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ARTICLE

Abiotic factors modulate interspecies competition mediated by the type VI secretion system effectors in *Vibrio cholerae*

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Vibrio cholerae, the etiological pathogen of cholera, employs its type VI secretion system (T6SS) as an effective weapon to survive in highly competitive communities. Antibacterial and anti-eukaryotic functions of the T6SS depend on its secreted effectors that target multiple cellular processes. However, the mechanisms that account for effector diversity and different effectiveness during interspecies competition remain elusive. Here we report that environmental cations and temperature play a key role in dictating cellular response and effector effectiveness during interspecies competition mediated by the T6SS of *V. cholerae*. We found that *V. cholerae* could employ its cell-wall-targeting effector TseH to outcompete the otherwise resistant *Escherichia coli* and the *V. cholerae* immunity deletion mutant Δ*tsiH* when Mg^{2+} or Ca^{2+} was supplemented. Transcriptome and genetic analyses demonstrate that the metal-sensing PhoPQ two-component system is important for Mg^{2+}-dependent sensitivity. Competition analysis in infant mice shows that TseH was active under in vivo conditions. Using a panel of *V. cholerae* single-effector active mutants, we further show that *E. coli* also exhibited variable susceptibilities to other T6SS effectors depending on cations and temperatures, respectively. Lastly, *V. cholerae* effector VasX could sensitize *Pseudomonas aeruginosa* to its intrinsically resistant antibiotic irgasan in a temperature-dependent manner. Collectively, these findings suggest that abiotic factors, that *V. cholerae* frequently encounters in natural and host environments, could modulate cellular responses and dictate the competitive fitness conferred by the T6SS effectors in complex multispecies communities.

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INTRODUCTION

Living in complex natural and host environments, microbes frequently compete for limited nutrients and ecological niches. To survive, microbes have evolved multiple effective weapons [1–4], one of which is the type VI protein secretion system (T6SS) commonly found in gram-negative bacteria [5–7]. The T6SS structure consists of a transmembrane complex (TssJLM), a baseplate (TssEFGK), and a double-tubular structure with the Hcp inner tube surrounded by the contractile outer sheath (TssB/C, also known as VipA/B) [8–11]. The Hcp tube is sharpened by a VgrG-PAAR spike complex [12–14]. Upon sheath contraction, the T6SS could translocate the Hcp tube-spike complex and the associated effectors to the extracellular environment or directly into neighboring recipient cells [15–17].

Effectors are T6SS-secreted toxins exhibiting antibacterial and anti-eukaryotic activities, and they collectively dictate the T6SS functions [17–20]. The antibacterial effectors include cell-wall disrupting enzymes, membrane-damaging toxins, nucleases, $NAD(P)^+$ hydrolase, ADP-ribosyl transferase, and (p)ppApp synthetase [21–26]. To confer self-protection, bacteria encode effector-cognate immunity proteins that can neutralize effector toxicities through specific protein–protein interactions [21, 27–29]. However, recent studies have demonstrated that such specific protection can be insufficient and bacteria require multiple stress response pathways that collectively provide innate-immunity-like general protection against effectors [30]. These include production of extracellular polysaccharides [18, 31], oxidative stress response [32, 33], envelope stress responses [18], acid and osmotic stress responses [19]. In addition, spatial separation at the community level can also protect susceptible cells from T6SS aggressors [34–38].

T6SS species often encode multiple effector modules which may be used synergistically or independently for killing a broad spectrum of competitors [39–42]. Effectors may also exhibit species-specific or condition-specific toxicities. For example, the cell-wall targeting effector TseH in *Vibrio cholerae* is highly toxic to *Aeromonas dhakensis* and several other waterborne species that *V. cholerae* may encounter in natural environments, but it shows little toxicity against *Escherichia coli* or the *V. cholerae* immunity gene deletion mutant Δ*tsiH* when it is delivered by the T6SS [18, 43]. Similarly, a pore-forming toxin Tse4 in *Pseudomonas aeruginosa* exhibits preference in high-salinity environments and synergy with other effectors [42]. The difference in susceptibility may be attributed to effector capabilities and nonspecific stress responses in bacteria [18–20], but the exact mechanisms of conditional toxicity remain elusive.

V. cholerae causes the pandemic cholera disease that poses a serious threat to public health globally [44]. Commonly transmitted through contaminated water, *V. cholerae* lives in a complex lifestyle of being motile in water and sessile in biofilm and adapts

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to near constantly changing conditions affected by nutrient availability, temperature, and stresses in the aquatic environments and in different hosts [45]. The T6SS is a crucial tool for *V. cholerae* to compete with other bacteria [46], defend against predatory eukaryotes [5], assimilate exogenous DNA [47], and colonize in the host gut [48]. Recent genomic research has suggested that the T6SS is likely a key factor enabling the dominance of the current 7th pandemic *V. cholerae* that has replaced the classical *V. cholerae* strains in global cholera outbreaks [49], highlighting the importance of the T6SS-mediated competition in understanding the evolution of cholera pandemics.

In this study, we report that a number of abiotic environmental factors, including cation, temperature, and antibiotic, play crucial roles in T6SS-mediated interspecies competition by modulating target-species cellular response to specific effectors. Using a panel of single-effector active *V. cholerae* V52 mutants and competition assays [17, 18], we show that trace amounts of divalent cations, Mg^{2+} and Ca^{2+}, could sensitize the otherwise resistant *E. coli* to TseH toxicity. Using transcriptome and mutational analyses, we further show that the PhoPQ two-component system, a known sensor of environmental Mg^{2+} [50], is important for *E. coli* survival against TseH. Competition assay in vivo shows that TseH was active during *V. cholerae* colonization in infant mice. We have also found that temperature is a crucial factor determining the susceptibility of *E. coli* to other *V. cholerae* effectors, VasX, VgrG3, and TseL [16, 29, 51]. In addition, the expression of VasX could abolish the intrinsic resistance of *P. aeruginosa* to irgasan in a temperature-dependent manner. Collectively, our data indicate that T6SS-mediated interspecies competition is not only dependent on the functions of T6SS effector proteins but also tightly modulated by environmental abiotic factors that dictate the cellular response and susceptibility of target species to effector toxicities.

RESULTS

T6SS-delivered TseH is conditionally toxic to *E. coli* depending on agar sources

We have previously reported that the *V. cholerae tseH*$^+$ only mutant, with all the other antibacterial effectors inactivated, could not outcompete *E. coli* due to the nonspecific protection conferred by the envelope stress response pathways [18]. However, we serendipitously found that TseH toxicity was largely affected by the source of agars used for competition. When agar source 1 was used, we reproduced the earlier observation and showed that the survival of *E. coli* competed with the *tseH*$^+$ only mutant was comparable to that competed with the T6SS-null mutant Δ*vasK* and the four-effector-inactive mutant *4eff*$_c$ (Fig. 1A, Supplementary Fig. 1A) [18]. However, when a different source of agar was used (source 2), *E. coli* survival was reduced by 10,000-fold in competition with the *V. cholerae tseH*$^+$ only mutant, relative to that competed with the *4eff*$_c$ and the Δ*vasK* mutant (Fig. 1A, Supplementary Fig. 1A). Survival of *E. coli* remained the same under both agar conditions for samples competed with wild-type *V. cholerae*, the Δ*vasK*, or the *4eff*$_c$ mutant, separately.

