations that the *B. thailandensis* T6SS4 has antioxidant activity and is subject to zinc regulation suggest that T6SS4 is also involved in Zn^{2+} transport for combating oxidative stress. To test this hypothesis, we measured the intracellular Zn^{2+} contents in bacteria challenged with CHP and H_2O_2 using inductively coupled plasmon resonance atomic absorption spectrometry (ICP-MS). As predicted, deletion of *clpV4* significantly reduced intracellular Zn^{2+} contents, and these defects were almost fully recovered by complementation of the *clpV4* gene (Figure 2A). Such defects did not result from potentially lower live bacterial cells because the 20-min treatment did not affect bacterial viability (Figure S1E). The role of T6SS4 in combating oxidative stress by importing Zn^{2+} was further corroborated by the observations that deletion of *clpV4* diminished the effect of exogenous Zn^{2+} on increasing the survival rate and reducing the intracellular reactive oxygen species (ROS) levels of bacteria under oxidative stress conditions (Figures 2B and 2C). Interestingly, the Zur-mediated repression of *hcp4* was eliminated upon CHP challenge (Figure S1F). Thus, oxidative stress relieved Zur-mediated repression of T6SS4 transcription; T6SS4 then exerted an antioxidant function via importing zinc.

T6SS4 Secretes a Zinc-Binding Protein Substrate

We previously reported that zinc transportation by *Y. pseudotuberculosis* T6SS4 can be achieved by secreting YezP, a Zn^{2+}-binding protein located in the T6SS4 gene cluster (Wang et al., 2015). Interestingly, a hypothetical protein (BTH_II1884) containing two zinc-finger motifs that is 39% identical with YezP was identified in the *B. thailandensis* T6SS4 gene cluster (Figures S3A–S3D). The disassociation constant (K_D) between BTH_II1884 and Zn^{2+} was 0.28 ± 0.05 μM, as measured by isothermal titration calorimetry (ITC). Mutation of the two zinc-finger motifs ($BTH_II1884^{H15A/Q16R/H76A/Q77R}$) markedly reduced its affinity to Zn^{2+} (K_D = 376.9 ± 17.5 μM) (Figure 3A). However, this protein did not seem to bind iron (Figure S4B). Although significant amounts of BTH_II1884 could be easily detected in

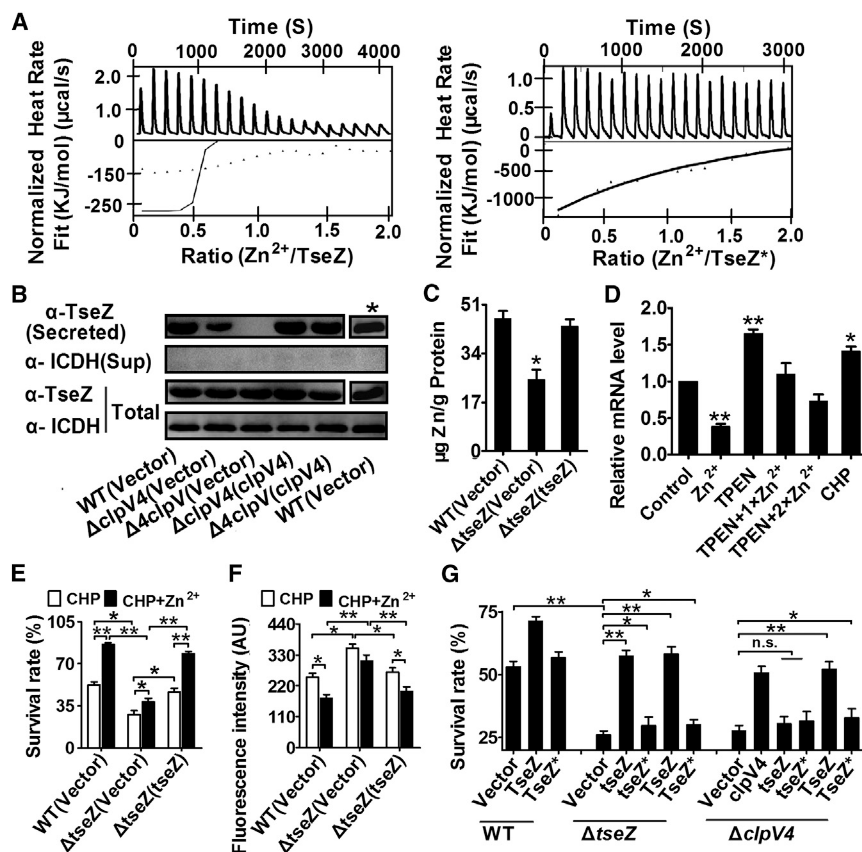

Figure 3. A Zn^{2+}-Binding Protein Translocated by T6SS4 Resisted Oxidative Stress

(A) The binding of Zn^{2+} to TseZ (left) or TseZ*(TseZ$^{H15A/Q16R/H76A/Q77R}$) (right) examined with ITC.

(B) TseZ is a secreted substrate of T6SS4. Proteins in culture supernatant of the relevant *B. thailandensis* strains were probed using a specific anti-TseZ antibody. The cytoplasmic protein ICDH was used as a loading control and lysis control. *, secretion of a known T6SS substrate, TseM, in the wild-type served as a positive control.

(C) TseZ is involved in Zn^{2+} acquisition. Stationary phase *B. thailandensis* strains were exposed to 0.25 mM CHP for 20 min in PBS containing 1 µM $ZnSO_4$. Zn^{2+} associated with bacterial cells was determined by ICP-MS.

(D) TseZ expression is inhibited at high Zn^{2+} concentrations. *B. thailandensis* wild-type cells were grown in LB containing 60 µM Zn^{2+}, 60 µM TPEN, 60 µM TPEN together with 60 µM Zn^{2+} (TPEN + 1 × Zn^{2+}), 60 µM TPEN together with 120 µM Zn^{2+} (TPEN + 2 × Zn^{2+}), or 100 µM CHP, respectively, and the expression of *tseZ* was measured by qRT-PCR.

