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Serine/threonine kinase PpkA coordinates the interplay between T6SS2 activation and quorum sensing in the marine pathogen *Vibrio alginolyticus*

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Summary

Type VI secretion systems (T6SS) are multiprotein secretion machines that can mediate killing of bacterial cells and thereby modify the composition of bacterial communities. The mechanisms that control the production of and secretion of these killing machines are incompletely understood, although quorum sensing (QS) and the PpkA kinase modulate T6SS activity in some organisms. Here we investigated control of the T6S in the marine organism *Vibrio alginolyticus* EPGS, which encodes two T6SS systems (T6SS1 and T6SS2). We found that the organism principally relies on T6SS2 for interbacterial competition. We further carried out a phosphoproteomic screen to identify substrates of the T6SS2-linked PpkA2 kinase. Substrates of PpkA2 encoded within the T6SS2 cluster as well as proteins that are apparently not linked to T6SS-related processes were identified. Similar to other organisms, PpkA2 autophosphorylation was critical for T6SS2 function. Notably, phosphorylation of a polypeptide encoded outside of the T6SS2 cluster, VtsR, was critical for T6SS2 expression and function because it augments the expression of *luxR*, a key regulator of QS, that also promotes T6SS2 gene expression. Thus, PpkA2 controls a phosphorylation cascade that mediates a positive regulatory loop entwining T6SS and QS, thereby coordinating these pathways to enhance the competitive fitness of *V. alginolyticus*.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

Quorum sensing; VtsR; T6SS; *Vibrio alginolyticus*; PpkA; phosphoproteome

Introduction

Bacteria often depend on a variety of specialized secretion machineries to interact with and adapt to their environmental niches. Type VI secretion systems (T6SSs) are widespread in Gram-negative bacteria (Mougous *et al.*, 2006; Pukatzki *et al.*, 2006). Some bacteria harbor more than one T6SS and these systems can carry out distinct functions (Cascales, 2008). These multi-component secretion machines can mediate contact dependent killing of prokaryotic as well as eukaryotic cells (Hood *et al.*, 2010; Silverman *et al.*, 2012; Böck *et al.*, 2017). Besides killing adjacent bacterial cells, recent investigations have revealed that T6SS play multiple non-killing functions, e.g. mediating intracellular pH homeostasis (Zhang *et al.*, 2013), Zn²⁺ transport (Wang *et al.*, 2015), manganese/iron scavenging and resistance to reactive oxygen species (Lin *et al.*, 2017; Si *et al.*, 2017; Wan *et al.*, 2017), facilitating bacterial stress adaptation to changing environmental conditions. Most of the killing and non-killing functions of T6SS are mediated by specific effector proteins propelled outside of bacterial cells and into adjacent recipient cells through the action of contractile T6SS (Basler and Mekalanos, 2012). Cells encoding T6SS also encode immunity proteins to prevent auto-intoxication from autologous effector proteins (Benz and Meinhart, 2014).

Canonical T6SS consist of 13 core subunits and several additional components, which are less conserved (Boyer *et al.*, 2009). Each T6S apparatus has three main modules: a contractile sheath-tube, a baseplate, and an anchor attached to the cytoplasmic membrane (Böck *et al.*, 2017). The contractile sheath-tube extends into the cytoplasm and consists of a polymer of TssBC (VipA/VipB) building blocks (Basler *et al.*, 2012; Kudryashev *et al.*, 2015). The assembly of Hcp (TssD) forms the secretory tube, which is surrounded by the VipA/VipB sheath (Mougous *et al.*, 2006; Basler *et al.*, 2012). TssH (ClpV) interacts with and disassembles the sheath after contraction and recycles VipA/VipB subunits (Kapitein *et al.*, 2013). VgrG (TssI) and some PAAR family proteins form the spike at the tip of the Hcp tube and can be decorated with various effector proteins (Shneider *et al.*, 2013). The anchor module is a large complex composed of TssJ, TssM, and TssL (DotU), which fixes the baseplate, the sheath tube assembly platform, and allows passage of the Hcp tube/VgrG spike (Zoued *et al.*, 2016; Böck *et al.*, 2017).

As an interbacterial ‘nanoweapon’, T6SS are tightly controlled by various regulatory systems acting at multiple levels. Multiple cues, such as surface contact, iron limitation, c-di-GMP, and chitin (Silverman *et al.*, 2012; Joshi *et al.*, 2017), are sensed by a variety of regulators to coordinate activation of T6S at the transcriptional, post-transcriptional, and post-translational levels. A subset of T6SS gene clusters harbor orthologs of PpkA (a Ser/Thr protein kinase), PppA (a phosphatase) and Fha (forkhead-associated domain-containing protein) that constitute Ser/Thr phosphorylation cascades to control T6S activity (Mougous *et al.*, 2007; Cascales, 2008; Silverman *et al.*, 2011; Fritsch *et al.*, 2013; Lin *et al.*,