Mg^{2+} and Ca^{2+} stimulate TseH-mediated killing against *E. coli*

To determine the difference between agar sources that affects the TseH killing efficiency, we used XRF (X-Ray Fluorescence) to compare element contents in these two agar samples. XRF results show that Ca and Mg levels were substantially higher in the source 2 agar (Supplementary Table 1). Using ICP-MS (Inductively Coupled Plasma Mass Spectrometry), we further quantified Mg and Ca contents. In the source 1 agar, Mg and Ca levels were 85.83 ± 2.40 mg/kg and 16.92 ± 0.30 mg/kg, respectively. However, in the source 2 agar, Mg and Ca levels were 515.29 ± 13.57 mg/kg and 200.93 ± 9.29 mg/kg, about sixfold and tenfold higher than their levels in the source 1 agar, respectively.

To test if the observed high levels of Mg and Ca are responsible for the enhanced TseH-mediated killing against *E. coli*, we added additional Mg^{2+} or Ca^{2+} to the source 1 agar and tested their effects on bacterial competition. Indeed, the addition of Mg^{2+} or Ca^{2+} significantly reduced *E. coli* survival upon TseH-mediated attack, with Ca^{2+} showing a stronger effect (Fig. 1B, Supplementary Fig. 1B). Notably, the combination of 0.27 mM Mg^{2+} and 0.07 mM Ca^{2+}, the amount comparable to the levels in source 2 agar, did not reduce *E. coli* survival to the same levels as in source 2 agar, suggesting other factors in the source 2 agar also contribute to the increased TseH toxicity.

To further confirm that metal ions play a critical role in stimulating TseH-mediated killing against *E. coli*, we washed the source 2 agar powder with a metal-chelator EDTA solution to remove divalent cations. Results showed that EDTA-treatment abolished TseH-mediated killing on the source 2 agar, while the deionized water (ddH_2O)-treated source 2 agar could still sustain TseH killing (Fig. 1C, Supplementary Fig. 1C). Either EDTA-treatment or water-treatment did not support TseH-mediated killing on source 1 agar plates.

We also tested competition between the *tseH*$^+$ mutant and *E. coli* using the defined M9 medium. Both source 1 agar and source 2 agar plates supported TseH-mediated killing, with the source 2 agar plate showing a stronger killing (Fig. 1D, Supplementary Fig. 1D). Because the M9 medium contains Mg^{2+} and Ca^{2+}, we also tested the effects when these two cations were omitted. Results show that only the source 2 agar supported TseH-mediated killing (Fig. 1D, Supplementary Fig. 1D). Collectively, these results demonstrate that TseH-mediated toxicity is largely dependent on environmental Mg^{2+} and Ca^{2+} levels.

Conditional toxicity of TseH is also applicable to *V. cholerae* immunity-defective mutant

Next, we tested whether cation supplement could enable TseH to kill its cognate immunity gene mutant Δ*tsiH*, which has been previously shown to be resistant due to the protection of immunity-independent defenses primarily mediated by the WigKR two-component system [18]. We constructed a Δ*tsiH* deletion mutant lacking the *paar2-tseH-tsiH* three-gene operon in the *4eff*$_c$ mutant background and used it as prey for competition against the *tseH*$^+$ strain or the *4eff*$_c$ strain, separately. Competition results show that, like *E. coli*, the Δ*tsiH* mutant was sensitive to the *tseH*$^+$ strain on source 2 agar plates but not on source 1 agar plates (Fig. 1E, Supplementary Fig. 1E). Supplement of 0.3 mM Ca^{2+} to the source 1 agar reduced the Δ*tsiH* mutant survival significantly, while a combination of 0.27 mM Mg^{2+} and 0.07 mM Ca^{2+}, or 0.3 mM Mg^{2} alone had minor effects. These results show that TseH-mediated killing in *V. cholerae* could be stimulated by Ca^{2+} but not Mg^{2+}.

The addition of Mg^{2+} and Ca^{2+} does not affect T6SS secretion or TseH activity

To test whether the stimulating effect of Mg^{2+} and Ca^{2+} on TseH-mediated killing is due to increased T6SS secretion, we used the secretion of Hcp as an indicator for T6SS activities [5]. The results show that Hcp was secreted to the same levels with or without Mg^{2+} and Ca^{2+} supplement for both the wild type and the *tseH*$^+$ samples, suggesting the addition of Mg^{2+} and Ca^{2+} does not increase the already highly active T6SS secretion (Fig. 2A, Supplementary Fig. 2A). We next determined whether TseH requires the supplement of Mg^{2+} and Ca^{2+} for its enzymatic activity. The recently solved TseH structure suggests it is a NlpC/P60-family cysteine endopeptidase with its catalytic residue blocked by a connecting loop structure [18]. Because TseH has been shown to be inactive in vitro [18], we used a TseH-sensitive prey *A. dhakensis* [18] and competition assays to evaluate TseH secretion and activity. Results show that, with and without Mg^{2+} and Ca^{2+}, the survival of *A. dhakensis* was reduced about 2-logs by the *tseH*$^+$ strain (Fig. 2B, Supplementary Fig. 2B), relative to that