(E) Alleviation of the sensitivity of *B. thailandensis* strains to CHP by exogenous Zn^{2+} and the TseZ protein. The viability of stationary phase *B. thailandensis* strains was determined after exposure to CHP in M9 medium with or without exogenously provided Zn^{2+} (1 µM).

(F) Deletion of *tseZ* leads to accumulation of intracellular ROS. The intracellular levels of ROS were determined with the 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe after exposure of stationary phase *B. thailandensis* strains to CHP in M9 medium with or without exogenously provided Zn^{2+} (1 µM).

(G) Rescue of the *tseZ* or *clpV4* mutant with recombinant TseZ or TseZ*. Recombinant TseZ or TseZ* protein (1 µM) was included in the bacterial survival assay mixtures before assessment of viability. Mutants complemented with the corresponding genes were used as controls.

The mean values and SDs from at least three replicates are shown. **$p < 0.01$; *$p < 0.05$; n.s., not significant. See also Figures S2 and S3.

culture supernatant of *B. thailandensis* wild-type by a specific antibody, the secretion of BTH_II1884 was largely abolished in the Δ*clpV4*, Δ*hcp4*, and Δ*icmF4* mutants and could be fully restored to the wild-type level by appropriate genetic complementation (Figure 3B; Figures S2B, S2C, and S4C). The secretion of BTH_II1884 was completely abrogated in the Δ*4clpV* mutant lacking four sets of T6SSs (T6SS1, T6SS2, T6SS4, and T6SS6), and the defects in secretion were completely rescued (thus, returned to the wild-type levels) upon complementation of *clpV4* (Figure 3B). These results suggest that BTH_II1884 is a T6SS substrate mainly secreted by T6SS4.

The observation that BTH_II1884 is a zinc-binding protein substrate of T6SS4 suggests that it plays a role in Zn^{2+} acquisition. As expected, deletion of the *bth_II1884* gene markedly reduced intracellular Zn^{2+} accumulation under oxidative stress conditions, and intracellular Zn^{2+} accumulation was restored to the wild-type level by complementation (Figure 3C). Consistent with its role in zinc acquisition, the expression of *bth_II1884* was induced by low zinc concentrations but repressed by high zinc concentrations (Figure 3D). Therefore, we designated this protein TseZ (type VI secretion system effector for zinc acquisition). Similar to T6SS4 component genes, the expression of *tseZ* was repressed by Zur (Figures S4D and S4E).

As a zinc-binding substrate of the oxidative stress-resistant T6SS4, TseZ is thought to be required for maximal bacterial survival under conditions of oxidative challenge. As shown in Figure 3E, the Δ*tseZ* mutant exhibited sensitivity to CHP at levels similar to those of T6SS4 mutants, and such sensitivity could be restored to the wild-type level by complementation with the *tseZ* gene from a plasmid. Moreover, deletion of *tseZ* resulted in accumulation of deleterious ROS in bacterial cells to levels similar to those observed in other T6SS4 mutants, and such accumulation could be eliminated by complementation (Figure 3F). In agreement with its role in zinc transport and survival oxidative stress, deletion of *tseZ* significantly abrogated the effect of exogenous Zn^{2+} (1 µM) on increasing the survival rate and reducing the intracellular ROS under oxidative stress conditions (Figures 3E and 3F).

Next we determined whether exogenously provided TseZ could restore the ability of relevant *B. thailandensis* mutants to resist oxidative stress. As shown in Figure 3G, inclusion of metal-free TseZ (1 µM) in cultures of the Δ*tseZ* mutant almost fully restored its resistance to CHP, but the exogenous zinc

binding-deficient variant TseZ*(TseZ$^{H15A/Q16R/H76A/Q77R}$) or expression of the *tseZ**(*tseZ*$^{H15A/Q16R/H76A/Q77R}$) mutant gene barely recovered the resistance. Moreover, exogenous TseZ protein, but not TseZ protein produced intracellularly by providing a plasmid expressing the *tseZ* gene, also protected the Δ*clpV4* mutant from oxidative stress (Figure 3G). These observations suggested that, after T6SS4-mediated translocation, Zn^{2+} uptake by TseZ occurred independently of the secretion system. These data indicate that T6SS4 secretes the zinc-binding effector TseZ for acquisition of Zn^{2+} under oxidative stress conditions.

TseZ Interacts with the TonB-Dependent Heme Receptor HmuR

After a zinc-binding effector that plays a role in mediating zinc acquisition was identified, the next challenge was to determine how zinc sequestered by this protein is delivered into the cell. We hypothesized that TseZ might interact with receptor proteins on the bacterial surface to transport Zn^{2+} into the cell. To identify such putative receptors, we performed a GST pull-down screening using GST-TseZ-coated beads against total cell lysates of wild-type *B. thailandensis* cells challenged with CHP. Proteins retained by GST-TseZ were detected by silver staining after SDS-PAGE. As shown in Figure 4A, several proteins were specifically retained by beads coated with GST-TseZ. Mass spectrometry identified an 85-kDa band as a member of the TonB-dependent heme receptor HmuR (BTH_II2139), and the other bands were identified as a chaperonin (BTH_I1458), an inner membrane permease (BTH_II2344), and the heme import ATP-binding protein termed HmuV (BTH_II2143) (Figure 4A). A specific interaction between TseZ and HmuR was confirmed by GST pull-down and cross-linking assays performed using purified GST-TseZ and His_6-HmuR proteins in vitro (Figure 4B; Figure S5A). Moreover, the specific interaction between TseZ and HmuR was competitively disrupted by heme (Figure S5B).

TonB-dependent receptors are bacterial outer membrane transporters that mediate the active uptake of iron siderophores, heme, and zinc (Kumar et al., 2012; Stork et al., 2010). The *B. thailandensis hmuR* gene is located in the *hmuRSTUV* gene cluster, which encodes a canonical heme acquisition system and shows high degrees of similarity to the identified heme uptake systems in *Pseudomonas aeruginosa*, *Serratia marcescens*, *Shigella dysenteriae*, and *Yersinia pestis* (Smith et al., 2015; Ghigo et al., 1997; Eakanunkul et al., 2005; Rossi et al., 2001; Figure 4C). The function of the *hmuR* gene in heme acquisition was confirmed by heme plate assay. As shown in Figure 4D, deletion of *hmuR* led to severe growth defects on plates with heme iron as the sole iron source provided on the heme-impregnated disc. The growth defect of the Δ*hmuR* mutant was completely restored upon complementation (Figure 4D). A role for HmuR in heme acquisition was further supported by the finding that *hmuR* expression was induced by low iron and heme levels, but it was repressed by higher concentrations of these materials (Figure 4E).

Consistent with the observation that HmuR is involved in heme acquisition, the intracellular iron concentration of the Δ*hmuR* mutant was markedly reduced compared with the wild-type and the complemented strains under both normal and oxidative stress conditions (Figure 4F). It is worth noting that both the wild-type and complemented strains showed markedly reduced intracellular iron contents under conditions of oxidative stress, suggesting that the HmuR protein is mainly functional in iron transportation under normal conditions but not oxidative stress conditions.

HmuR Is Involved in Zinc Transportation under Oxidative Stress Conditions

The direct interaction of HmuR with TseZ may allow HmuR to transport zinc. Moreover, HmuR is evolutionarily close to the outer membrane zinc transporter ZnuD of *N. meningitidis* (Kumar et al., 2012), further suggesting a role in zinc acquisition (Figure S4F). This hypothesis was confirmed by measuring the total zinc contents in bacterial cells using ICP-MS. As shown in Figure 5A, deletion of *hmuR* significantly reduced intracellular zinc levels under conditions of CHP challenge, and such defects could be fully restored by complementation. However, deletion of *hmuR* had a marginal effect on intracellular zinc accumulation under normal conditions. These results indicated that the HmuR protein plays a role in zinc transportation, especially under oxidative stress conditions.

Consistent with its role in zinc transportation, the expression of HmuR was repressed by Zur (Figure 5B) and induced by low levels of Zn^{2+} but repressed by high levels of Zn^{2+} (Figure 5C). The role of HmuR in zinc transportation was further corroborated by the observation that HmuR directly bound zinc (Figure S6A).

Interestingly, the expression of HmuR was also induced by CHP (Figure 5C), suggesting a possible role in the oxidative stress response. As expected, the *hmuR* mutant was more sensitive to CHP than the wild-type and complemented strains (Figure 5D). Moreover, deletion of *hmuR* significantly abrogated the effect of exogenous Zn^{2+} (1 μM) on increasing the survival rate under conditions of oxidative stress (Figure 5D). These results indicated that the TonB-dependent heme receptor HmuR is involved in zinc acquisition and plays important roles in oxidative stress resistance.

The Substrate Switch of HmuR Is Regulated by Formation of an Intramolecular Disulfide Bond

The finding that HmuR is involved in the transport of heme-iron and zinc under different conditions raises questions regarding how the substrate specificity of HmuR is regulated. The cysteine residue is sensitive to oxidative stress and was reported to serve as an oxidative stress sensor by reversible oxidation in different proteins (Georgiou, 2002; Wood et al., 2004; Aichem et al., 2006). Interestingly, HmuR contains two cysteines residues, Cys^{692} and Cys^{697}, located on the surface of the outer membrane (Figure S4G). To investigate whether these cysteines act as oxidative stress sensors, we purified His_6-HmuR expressed in *B. thailandensis* with or without CHP treatment. MS analysis of the His_6-HmuR protein from CHP-treated *B. thailandensis* led to the detection of a 2383.9-Da peptide consisting of peptic cleavage products (^{686}DVSSAACR^{694} and ^{696}TCFTPPSSFVVDLR^{709}) linked by a disulfide bond between Cys^{692} and Cys^{697} (Figure 6A). However, both Cys^{692} and Cys^{697} in His_6-HmuR purified from CHP-untreated *B. thailandensis* were maintained in the reduced state (Figure S6B). The formation of the intracellular disulfide