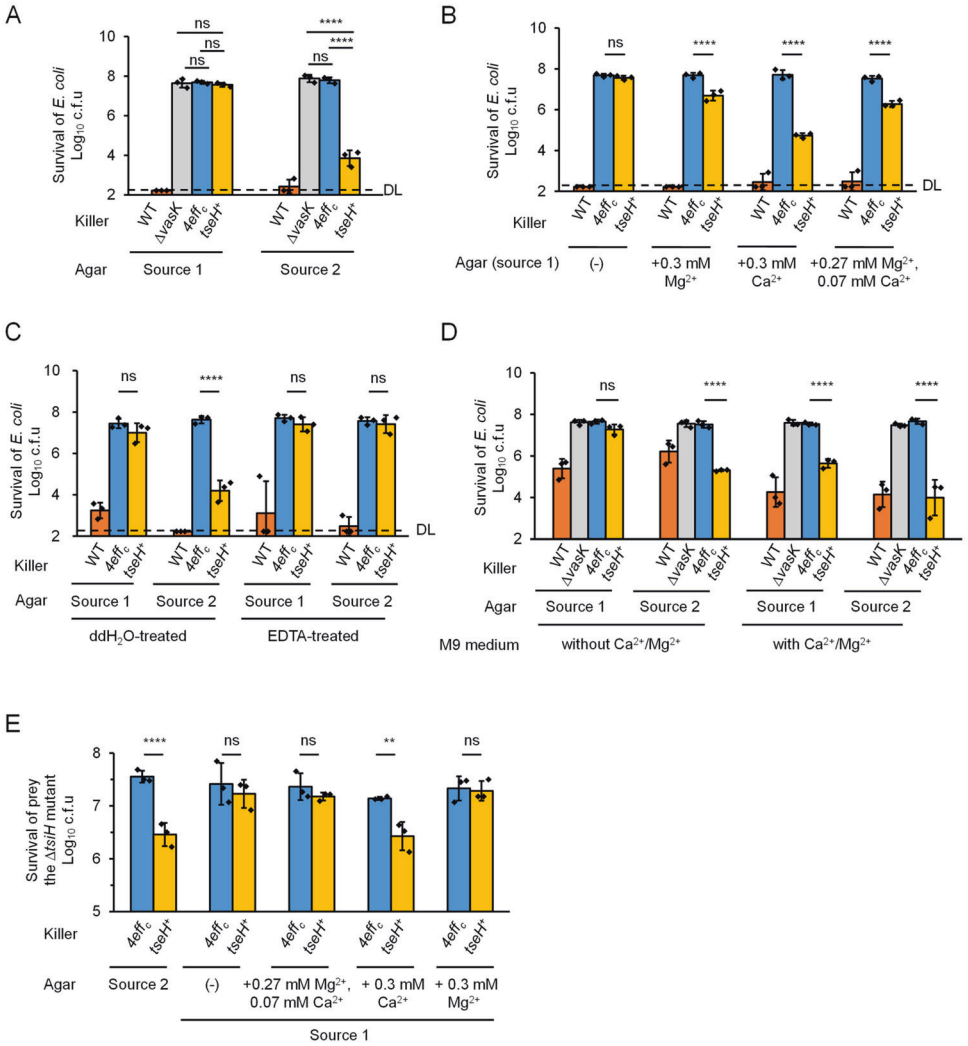


Fig. 1 Mg^{2+} and Ca^{2+} stimulate TseH-mediated killing against *E. coli* and the *V. cholerae* Δ*tsiH* mutant. A Survival of *E. coli* after competition with *V. cholerae* strains on LB plates of two different agar sources. **B** Survival of *E. coli* competed with *V. cholerae* strains on LB plates of agar source 1, supplemented with Mg^{2+} and Ca^{2+}. (−), no cations addition. **C** Effect of EDTA-treatment on survival of *E. coli* competed with *V. cholerae* strains. LB plates of ddH_2O (deionized water)-treated agar were used as the control. **D** Survival of *E. coli* competed with *V. cholerae* strains on M9 source-1-agar or source-2 agar-plates. **E** Survival of the *V. cholerae* Δ*tsiH* mutant after competition with the $4eff_c$ and $tseH^+$ strain. (−), no cations addition. For (**A**–**E**) killer strains are indicated at the bottom of each panel. WT wild type, Δ*vasK* the T6SS-null Δ*vasK* mutant, $4eff_c$ the 4-antibacterial-effector-inactive mutant, $tseH^+$ the TseH-active mutant. Survival of prey cells was enumerated by serial plating on the selective medium. Killer survival was shown in Supplementary Fig. 1A–E respectively. Error bars indicate the mean ± standard deviation of three biological replicates. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test. $^{**}p < 0.01$; $^{****}p < 0.0001$. DL detection limit.

competed with the $4eff_c$ mutant and the Δ*vasK* mutant. Therefore, TseH toxicity is independent of Mg^{2+} and Ca^{2+} in *A. dhakensis*. Taken together, these results suggest that the supplement of Mg^{2+} and Ca^{2+} does not affect T6SS secretion or TseH activity but likely changes the cellular response of *E. coli*.

PhoPQ two-component system protects *E. coli* against T6SS-delivered TseH

To understand why the supplement of divalent Mg^{2+}/Ca^{2+} cations would sensitize *E. coli* to TseH, we tested the effects of the PhoPQ two-component system that is a key regulatory system activated in response to Mg^{2+} starvation in *E. coli* [52, 53]. As a transmembrane histidine kinase, PhoQ senses low Mg^{2+} and activates PhoP and its regulated genes [54]. We constructed the Δ*phoQ* mutant and the $phoQ^{D179L}$ mutant in *E. coli*, the latter a PhoQ constitutively active mutant [55]. Competition assays with the $tseH^+$ strain show that the deletion of *phoQ* rendered *E. coli* significantly more sensitive to TseH with or without supplementing Mg^{2+} and Ca^{2+} (Fig. 3A, Supplementary Fig. 3A). Although the survival of both wild type and the two *phoQ* mutants was reduced when Mg^{2+} and Ca^{2+} were added, the $phoQ^{D179L}$ mutant survived significantly better than the wild type *E. coli*. Notably, the reduced survival of the $phoQ^{D179L}$ mutant by cation supplement also suggests that activation of the PhoPQ regulon is insufficient for full protection against the TseH-mediated killing.

Because prey cell survival may be affected by the efficiency of direct contact between T6SS killer and prey cells during the competition assays, we next compared the survival of *E. coli* wild type and the Δ*phoQ* mutant when TseH was periplasmically expressed with a Tat (twin-arginine translocation) secretion signal [18, 43]. The results show a nearly 100-fold reduced survival of the *E. coli* Δ*phoQ* mutant expressing wild-type TseH relative to that expressing $TseH^{H64A}$ (the catalytically inactive mutant of TseH [18]), while there was about a tenfold reduced survival when TseH and $TseH^{H64A}$ were individually expressed in *E. coli* wild type (Fig. 3B, Supplementary Fig. 3B). These results collectively suggest

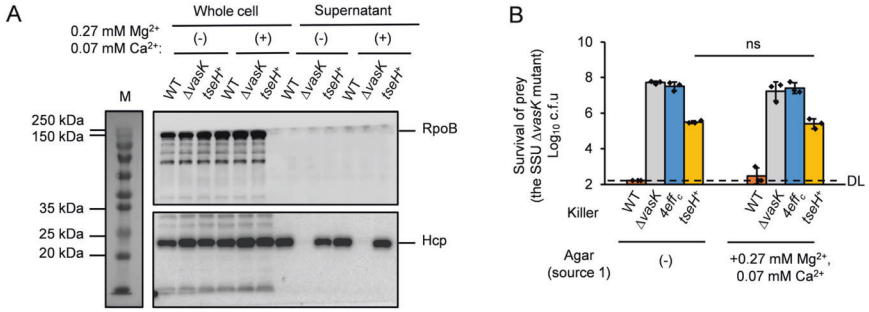


Fig. 2 Addition of Mg^{2+} and Ca^{2+} does not affect T6SS secretion. A Secretion analysis of Hcp in *V. cholerae* strains with or without Mg^{2+} and Ca^{2+}. WT wild type, Δ*vasK* the T6SS-null Δ*vasK* mutant, *tseH*$^{+}$ the TseH-active mutant. (−), no cations addition; (+), addition of 0.27 mM Mg^{2+} and 0.07 mM Ca^{2+}. RpoB, the beta subunit of DNA-directed RNA polymerase, serves as cell autolysis and loading control. Full images were shown in Supplementary Fig. 2A. **B** Survival of the *Aeromonas dhakensis* SSU Δ*vasK* mutant after competition with *V. cholerae* strains, as indicated. WT wild type, Δ*vasK* the T6SS-null Δ*vasK* mutant, *4eff*$_c$ the 4-antibacterial-effector-inactive mutant, *tseH*$^{+}$ the TseH-active mutant. Survival of prey strains was enumerated by serial plating on selective medium. Killer survival was shown in Supplementary Fig. 2B. Error bars indicate the mean ± standard deviation of three biological replicates. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test. ns not significant, DL detection limit.

that the PhoPQ system is important for protecting *E. coli* against TseH under low Mg^{2+} conditions, representing an additional immunity-independent mechanism to the recently reported envelope stress response systems [18].

Transcriptome analysis reveals induction of stress response genes by T6SS-delivered TseH

To understand how *E. coli* responds to cation supplement and how the PhoPQ regulation confers protection against TseH, we employed transcriptome analysis to examine the effect of cation supplement in *E. coli* with and without *V. cholerae* competitors. We first tested how the supplement of Mg^{2+} affects gene expression in *E. coli* by growing *E. coli* on LB source 1 agar with and without 0.3 mM Mg^{2+}. Exponentially growing *E. coli* cells were plated on LB plates for 30 min prior to RNA extraction. RNA-seq analysis shows near-identical transcriptomes (Supplementary Fig. 4A, B, Dataset 1). These results indicate that *E. coli* cells do not exhibit drastic changes in response to Mg^{2+} supplement under the conditions tested.