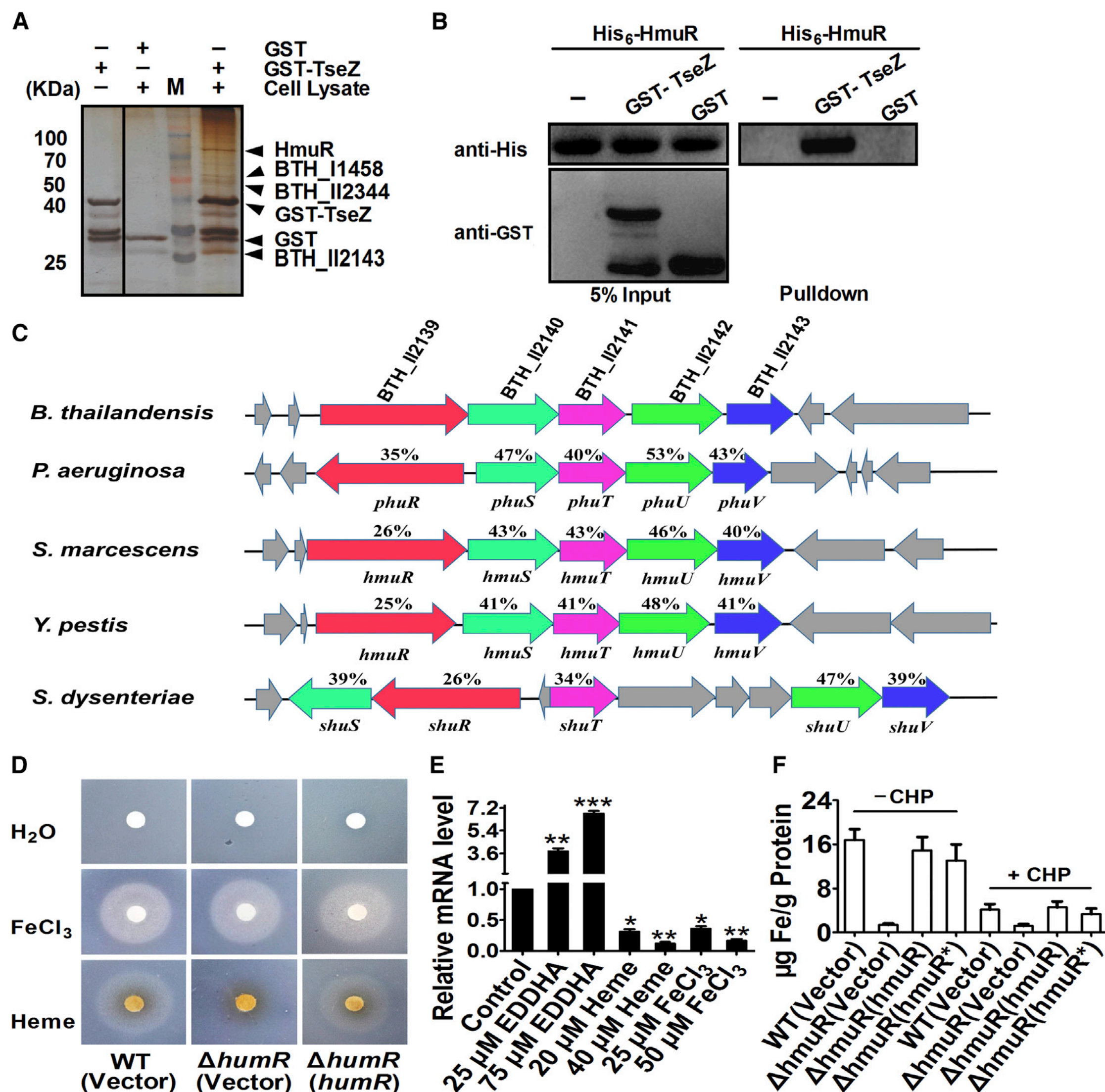

Figure 4. TseZ Interacts with the Heme Transporter HmuR

(A) HmuR was retained by agarose beads coated with GST-TseZ. GST-Bind beads coated with GST-TseZ (lane 4) or GST (lane 2) were incubated with CHP-treated *B. thailandensis* lysates. After washing with Tris/EDTA/NaCl (TEN) buffer, the proteins resolved by SDS-PAGE were visualized using silver staining, and bands specifically retained by GST-TseZ-coated beads were identified by mass spectrometry.

(B) Binding between TseZ and HmuR using an in vitro GST pull-down assay. His_6-HmuR was incubated with GST-TseZ or GST, and the protein complexes captured with glutathione beads were detected by western blotting. −, beads-only control.

(C) Comparison of HmuRSTUV amino acid sequences from different species. The accession numbers of HmuRSTUV from different species are as follows: *Burkholderia thailandensis* (SwissProt: gi:83651628, 83650953, 83651697, 83650138, and 83651699), *Pseudomonas aeruginosa* (SwissProt: gi:670356699, 670356698, 670356697, 670356696, and 670356695), *Serratia marcescens* (SwissProt: gi:560173286, 560173285, 560173284, 560173283, and 560173282), *Yersinia pestis* (SwissProt: gi:167054836, 167054838, 167054860, 167054866, and 167054904), and *Shigella dysenteriae* (SwissProt: gi:81242819, 81242818, 81242816, 81242813, and 81242812).

(D) Heme-dependent growth assay. Overnight cultures of relevant *B. thailandensis* strains were resuspended in soft agar and poured onto plates supplemented with 125 μM ethylenediamine-di-o-hydroxyphenylacetic acid (EDDHA). Aliquots (10 μL) of Milli-Q water, 10 mM Fe^{3+}, and 10 mM heme solution were applied to paper discs.

(E) HmuR expression is inhibited by heme and Fe^{3+}. *B. thailandensis* wild-type cells were grown in LB containing EDDHA (25 μM or 75 μM), heme (20 μM or 40 μM), or Fe^{3+} (25 μM or 50 μM), and the expression of HumR was measured by qRT-PCR.

(F) HmuR exhibited heme-iron transport activity under normal conditions. Stationary phase *B. thailandensis* strains were exposed to PBS containing 1 μM heme and 0.25 mM CHP or 1 μM heme alone for 20 min. Iron ions associated with bacterial cells were determined by ICP-MS. *hmuR**, *hmuR*$^{C692A/C697A}$.

The mean values and SDs from at least three replicates are shown. ***p < 0.001,**p < 0.01, *p < 0.05. See also Figures S4 and S5.

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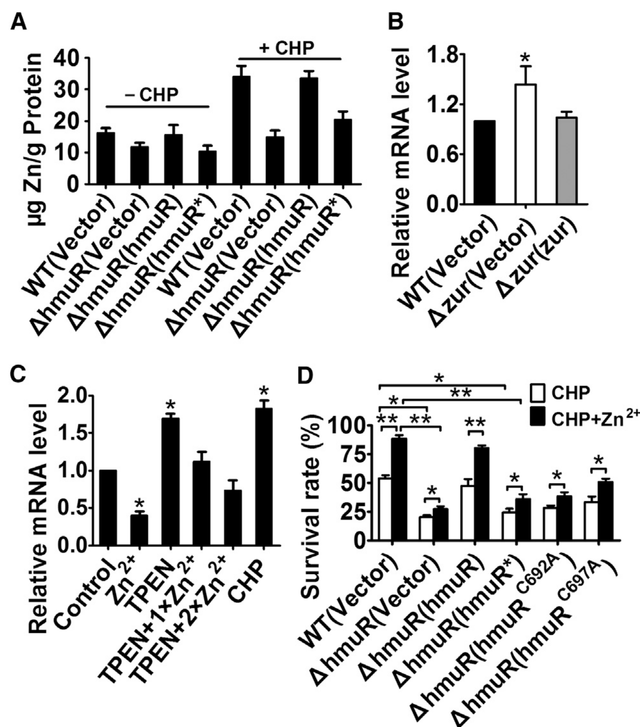

Figure 5. HmuR Is Involved in Zn^{2+} Transport under Oxidative Stress Conditions

(A) HmuR exhibited Zn^{2+} transport activity under oxidative stress. Stationary phase *B. thailandensis* strains were exposed to PBS containing 1 μM $ZnSO_4$ or 1 μM $ZnSO_4$ with 0.25 mM CHP for 20 min. Zn^{2+} associated with bacterial cells was determined by ICP-MS. *hmuR**, *hmuR*$^{C692A/C697A}$.