To understand how *E. coli* responds to T6SS-delivered TseH, we performed transcriptome analysis of *E. coli* competed with the *V. cholerae 4eff*$_c$ or *tseH*$^{+}$ mutants on LB source 1 agar plates with or without 0.3 mM Mg^{2+} or 0.3 mM Ca^{2+} (Supplementary Table 2, Dataset 2). To maximize the delivery of TseH from *V. cholerae* to *E. coli*, we used a mixing ratio of *V. cholerae* to *E. coli* at 10 to 1 and co-incubated the mixture for variable times until the survival of *E. coli* was reduced by 10-fold (Supplementary Fig. 3C, D). Specifically, because Mg^{2+} supplement had a weaker effect than Ca^{2+} on TseH-mediated killing (Fig. 1B, E), we performed competition under Mg^{2+}-supplemented conditions for 3 h co-incubation and used 1.5 h co-incubation for Ca^{2+} conditions. Transcriptome data show that multiple genes responsible for maintaining cell-envelope integrity were upregulated in *E. coli* cells attacked by the *V. cholerae tseH*$^{+}$ mutant under all conditions (Supplementary Fig. 5A–D, Dataset 2). These include the BaeR regulon genes *spy*, *osmB*, *wza*, *wzc*, the RcsB regulon gene *rcsA*, and a periplasmic protease gene *degP*, which are consistent with the previous report [18]. In addition, genes induced by osmotic pressure such as *osmY* and *osmE* were also upregulated, suggesting TseH toxicity may disturb the osmotic balance.

When comparing the effect of Mg^{2+} on gene expression in *E. coli* attacked by the *tseH*$^{+}$ *V. cholerae*, we found two PhoPQ regulon genes *mgtA* and *rstA* [53, 56] were significantly upregulated in samples without Mg^{2+} supplement but they were not upregulated in *E. coli* samples competed with the effector-inactive *4eff*$_c$ mutant with or without Mg^{2+} (Fig. 3C, D). Considering that deletion of *phoQ* rendered *E. coli* more sensitive to TseH (Fig. 3A), these data collectively suggest that TseH-mediated toxicity may elicit a specific protective response of the PhoPQ regulon in *E. coli* under low Mg^{2+} conditions.

Effect of Ca^{2+} is independent of the PhoPQ pathway

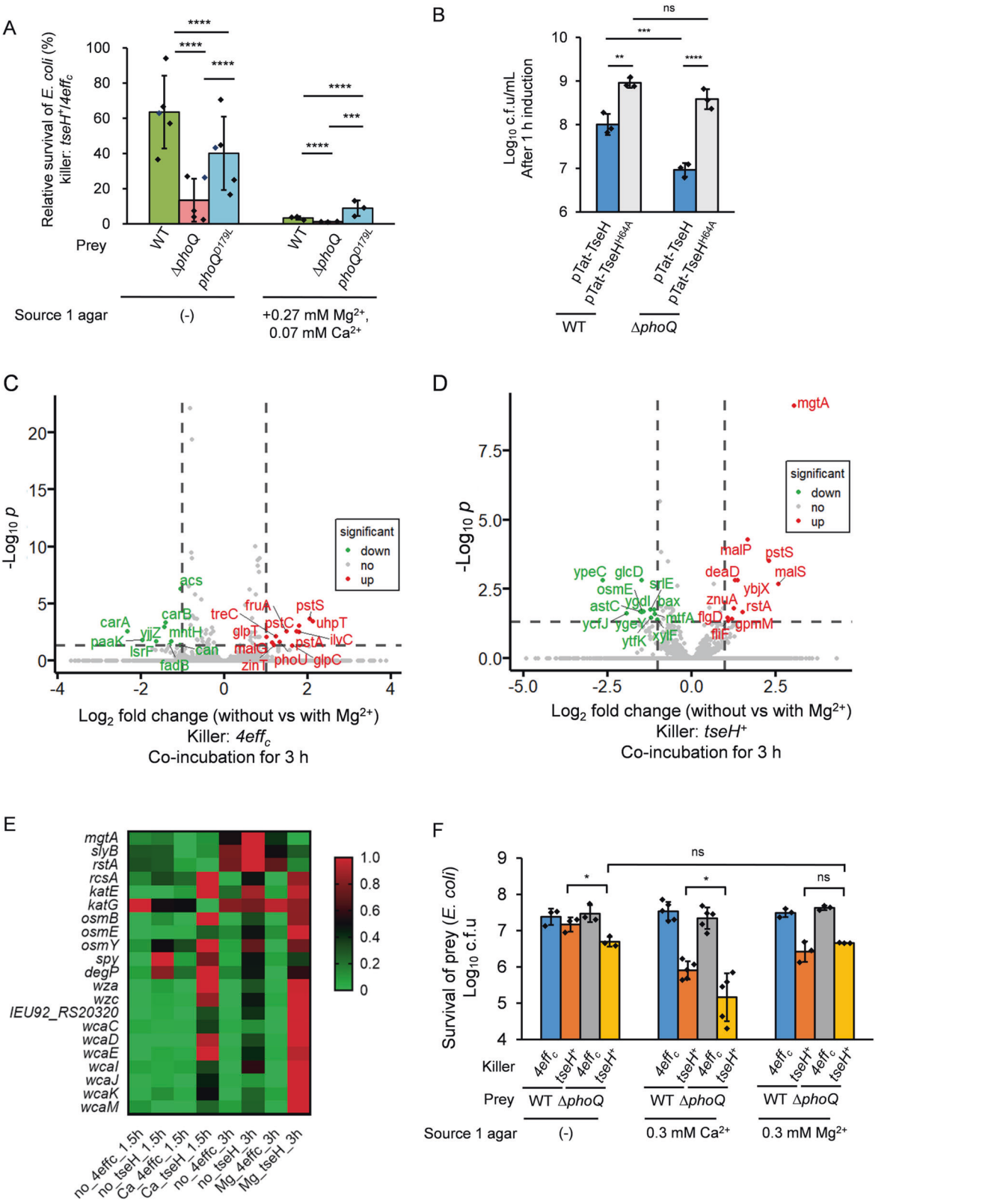
Similar to Mg^{2+} treatment, Ca^{2+} supplement significantly increased the number of differentially expressed genes in *E. coli* between the *tseH*$^{+}$ and the *4eff*$_c$ samples, while the transcriptomes of *E. coli* in these 1.5 h co-incubation samples differed by only 6 genes with moderate changes without supplemented Ca^{2+} (Supplementary Fig. 5A, B). Highly induced genes under Ca^{2+} conditions by the *tseH*$^{+}$ competition include a number of known stress response genes, *rcsA*, *katE, osmY*, and *osmB*, indicating stress response pathways were activated (Dataset 2, Fig. 3E). Notably, genes belonging to the PhoPQ regulon were not differentially expressed under Ca^{2+}-supplemented conditions (Fig. 3C–E, Supplementary Fig. 5E, F), which might be attributed to the differential responses of PhoPQ to Mg^{2+} and Ca^{2+} [57]. Accordingly, using competition assays against the *V. cholerae tseH*$^{+}$ mutant, we found that deletion of *phoQ* did not further reduce *E. coli* survival in the presence of Mg^{2+} but significantly reduced *E. coli* survival with Ca^{2+} supplement or when no cation was added (Fig. 3F, Supplementary Fig. 3E). These results suggest that the addition of Mg^{2+} but not Ca^{2+} could suppress the PhoPQ system under the conditions tested.