(B) HmuR expression was negatively regulated by Zur. Cells of relevant *B. thailandensis* strains were grown in LB medium, and the expression of *hmuR* was measured by qRT-PCR.

(C) HmuR expression was inhibited by high-level Zn^{2+} and induced by CHP and TPEN. Cells of *B. thailandensis* wild-type were grown in LB medium containing 60 μM Zn^{2+}, 60 μM TPEN, 60 μM TPEN together with 60 μM Zn^{2+} (TPEN + 1 × Zn^{2+}), or 60 μM TPEN together with either 120 μM Zn^{2+} (TPEN + 2 × Zn^{2+}) or 100 μM CHP, respectively. The expression of *hmuR* was measured by qRT-PCR.

(D) Alleviation of the sensitivity of *B. thailandensis* strains to CHP by exogenous Zn^{2+} (1 μM) requires HmuR. The viabilities of various stationary phase *B. thailandensis* strains were determined after exposure to CHP or CHP with 1 μM Zn^{2+} for 40 min.

The mean values and SDs from at least three replicates are shown. **$p < 0.01$, *$p < 0.05$. See also Figure S6.

bond was confirmed by 4-acetamido-4’maleimidyldystilbene-2, 2’-disulfonic acid (AMS) assay. AMS covalently modifies free thiol groups, retarding electrophoretic mobility in proportion to the number of free thiol groups in proteins (Ehira and Ohmori, 2012). As shown in Figure 6B, HmuR treated with CHP and modified with AMS migrated faster than its non-CHP-treated AMS-modified form and the same as the CHP-treated non-AMS-modified form, indicating the formation of an intramolecular disulfide bond between Cys^{692} and Cys^{697} in the CHP-treated protein. Consistently, CHP-treated HmuR exhibited more marked zinc-binding activity than DTT-treated HmuR (Figure 6C).

To investigate the functional difference between Cys^{692} and Cys^{697}, we generated HmuR^{C692A} and HmuR^{C697A} variants that were then treated with NBD-Cl (4-chloro-7-nitrobenzo-2-oxa-1,3-diazole) with and without previous exposure to CHP. NBD-Cl can exclusively react with thiol groups and sulfenic acids, and the covalent attachment of NBD-Cl generated an absorption peak at about 420 nm upon reaction with thiol groups, whereas it peaked at about 347 nm upon reaction with sulfenic acids (Baker and Poole, 2003). Following the reaction with NBD-Cl, the absorption spectrum of the HmuR^{C692A} variant was unchanged before and after exposure to CHP (Figure 6D), exhibiting only the peak at 420 nm. The HmuR^{C697A} variant with CHP treatment showed Soret bands at 347 nm and 420 nm, indicating the simultaneous reaction of NBD-Cl with sulfenic acids and free thiol groups. Cys oxidation was also quantified by measuring the free thiol content per HmuR monomer by 5,5-dithiobis(2-nitrobenoic acid) (DTNB) assay (Figure 6E). As expected, the HmuR^{C692A} variant showed one thiol per monomer before and after CHP treatment. However, the HmuR^{C697A} and HmuR wild-type proteins with CHP treatment lost one and two thiol groups, respectively, compared with the thiol content of DTT-treated states (Figure 6E). These results suggest that the Cys^{692} residue is more susceptible to oxidation.

Indeed, these two Cys residues are essential for zinc but not heme acquisition. Although the *hmuR*$^{C692A/C697A}$ mutant gene was as efficient as the wild-type *hmuR* gene in recovering intracellular iron accumulation in the Δ*hmuR* mutant under normal conditions (Figure 4F), it failed to recover intracellular zinc accumulation in the Δ*hmuR* mutant under oxidative stress conditions (Figure 5A). Consistent with these observations, complementation of the *hmuR*$^{C692A/C697A}$, *hmuR*^{C692A}, and *hmuR*^{C697A} mutant genes had marginal effects on recovery of the survival rates of the Δ*hmuR* mutant under oxidative stress conditions (Figure 5D). These data indicate that the heme receptor HmuR switches to transportation of zinc by forming an intramolecular disulfide bond upon sensing an extracellular oxidative stress challenge.

The Zinc Transport Activity of T6SS4 Depends on HmuR

To determine the role of HmuR in T6SS4-mediated zinc uptake, we generated Δ*hmuR*Δ*tseZ* and Δ*hmuR*Δ*clpV4* double mutants. Both mutants showed very low survival under conditions of CHP challenge compared with the wild-type (Figure 7A). Although complementation of the *tseZ* gene completely rescued the sensitivity of the Δ*tseZ* mutant to CHP (Figure 3E), complementation of the *tseZ* gene had no effect on rescue of the sensitivity of the Δ*hmuR*Δ*tseZ* double mutant (Figure 7A). Moreover, although exogenous provision of metal-free TseZ (1 μM) significantly increased the survival rate of the wild-type and the Δ*tseZ* mutant under conditions of CHP challenge (Figure 3G), this effect was completely abrogated in the Δ*hmuR*Δ*tseZ*(Vector) and Δ*hmuR*Δ*tseZ*(*tseZ*) strains. However, exogenous provision of TseZ efficiently enhanced the survival rate of Δ*hmuR*Δ*tseZ* (*hmuR*) under conditions of CHP challenge (Figure 7A). Similarly, complementation of *clpV4* failed to restore the resistance of the Δ*hmuR*Δ*clpV4* double mutant to CHP, and exogenous provision of TseZ protected Δ*hmuR*Δ*clpV4*(*hmuR*), but not Δ*hmuR*Δ*clpV4*(Vector) or Δ*hmuR*Δ*clpV4*(*clpV4*), from CHP toxicity (Figure 7A).