TseH is active under environmental and in vivo conditions

Due to the prevalence of Mg^{2+} and Ca^{2+} in the environment, we postulated that TseH is likely important for T6SS-mediated competitive fitness of *V. cholerae* under diverse conditions. Using crude agar extracts from algae to mimic a natural surface *V. cholerae* may grow on in the environment, we found that *V. cholerae* could use TseH to effectively outcompete *E. coli* or the Δ*tsiH* mutant of *V. cholerae* (Fig. 4A, B, Supplementary Fig. 6A, B). Because the T6SS is shown to be important for *V. cholerae* colonization in the host [48], we also tested whether TseH is active in vivo using an infant mouse infection model. Competition assays show that survival of the Δ*tsiH* mutant was significantly reduced by the *V. cholerae tseH*$^{+}$ mutant in comparison with its survival against the effector-inactive *4eff*$_c$ mutant (Fig. 4C), indicating TseH contributes to the in vivo fitness of *V. cholerae*.

Conditional sensitivity is generally applicable to other T6SS effectors

Having established that environmental cations affect *E. coli* sensitivity to TseH, we next examined whether similar effects are applicable to the other three antibacterial effectors in *V. cholerae*,



the lipase TseL, the colicin-like VasX, and the lysozyme VgrG3 [16, 29, 51]. Through screening a panel of single-effector active mutants of *V. cholerae* in competition with *E. coli* under different conditions, we made two key observations. First, *E. coli* grown on the source 2 agar was substantially more sensitive to the lipase effector TseL than *E. coli* on the source 1 agar (Fig. 5A, Supplementary Fig. 7A). Treatment of agar sources with EDTA abolished such difference and increased *E. coli* survival on source 2 agar (Fig. 5B, Supplementary Fig. 7B). By supplementing the source 1 agar with 0.3 mM Mg^{2+}, Ca^{2+}, Ni^{2+}, and Cu^{2+}, separately,

Fig. 3 The PhoPQ two-component system protects *E. coli* against TseH in the absence of Mg^{2+}. **A** Relative survival of *E. coli* strains competed with the *V. cholerae* $4eff_c$ and $tseH^+$ strain ($tseH^+/4eff_c$) with or without 0.27 mM Mg^{2+} and 0.07 mM Ca^{2+}. (−), without any addition. WT wild type, Δ*phoQ* the *phoQ* deletion mutant, $phoQ^{D179L}$, a constitutively active *phoQ* mutant. **B** Survival of *E. coli* wild type and Δ*phoQ* mutant expressing Tat-TseH or Tat-$TseH^{H64A}$ after 1 h induction. Survival of *E. coli* strains before induction was shown in Supplementary Fig. 3B. **C**, **D** Volcano plot of *E. coli* RNA-seq results (without/with 0.3 mM Mg^{2+}) competed with the *V. cholerae* $4eff_c$ mutant (**C**) or the $tseH^+$ mutant (**D**). DEGs were screened by setting cut-off value with Log_2 fold change > 1, p value < 0.05. Up and down-regulated genes were indicated in red and green, respectively. Genes with no significant change were indicated in gray. **E** Heat map of some important differential expression mRNAs in *E. coli* samples competed with *V. cholerae* strains. Scale means the normalized FPKM of each sample. **F** Survival of *E. coli* strains competed with the *V. cholerae* $4eff_c$ and $tseH^+$ strain with or without 0.3 mM Mg^{2+} or Ca^{2+}. (−), without any addition. For (**A**, **F**) killer survival was shown in Supplementary Fig. 3A, E respectively. For (**A**, **B**, **F**) survival of each strain was enumerated by serial plating on the selective medium. Error bars indicate the mean ± standard deviation of at least three biological replicates. Statistical significance was calculated using two-way ANOVA with Tukey's multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns not significant.

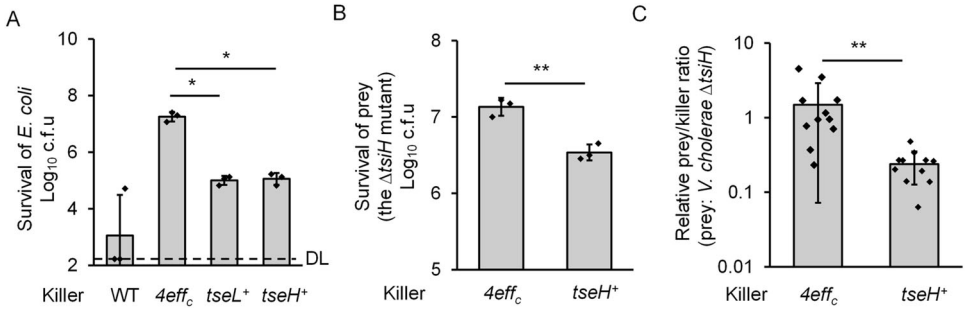


Fig. 4 TseH is active under environmental and in vivo conditions. **A**, **B** Survival of prey *E. coli* (**A**) or the *V. cholerae* Δ*tsiH* mutant (**B**) when competed with *V. cholerae* strains on LB raw-agar plates, as indicated. **C** Survival of the *V. cholerae* Δ*tsiH* mutant normalized to killer strains (as indicated) in infant mouse infection model. For (**A**, **B**) killer survival was shown in Supplementary Fig. 6A, B respectively. For (**A**–**C**) killer strains are indicated at the bottom. WT wild type, $4eff_c$ the 4-antibacterial-effector-inactive mutant, $tseL^+$ the TseL-active only mutant, $tseH^+$ the TseH-active only mutant. Survival of prey cells was enumerated by serial plating on the selective medium. Error bars indicate the mean ± standard deviation of at least three biological replicates. Statistical significance was calculated using one-way ANOVA with Tukey's multiple comparison test for (**A**) and two-tailed Student's t test for (**B**, **C**). *$p < 0.05$; **$p < 0.01$; DL detection limit.

we found that none of the cations increased *E. coli* sensitivity to TseL-toxicity (Fig. 5C, Supplementary Fig. 7C). These results suggest that *E. coli* sensitivity to TseL differs from that to TseH but is likely also modulated by EDTA-chelated cations. Second, *E. coli* displayed temperature-dependent sensitivity to effectors. Competition results show that the relative survival of *E. coli* was significantly reduced at 30 °C in comparison to that at 37 °C when *E. coli* was competed with the $vasX^+$ and the $vgrG3^+$ mutants, separately (Fig. 5D, Supplementary Fig. 7D). In contrast, the survival of *E. coli* was significantly increased at 30 °C relative to that at 37 °C when *E. coli* was competed with the $tseL^+$ strain (Fig. 5D, Supplementary Fig. 7D). These results clearly demonstrate that the T6SS-mediated interspecies competition is modulated by environmental conditions.

Expression of VasX sensitizes *P. aeruginosa* to irgasan in a temperature-dependent manner

We have previously reported that periplasmic expression of VasX using a Tat secretion signal is highly toxic in *E. coli* but not in *Pseudomonas aeruginosa* PAO1, an important opportunistic pathogen [19]. We thus tested whether the resistance of *P. aeruginosa* to VasX is temperature-dependent. First, we confirmed this resistance phenotype by comparing the survival of PAO1 expressing plasmid-borne Tat-VasX or its inactive mutant Tat-$VasX^{\Delta C16}$ [17] at 37 °C on LB plates containing irgasan and gentamycin, two antibiotics that select for intrinsic resistance of PAO1 and the plasmid vector, respectively (Fig. 5E). However, when Tat-VasX was induced at 30 °C, we found that survival of PAO1 was reduced to undetectable levels (Fig. 5E). Interestingly, the killing of PAO1 by Tat-VasX was dependent on the presence of irgasan, since survival of PAO1 was unaffected by Tat-VasX on LB plates containing only gentamycin at both 30 and 37 °C (Supplementary Fig. 7E). These results suggest that expression of VasX at 30 °C, albeit insufficient to cause cell death directly, can disturb the intrinsic resistance of *P. aeruginosa* to irgasan (Fig. 5E, Supplementary Fig. 7E).