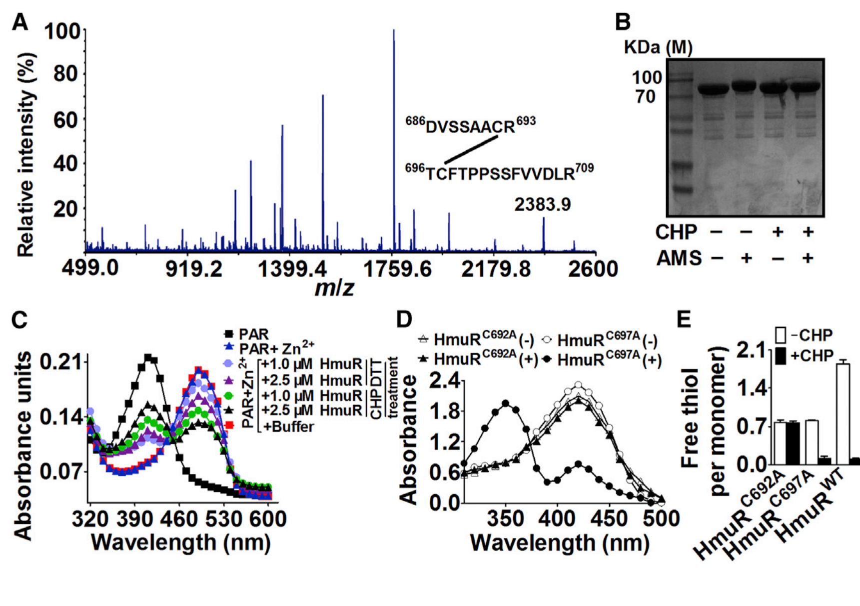

Figure 6. The Substrate Switch of HmuR Is Regulated by Forming an Intramolecular Disulfide Bond

(A) Analysis of CHP-treated HmuR by MS. HmuR protein purified from CHP-treated Δ*hmuR* (pM6032-His$_6$-*hmuR*) cells was resolved on non-reducing gel. The band was then excised, digested, and subjected to MS analysis. Only the relevant portion of the mass spectrum is shown.

(B) Redox response of HmuR. Proteins from CHP-treated or untreated Δ*hmuR*(pM6032-His$_6$-*hmuR*) cells grown in the presence or absence of AMS were separated by 10% non-reducing SDS-PAGE.

(C) The Zn^{2+}-binding activity of HmuR is enhanced upon CHP treatment. HmuR protein (10 μM) treated with 50 mM DTT was further incubated with or without CHP (10 mM). The Zn^{2+}-binding activities of the resulting proteins were determined by 4-(2-pyridylazo)-resorcinol (PAR) assay.

(D) Spectrophotometric analysis of the NBD-labeled HmuR variant. Proteins treated with or without CHP were modified with NBD-Cl. The resulting proteins were analyzed spectrophotometrically at 200–600 nm.

(E) Quantification of free HmuR thiol levels in reduced and oxidized proteins. CHP- and DTT-treated proteins (10 μM) were admixed with DTNB (2 mM) in 50 mM Tris-HCl buffer (pH 8.0), and the absorbance at 412 nm was measured.

See also Figure S6.

To further determine the mechanisms of zinc transportation mediated by HmuR and T6SS4/TseZ, we performed intrabacterial growth competition assays between relevant *B. thailandensis* strains under conditions of CHP challenge. Strikingly, neither Δ*tseZ* nor Δ*clpV4* showed a growth disadvantage when competing with the wild-type. In contrast, both Δ*tseZ* and Δ*clpV4* showed a 1.8- to 2.2-fold growth advantage compared with the Δ*hmuR* mutant (Figure 7B). These data suggest that, although Δ*tseZ* and Δ*clpV4* cannot secrete TseZ to scavenge zinc, they can still use HmuR to recruit TseZ secreted by other strains. Consistent with this suggestion, deletion of *hmuR* in the Δ*tseZ* and Δ*clpV4* mutant abrogated their growth advantage relative to the Δ*hmuR* mutant (Figure 7B). Taken together, these results established that the zinc transport activity of T6SS4 and TseZ was mediated by the outer membrane transporter HmuR.

T6SS4 Is Involved in Contact-Independent Interbacterial Competition

The participation of T6SS in the acquisition of zinc predicts that it would play a role in cell contact-independent competition for essential nutrients. To test this hypothesis, we quantified the magnitudes of T6SS4 and TseZ-mediated zinc uptake effects on *B. thailandensis* fitness in competition with other bacteria in zinc-limited liquid medium under conditions of challenge with CHP. As shown in Figure 7C, although wild-type *B. thailandensis* was highly competitive against the *E. coli* K12 competitor (21.5:1), the competitive advantage was abolished in the Δ*tseZ*, Δ*clpV4*, and Δ*hmuR* mutants. Similar results were obtained when Gram-negative *Pantoea alhagi* and Gram-positive *Staphylococcus aureus* were used as competitors.

We postulated that heterologous expression of HmuR in *E. coli* K12 and *P. alhagi* would improve their zinc acquisition capability and growth competition with other bacteria because it is an outer membrane TonB-dependent zinc receptor. As expected, the expression of HmuR increased intracellular zinc accumulation in *E. coli* K12 and *P. alhagi*, especially when exogenous TseZ protein was provided (Figure S6C). Furthermore, expressing HmuR diminished the competitive advantage of wild-type *B. thailandensis* with regard to *E. coli* K12 and *P. alhagi* competitors (Figure 7D). Moreover, the *E. coli* K12 and *P. alhagi* competitors expressing HmuR out-competed the *B. thailandensis* Δ*hmuR* mutant. Indeed, the *B. thailandensis* wild-type, Δ*tseZ*, and Δ*clpV4* strains exhibited competitive abilities equivalent to those of *E. coli* K12(pME6032-*hmuR*) and *P. alhagi*(pME6032-*hmuR*) (Figure 7D). These data indicate that the T6SS4/HmuR-mediated zinc transport pathway confers a contact-independent competitive advantage.