DISCUSSION

In this study, we demonstrate how abiotic environmental factors, cations and temperature, can have profound effects on the outcome of T6SS-mediated competition. We have delineated effector-specific effects using a panel of *V. cholerae* single-effector-active mutants, which enable target cells to respond and survive single-effector attacks rather than being overwhelmed by multiple and simultaneously delivered effectors by wild-type T6SS cells. Of the four *V. cholerae* antibacterial effectors, TseH and TseL are influenced by divalent cations while TseL, VgrG3, and VasX exhibit temperature-dependent efficacies in killing *E. coli*. We show that the Mg^{2+}-sensing PhoPQ system in *E. coli* plays a key protective role while the supplement of Mg^{2+} likely suppresses such protection. Periplasmic expression of the colicin-like VasX does not directly kill *P. aeruginosa* but disturbs the intrinsic resistance of *P. aeruginosa* to irgasan in a temperature-dependent manner. Collectively, these findings not only reveal the importance of abiotic factors in modulating cellular responses to specific effectors in the context of interspecies competition but also implicate the prevalence and diverse mechanisms of conditional sensitivity to effectors in different organisms.

Almost all T6SS species secrete multiple effectors that may act synergistically to contribute to T6SS-mediated competitive fitness [34, 40, 42]. However, many effectors are phenotypically inactive, hindering the full understanding of the ecological functions of the T6SS. Our previous study has shown that effector functions may be masked due to the protection of envelope stress responses [18]. Here, we show that effector efficacy can be activated by Mg^{2+} or Ca^{2+} cations or a temperature shift, which *V. cholerae* likely commonly encounters during its natural host-environment

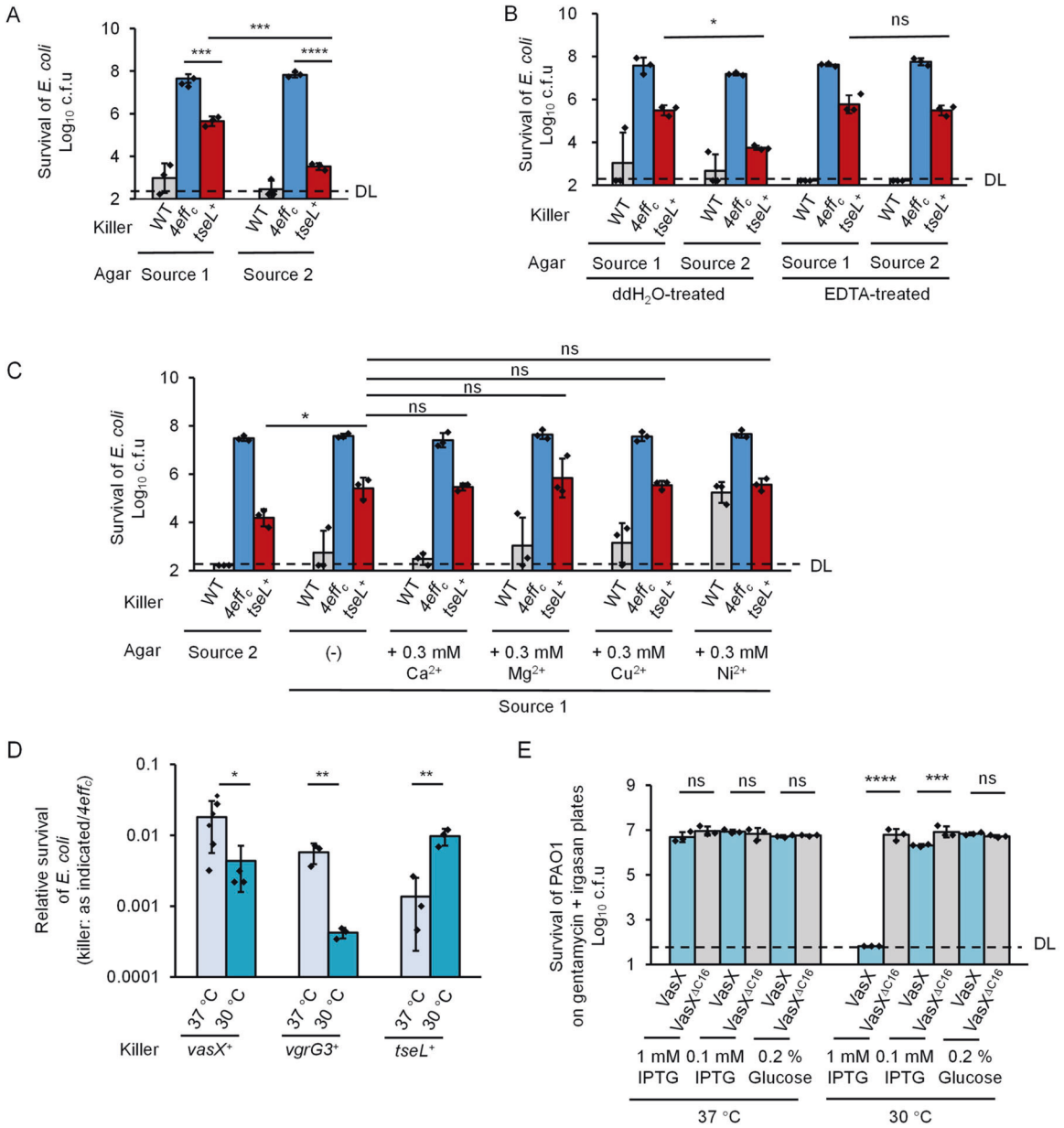


Fig. 5 Effects of cations and temperature on killing by other effectors. A Survival of *E. coli* after competition with *V. cholerae* TseL-active mutant *tseL*$^+$ on LB source-1-agar or source-2-agar plates. **B** Effect of EDTA-treatment on survival of *E. coli* competed with *V. cholerae tseL*$^+$. The ddH$_2$O-treated agar plates serve as the control. **C** Effect of cation supplement on TseL-mediated *E. coli* killing. LB source-1-agar plates were supplemented with 0.3 mM Ca^{2+}, Mg^{2+}, Cu^{2+} or Ni^{2+}, respectively. (−), without any addition. **D** Relative survival of *E. coli* after competed with *V. cholerae* strains at 30 °C or 37 °C (normalized to *4eff$_c$*), as indicated. For (**A**–**D**) WT wild type, *4eff$_c$* the 4-antibacterial-effector-inactive mutant, *tseL*$^+$ the TseL-active only mutant, *vasX*$^+$ the VasX-active only mutan, *vgrG3*$^+$ the VgrG3-active only mutant. Survival of *E. coli* strains was enumerated by serial plating on the selective medium. Survival of killer strains was shown in Supplementary Fig. 7A–D, respectively. **E** Toxicity assay of *P. aeruginosa* PAO1 strains ectopically expressing Tat-VasX and its colicin-inactivated mutant Tat-VasX$^{\Delta C16}$ on plates with gentamycin and irgasan at 30 or 37 °C. For (**A**–**E**) error bars indicate the mean ± standard deviation of at least three biological replicates. Statistical significance was calculated using two-tailed Student's *t* test for (**D**) and two-way ANOVA with Tukey's multiple comparison test for (**A**, **B**, **C**, **E**). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; ns not significant. DL detection limit.

transmission cycle. The efficacy of effectors in *P. aeruginosa* also varies depending on salinity, pH, and aerobic conditions [42]. The observations that VasX could sensitize *P. aeruginosa* to irgasan implicate a potential synergy between the T6SS effectors and other antimicrobial compounds within complex polymicrobial communities. Considering the prevalence of T6SS organisms, including plant and host pathogens, as well as the diversity of effectors in each species, these results highlight the necessity of considering the ecologically relevant environmental factors when studying the numerous cryptic effectors of protein secretion systems, especially for those causing nonlethal yet critical physiological changes.

Mg^{2+} and Ca^{2+} are known to have critical cellular functions, but their effects on dictating cellular survival in the context of T6SS-mediated interspecies competition have been largely unknown. As the most abundant divalent cation in living cells, Mg^{2+} neutralizes the negative charges of biomolecules, including nucleotides and nucleic acids, membrane phospholipids, as well as serving as an indispensable cofactor in various enzymes [50, 58, 59]. Ca^{2+} also plays a key role in multiple cellular processes

including motility and cell division processes [60, 61]. Due to the critical role Mg^{2+} and Ca^{2+} play in bacteria, their concentrations are tightly regulated [59, 62]. Under low Mg^{2+} conditions, the PhoPQ system is activated to regulate a large number of genes involved in virulence, metal uptake, and other important functions [50, 59]. In *Klebsiella pneumoniae*, the PhoPQ system is important for activating the T6SS expression upon attacks by T6SS active competitors or by the antibiotic colistin [33]. Here, we show that PhoPQ confers key protection to *E. coli* against TseH toxicity under low Mg^{2+} conditions. Because activation of the PhoPQ system is known to strengthen the outer-membrane (OM) barrier by modifying OM components including lipopolysaccharides, glycerophospholipids, and OM-bound proteins [63–67], the fortified and stiff OM barrier may protect cells not only from external T6SS attacks, as the exopolysaccharides matrix does in *V. cholerae* [31], but also from cell-wall damages elicited by effectors [68]. Collectively, these results suggest a strong connection between environmental Mg^{2+} and T6SS-mediated competition through the PhoPQ system in diverse bacterial species.

Microbes have been found in almost all ecological niches on earth and contribute to a variety of functions ranging from macroscale carbon recycling and waste removal to individual-level host health and disease. These functions are often determined by polymicrobial communities with extensive interspecies interactions. The outcome of T6SS-mediated competition is determined by diverse factors including the number and type of the secreted effectors [34], the frequency of T6SS firing correlated with energy state [34], the immunity protein-dependent specific protection [21], the nonspecific stress response pathways in killer and prey cells [18, 20], and the availability of nutrients [69, 70]. Environmental factors have previously been implicated in modulating effector efficacy and synergy in T6SS killer cells [42], but here we show that cation and temperature play a major role in prey cell susceptibility to effector toxicities, thereby affecting T6SS-mediated competition which may have significant ecological impact on community composition and pathogen evolution, the latter exemplified in the sequential displacement of *V. cholerae* pandemic strains [49].

Although we have yet to determine the exact physiological response in prey cells that accounts for the temperature and cation-dependent sensitivity to different effectors, we speculate numerous factors could be involved including membrane composition, envelope stability, stress response, and enzyme stability, which may be directly or indirectly influenced by cation and temperature changes. Nonetheless, these findings demonstrate even a quite narrow difference in common environmental parameters could have determined the life-or-death fate of bacterial cells. Our study may serve as an alluding primer to better understand the T6SS-mediated functions in broad ecological contexts, including dynamics of polymicrobial communities and the evolution of their defense and attack mechanisms, as well as the complex roles of seemingly redundant yet finetuned sensing and regulatory pathways for cells to survive in diverse ecological niches.

METHODS

Strains and growth conditions

Strains and plasmids used in this study are described in Supplementary Table 3. All constructs were verified by sequencing. Primers are available in Dataset 3. Bacteria were grown in LB([w/v] 1% tryptone, 0.5% yeast extract, 0.5% NaCl) at 37 °C aerobically. Antibiotics and inducers were used at following concentrations: kanamycin (25 μg/mL for *E. coli*, 50 μg/mL for *V. cholerae* and *A. dhakensis*), streptomycin (100 μg/mL), chloramphenicol (2.5 μg/mL for *V. cholerae* and 25 μg/mL for *E. coli*), irgasan (25 μg/mL), gentamycin (20 μg/mL), L-arabinose ([w/v] 0.1% or 0.01% as indicated), IPTG (0.1 mM or 1 mM as indicated). Tryptone (BIO BASIC, TG217(G211)), yeast extract (BIO BASIC, G0961), source-1-agar (BIO BASIC, FB0010), source-2-agar (Sangon Biotech, A505255-0250).

EDTA treatment of agar powder

Agar powder was washed with 0.1 M EDTA (Ethylenediaminetetraacetic acid) solution for 5 min with vortex, followed by deionized water washing 5 times to remove residual EDTA. Then the treated agar was dried and could be used for bacterial competition experiments.

Bacterial competition assay

Competing strains were grown overnight in LB with appropriate antibiotics. Killer cells were then sub-cultured to $OD_{600} = 1$. For the interspecies competition of *V. cholerae* with *E. coli*, killer and prey cells were mixed at a ratio of 5:1 and co-incubated on LB-agar plates or M9-agar (6 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, [w/v] 0.1% glucose, 1.3% agar) plates at 37 °C or 30 °C (as indicated) for 3 h. For the intraspecies competition between *V. cholerae* cells, killer and prey cells were mixed at a ratio of 20:1 (because *V. cholerae* was more resistant to TseH than *E. coli*) and co-incubated on LB-agar plates at 37 °C for 20 h (or 3 h when using the raw agar). After co-incubation, the survival of killer and prey cells were enumerated by 10-fold serial plating on LB plates with selective antibiotics. The mean Log_{10} c.f.u of the recovered killer and prey strains was plotted and error bars show the mean ± standard deviation of at least three biological replicates. Two-tailed Student's *t* test or ANOVA with Tukey's multiple comparison test was used to determine *p* values.

XRF and ICP analyses

XRF (X-Ray Fluorescence) and ICP (Inductively Coupled Plasma Mass Spectrometry) analyses were done by the Instrumental Analysis Center in Shanghai Jiao Tong University. For XRF analysis, the agar powder was pressed into disks (30 mm diameter) and performed with an XRF (XRF-1800, SHIMADZU). For ICP analysis, the two agar samples were digested with 5 mL concentrated nitric acid and diluted to 50 mL with ultrapure water. After samples were prepared, elemental analysis was performed with an ICP (Avio 500, PerkinElmer).

Protein secretion assay

Strains were grown overnight in LB with appropriate antibiotics. Cells were then sub-cultured to $OD_{600} = 1$ with or without Mg^{2+} and Ca^{2+}. Then, 2 ml of OD_{600}~1 cells were collected by centrifugation at 2500 × *g* for 3 min and resuspended in 1 mL fresh LB. Resuspended cells were placed at 30 °C for 1 h and centrifuged at 10,000 × *g* for 2 min at room temperature (RT). The cell pellet was used as the whole-cell sample. The supernatant was centrifuged at 10,000 × *g* for 2 min again as the secretion sample. All samples were mixed with SDS-loading dye, boiled at 98 °C for 10 min, followed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis and Western blotting analysis.