B. thailandensis Mutants Lacking *clpV4*, *tseZ*, or *hmuR* Are Defective in Virulence in the *Galleria mellonella* Larva Infection Model

To investigate the role of the T6SS4-dependent Zn^{2+} transport system in pathogenesis, *Galleria mellonella* (wax moth) larvae were infected with different *B. thailandensis* strains to determine their ability to kill the larvae. Those infected with wild-type *B. thailandensis* showed the lowest survival rate, with an average of 27%, whereas challenge with mutants lacking *clpV4*, *tseZ*, or *hmuR* resulted in mean survival rates of approximately 70%, 65%, and 80% of larvae, respectively (Figure S6D). Prior to the onset of paralysis and death, the number of bacteria (after 10 hr post-infection with 50 μL volumes containing 10^5 colony-forming units [CFUs]) was enumerated. Wild-type *B. thailandensis* showed increased bacterial numbers relative to mutants lacking *clpV4*, *tseZ*, or *hmuR* (Figure S6E). These observations suggest that the T6SS4-HmuR-mediated Zn^{2+} transport system is important for virulence.

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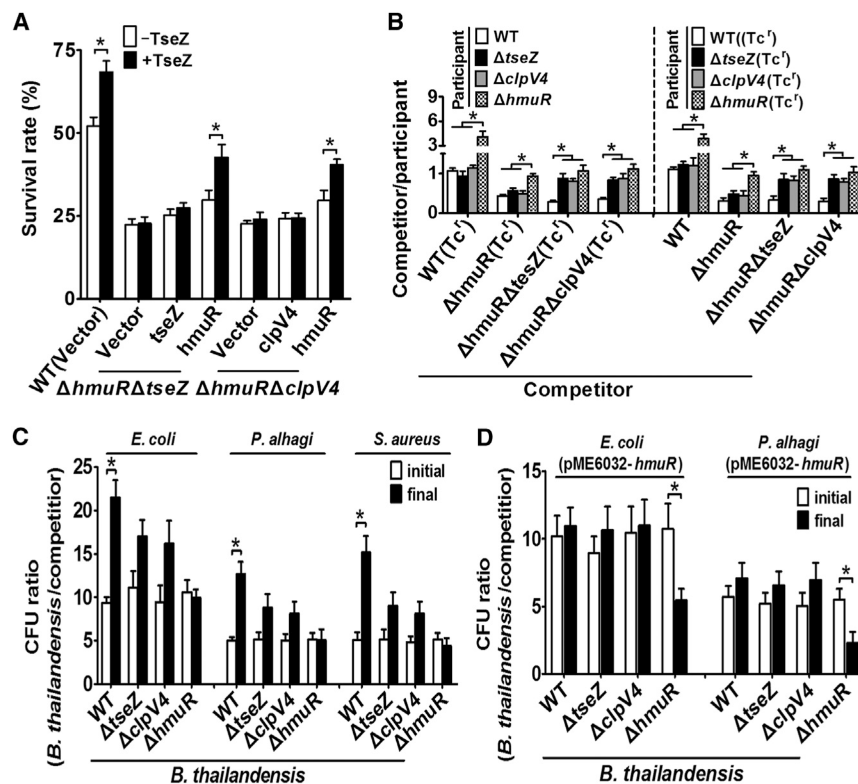

Figure 7. Intrabacterial and Interbacterial Growth Competition Assays

(A) The effect of apo-TseZ on survival. The indicated bacterial strains grown to stationary phase were exposed to CHP in M9 medium with or without apo-TseZ (1 μM), and the viability of the cells was determined.

(B) Intrabacterial growth competition. Assays comparing the indicated competitors (x axis) and other strains were performed via co-incubation at 37°C for 12 hr in M9 medium containing 20 μM CHP. The competitive index result was calculated as the final CFU ratio (competitor/participant) divided by the initial ratio.

(C) Growth competition assays between various organisms. The competition between the indicated organisms was examined in M9 medium containing 50 μM CHP at 37°C for 12 hr. The CFU ratio of the relevant *B. thailandensis* strain versus the competitors is plotted.

(D) HmuR expression in *E. coli* K12 and *Pantoea alhagi* engaging in growth competition.

The mean values and SDs from at least three replicates are shown. $^{*}p < 0.05$. See also Figure S6.

DISCUSSION

Although most nutrients can pass the outer membrane by passive diffusion through porins, diffusion is not effective when the extracellular concentration of a nutrient is low. In mammalian hosts, essential micronutrients such as iron and zinc are strictly restricted by a defense mechanism termed nutritional immunity (Hood and Skaar, 2012). Consequently, pathogenic bacteria have developed many effective means to obtain scarce nutrients in an energy-dependent manner. Although bacterial mechanisms of iron acquisition have been studied extensively, the mechanisms of zinc acquisition are not as well understood. Here we describe a sophisticated bacterial zinc-scavenging pathway consisting of a redox-regulated, dual-functional outer membrane heme transporter, HmuR, and a T6SS-secreted proteinaceous zincophore, TseZ.

The role of the TonB-dependent outer membrane heme transporter HmuR in Zn^{2+} uptake was supported by several lines of evidence. First, the *hmuR* mutant was markedly deficient in Zn^{2+} accumulation under oxidative stress conditions (Figure 5A). Second, deletion of *hmuR* abolished the protective effect of exogenous Zn^{2+} on oxidative stress resistance (Figure 5D). Finally, consistent with its role in zinc uptake, the expression of *hmuR* was repressed by Zur (Figure 5B) and was induced by low concentrations but repressed by high concentrations of Zn^{2+} (Figure 5C). The role of HmuR in Zn^{2+} uptake was further supported by its ability to bind zinc (Figure S6A). The dual role of HmuR in both heme iron and zinc uptake is not totally unexpected. Indeed, ZnuD, a Zn-regulated TonB-dependent outer membrane receptor, shows sequence similarity with the heme transporter HumA from *Moraxella catarrhalis* and participates in both Zn^{2+} and heme acquisition (Kumar et al., 2012).