Western blotting analysis

After electrophoresis in a 12% SDS-PAGE gel, proteins were transferred to a PVDF membrane (Bio-Rad). Then, the protein-bound membrane was blocked with 5% [w/v] non-fat milk in TBST (50 mM Tris, 150 mM NaCl, 0.1% [v/v] Tween-20, pH 7.6) buffer for 1 h at RT. After blocking, the membrane was sequentially incubated with primary and secondary HRP (horseradish peroxidase)-conjugated antibodies in TBST with 1% [w/v] milk for 1 h at RT. Signals were detected using the Clarity ECL solution (Bio-Rad). The monoclonal antibody to RpoB, the beta subunit of RNA polymerase, was purchased from Biolegend (RpoB, Product # 663905). The polyclonal antibody to Hcp was custom-made by Shanghai Youlong Biotech. The HRP-linked secondary antibodies were purchased from ZSGB-Bio (Product # ZB-2305 (mouse) and # ZB-2301 (rabbit)). The Hcp antibody was used at 1:10000 dilution, while others at 1:20000 dilution.

RNA sample preparation and extraction

For transcriptome analysis of *E. coli* with or without Mg^{2+}, exponential-phase-growing *E. coli* cells were collected and transferred onto LB plates with or without 0.3 mM Mg^{2+} for 30 min prior to RNA extraction. For transcriptome analysis of *E. coli* competed with *V. cholerae*, killer and prey strains were grown in LB overnight. Killer strains were then sub-cultured to OD_{600}~1. Killer and prey cells were mixed at a ratio of 10:1 and co-incubated on appropriate LB plates with/without cations. A pilot time-course experiment was performed by monitoring *E. coli* survival during competition and the following time points prior to substantial cellular death of *E. coli* were established, 1.5 h co-incubation for RNA extraction in the presence of Ca^{2+} and 3 h in the presence of Mg^{2+}.

For RNA extraction, cells were retrieved from the plate surface and resuspended in 800 µL 1× lysis buffer (1% [w/v] SDS, 2 mM EDTA) by vortex for 1 min. Then 800 µL prewarmed hot acidic phenol (65 °C) was added and mixed by inverting immediately. Tubes were incubated at 65 °C for 5 min with mixing briefly every 1 min. After putting on ice for 10 min, the mixture was centrifuged at 13,000 × *g* for 2 min. The top supernatant was carefully transferred to a new tube and an equal-volume of absolute ethanol was added. Then the crude RNA was purified by RNA prep Pure Cell/Bacteria Kit (TIANGEN, Product #DP430) and genomic DNA was removed by DNase I (NEB, Product B0303S) treatment at 37 °C for 30 min. After DNase I treatment, RNA samples were then purified with the RNA clean Kit (TIANGEN, Product #DP412). Purified RNA was electrophoresed on 1% [w/v] agarose gel to monitor the integrity and contaminants.

Transcriptome analysis

RNA-seq library preparation and sequencing were performed by Novogene. Briefly, rRNA was removed from total RNA samples by using probes. Then obtained mRNA was fragmented by divalent cations. The first-strand cDNA was synthesized by M-MuLV Reverse Transcriptase using random hexamers as primers. The RNA strand was degraded by RNase H. The second strand of cDNA was synthesized using dUTP to replace dTTP in the dNTP mixture. The purified double-stranded cDNA was end-repaired, added with an A and ligated to the sequencing adapter. USER enzyme (NEB, USA) was used to degrade the second strand of cDNA containing U. After using AMPure XP beads to screen cDNA with 370~420 bp and PCR amplification of these fragments, the final library was obtained with another cycle of AMPure XP beads purification. The clustering of the index-coded samples was performed on a cBot Cluster Generation System following the manufacturer's instructions, and the sequencing was performed using the HiSeq (Illumina) platform with pair-end 150 base reads. Raw data were filtered according to the following standards: (1) removing reads with unidentified nucleotides (N); (2) removing reads with low sequencing quality (>50% bases having Phred quality scores of ≤20); (3) removing reads with the adapter. The obtained clean data were used for downstream analysis. Bowtie2-2.2.3 was used to build an index of the reference genome and align clean reads to the reference genome. The gene expression level was calculated and further normalized by HTSeq v0.6.1. FPKM, fragments per kilobase of transcript sequence per millions base pairs sequenced, was used to demonstrate gene expression level here. Differential expression analysis of each two conditions was performed by DESeq R package (1.18.0). The resulting *p* values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Differential expression genes (DEGs) were identified by setting cut-off value as Log_2 fold change > 1, p value < 0.05. The bioinformatics analysis of RNA-seq results was performed and plotted by R and GraphPad Prism.

qRT-PCR analysis

Primers for qPCR were designed by PerlPrimer. The 16S rRNA gene was used as the reference. Reverse transcription was done using PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (Takara, #RR047A). qPCR reaction was prepared by TB Green Premix Ex Taq (Tli RNase H Plus) (Takara, #RR420A) and detected by CFX Connect Real-Time PCR Detection System (BIO-RAD, #1855201). Analysis of relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method. Each sample was measured in triplicate and repeated at least three times.

Infant mouse competition assay

In vivo competition assay was performed as previously described with some modifications [71]. Briefly, 4–5-day-old BALB/c infant mice from Guangdong Medical Laboratory Animal Center were kept at a constant temperature of 28 °C for 6 h since received. *V. cholerae* strains were cultured to OD_{600}~1 and diluted to 10^8 CFU/mL with sterile LB, killer (*V. cholerae* the *tseH*$^+$ or *4eff*$_c$ strain) and prey (*V. cholerae* the Δ*tsiH*, Δ*lacZ* strain) cells were mixed with 1:1. Each baby mouse was gavaged with 50 µL mixture (25 µL killer cells + 25 µL prey cells). After 24 h, mice were sacrificed and the small intestines were isolated and homogenized in 1 mL sterile PBS. A tenfold serial dilution was plated on LB plates with appropriated antibiotics and 40 µg/mL X-Gal. After incubation at 37 °C overnight, killer and prey cells were numerated by blue and white colonies in the plates and the prey/killer ratio was calculated.

Toxicity assay

For TseH toxicity assay in *E. coli*, plasmid encoding arabinose-inducible Tat-tagged (periplasm-directed) TseH or TseH^{H64A} was electroporated into *E. coli* strains and maintained on 0.4% [w/v] glucose to repress expression. Fresh colonies were resuspended in LB medium with 0.4% [w/v] glucose and sub-cultured to OD_{600}~1. Cells were collected and resuspended in LB with 0.1% [w/v] arabinose followed by 1 h induction. The survival of *E. coli* before and after induction was enumerated by tenfold serial dilutions on LB plates containing 0.4% [w/v] glucose and appropriate antibiotics.

For VasX toxicity assay in PAO1, overnight cells carrying pPSV37 constructs were grown in LB with appropriate antibiotics and 0.2% [w/v] glucose at 37 °C. Cells were then collected and resuspended in fresh LB. A serial dilution was plated on LB plates containing 0.1 mM, 1 mM IPTG or 0.2% [w/v] glucose with antibiotics as indicated.

DATA AVAILABILITY

The data that support the findings of this study are available within the paper or available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

TD conceived the project. MT performed most of the experiment and data analysis. TP, ZW, and HL performed experiments. QX and YF performed animal infection. XW contributed to RNA-seq data analysis. MT prepared the first draft. TD contributed to the revision with assistance from MT and TP.

COMPETING INTERESTS

The authors declare no competing interests.

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