Although a number of outer membrane metal transporters, such as ZnuD and FrpB4, have been reported to transport more than one substrate (Schauer et al., 2007; Kumar et al., 2012), whether these transporters transport different substrates simultaneously or in a mutually exclusive manner and how their substrate specificities are determined remains largely unknown. Here we demonstrated that HmuR preferentially transports heme under normal conditions and transports zinc under oxidative stress conditions. HmuR switches its substrate specificity by forming an intramolecular disulfide bond between Cys^{692} and Cys^{697}, located on the surface of the bacterial cells. The formation of the intramolecular disulfide bond under oxidative stress conditions was confirmed by MS analysis and AMS assay (Figures 6A and 6B). The formation of the intramolecular disulfide bond is crucial for zinc acquisition and oxidative stress resistance because mutation of the two Cys residues abolished the zinc acquisition and oxidative stress resistance of HmuR (Figures 5A and 5D). However, mutation of the two Cys residues had no effect on heme acquisition (Figure 4F). Based on these observations, we conclude that the substrate specificity of HmuR is regulated by formation of an intramolecular disulfide bond upon sensing extracellular oxidative stress.

Intramolecular disulfide bond formation is a versatile post-translational modification used to modulate the activities of a large number of redox-regulated proteins in response to oxidative stress. For example, disulfide bond formation in the *Saccharomyces cerevisiae* transcriptional activator Yap1p leads to its nuclear accumulation, leading to activation of antioxidant gene expression (Wood et al., 2004). Triggered by intramolecular

disulfide bond formation under conditions of severe oxidative stress, the *E. coli* Hsp33 chaperone undergoes a large conformational transition to a state that can bind unfolded protein substrates and rescue them from aggregation (Jakob et al., 1999). Other redox-regulated proteins, such as Keap1 and protein kinase C (PKC), are involved in signal transduction cascades (Zhang et al., 2004; Aichem et al., 2006). In contrast to these known intramolecular disulfide bond-activated proteins, which are oxidized by sensing intracellular oxidative stress challenge, HmuR is a dual-functional outer membrane transporter that, triggered by forming an intramolecular disulfide bond upon sensing extracellular oxidative stress challenge, switches its substrate from one metal ion to another. Thus, HmuR acts as a "redox sensor" for directly sensing extracellular oxidative signals.

Such functional switching by HmuR ensures the development of an immediate response to extracellular oxidative signals; no new antioxidant proteins need to be synthesized, suggesting that HmuR serves as a first line of defense against oxidative stress. Moreover, the HmuR response is finely tuned, facilitating a gradual response to oxidative stress. Although HmuR is used mainly for the transport of heme under normal conditions, it switches to transportation of zinc upon sensing extracellular oxidative stress, triggered by the formation of an intramolecular disulfide bond. Under conditions of mild oxidative stress, HmuR-mediated zinc transportation alone is sufficient for maintaining intracellular redox homeostasis, thus abrogating the need to induce the expression of other costly antioxidant systems. When the bacterium encounters a severe oxidative stress challenge, the increased levels of intracellular ROS could act as a messenger to activate the redox-sensitive regulator (i.e., OxyR), leading to induction of the expression of a wide range of antioxidant systems, including T6SS4. Under these conditions, induced T6SS4 secretes the proteinaceous zincophore TseZ to scavenge extracellular Zn^{2+}. The efficiency of HmuR in zinc transport was markedly enhanced by TseZ, which delivers its Zn^{2+} load via direct interaction with HmuR. The TseZ-HmuR-mediated active zinc transport system not only fulfills the increased cellular demand for Zn^{2+} under conditions of severe oxidative stress but also simultaneously reduces iron uptake to attenuate the ROS-producing Fenton reaction.

The TseZ/HmuR-mediated zinc transport system confers a competitive advantage when bacteria seek to survive in polymicrobial communities (Figure 7). Our findings support the hypothesis that the T6SSs were evolutionarily important in shaping the compositions of microbial populations; bacteria expressing such systems either directly killed competing cells via contact-dependent translocation of toxins or competed for essential nutrients via contact-independent secretion of proteinaceous metallophores (Si et al., 2017). Importantly, the TseZ/HmuR-mediated zinc transport system may play roles in other bacteria; both TseZ and HmuR homologs are evident in many bacterial species (Figure S7).

In conclusion, our results reveal a complex zinc acquisition pathway involving T6SS and a redox-regulated, dual-functional outer membrane transporter, HmuR, that enhances bacterial survival in polymicrobial communities in harsh environments and/or during its interactions with hosts and provides a special perspective for understanding the evolutionary role of T6SS in bacterium-bacterium competition.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Growth Conditions

All bacterial strains and plasmids used in this study are listed in Table S1. Details of strains, primers, plasmid constructions, and growth condition of bacteria are described in the Supplemental Experimental Procedures and Tables S1 and S2.

Growth Competition Assays

Intra- and inter-species growth competition assays were performed as described previously (Si et al., 2017). Detailed information can be found in the Supplemental Experimental Procedures.

Determination of Intracellular Ion Content

Intracellular ion content was determined as described previously (Wang et al., 2015). Detailed information can be found in the Supplemental Experimental Procedures.

Zn^{2+} Binding Assays

Zn^{2+} binding was measured using ITC at 25°C with a NANO-ITC 2G microcalorimeter (TA Instruments) (Schilling et al., 2005). Full details can be found in the Supplemental Experimental Procedures.

Statistical Analysis

Survival analyses, intracellular ion content levels, ROS levels, and expression data were compared using paired two-tailed Student's t test. All statistical analyses were performed with the aid of GraphPad Prism software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.081.

AUTHOR CONTRIBUTIONS

M.S., Y.W., and X.S. conceived the project. M.S., Y.W., B.Z., C.Z., Y.K., H.B., D.W., and L. Zhu performed the experiments. M.S., Y.W., L. Zhang, T.G.D., and X.S. analyzed the data. M.S., Y.W., T.G.D., and X.S. wrote the paper.

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