2014). A Thr phosphorylation regulatory mechanism was first implicated in control of T6S in *Pseudomonas aeruginosa*, where PpkA, a type II membrane-spanning Hanks-type threonine kinase, and the cognate phosphatase PppA can reciprocally regulate Thr phosphorylation of Fha and thus positively and negatively modulate Hcp secretion (Mougous *et al.*, 2007). In *Agrobacterium tumefaciens*, PpkA directly phosphorylates TssL (DotU) to activate T6SS, which differs from the paradigm in *P. aeruginosa* and *Serratia marcescens* (Fritsch *et al.*, 2013; Lin *et al.*, 2014). These studies suggest that the phosphorylation cascades mediated by PpkA proteins have been adapted to integrate diverse signaling systems to coordinate the temporal-spatial function of T6SS.

Vibrio alginolyticus, a Gram-negative mesophilic and moderately halophilic bacterium, is abundant in marine environments, and is also an important pathogen causing vibriosis in marine animals including coral, resulting in significant damage to marine ecosystems as well as aquaculture industries worldwide (Liu *et al.*, 2004; Xie *et al.*, 2013). Furthermore, *V. alginolyticus* can also cause intestinal and extraintestinal infections in humans as an opportunistic pathogen (Austin, 2010; Jacobs *et al.*, 2017). *V. alginolyticus* pathogenicity is regulated by a *V. harveyi*-like quorum sensing (QS) system, a bacterial cell-cell communication system controlled by three sets of autoinducers (AIs) (Liu *et al.*, 2017). LuxO and LuxR play pivotal roles in translating the signals from AIs and other environmental cues, e.g. temperature fluctuations, into regulation of the expression of diverse genes (Wang *et al.*, 2007; Rui *et al.*, 2008; Gu *et al.*, 2016).

V. alginolyticus encodes two T6SS gene clusters (T6SS1 and T6SS2), each of which encodes homologs of the 13 characteristic core T6SS proteins (Sheng *et al.*, 2013; Salomon *et al.*, 2015). Here, we found that a fish isolate, *V. alginolyticus* EPGS (Liu *et al.*, 2011), employs its T6SS2 to kill several Gram-negative bacterial species. Phosphoproteomic analysis revealed that PpkA2 and DotU2, as well as several non-T6SS proteins, are phosphorylated by PpkA2. Notably, we found that phosphorylation of a novel PpkA2 target, termed VtsR (*Vibrio* type six secretion regulator, EPGS_00471), controls QS gene expression by regulating *luxR* expression. Since LuxR is required for T6SS2 expression, our findings reveal that the PpkA2 phosphorylation cascade coordinates control of *V. alginolyticus*' T6SS and QS pathways. These results indicated that PpkA-mediated regulatory circuit may facilitate *V. alginolyticus*' capacity to thrive in marine environments.

Results

The interbacterial killing ability of *V. alginolyticus* EPGS is mediated by T6SS2

We found that *V. alginolyticus* EPGS (referred to as EPGS below) killed *E. coli* K12 strain MC4100 in co-culture (Supporting information Fig. S1). Various culture conditions were used to maximize killing (Supporting information Fig. S1). These experiments showed that EPGS reduced the starting CFU of *E. coli* ~1000× (colony forming unit (CFU) numbers were $\sim 9.13 \times 10^9$ and $\sim 4.97 \times 10^6$ for 1 ml *E. coli* culture mixed without or with co-culture with EPGS, respectively) when the starting ratio of EPGS to *E. coli* was 4:1 and the strains were co-cultured at 30°C for 4 h on LB plus 3% NaCl (LBS) agar plates (Supporting information Fig. S1a–d). EPGS' toxicity was not limited to *E. coli*; this *V. alginolyticus* strain was also highly toxic toward several other marine bacteria, including other *V.*

alginolyticus strains ATCC 33787 and MVP01, and *Edwardsiella* strains EIB202 and ET080813 (Supporting information Fig. S2a–d). In all these and the following co-culture assays, regardless of the prey bacteria, EPSG and its T6SS related mutants grew to similar densities (Supporting information Fig. S2e and S3).

To investigate whether T6SS is required for interbacterial killing, mCherry-tagged *E. coli* was co-cultured with GFP-labeled EPSG or a *hcp1 hcp2* derivative. The latter strain lacks *hcp1* and *hcp2* genes that are predicted to be essential for T6SS1 and T6SS2 activity, respectively (Mougous *et al.*, 2006). After 4 h of exposure to EPSG, *E. coli* was barely detectable; in contrast, the growth of *E. coli* was significant when co-cultured with the *hcp1 hcp2* strain as compared to with wt (Fig. 1a). Additional EPSG/*E. coli* mixing experiments, where CFU counting was used to quantify the killing of the *E. coli* prey, confirmed that the out-competition of *E. coli* by *V. alginolyticus* was mainly T6SS-dependent, although the CFU numbers of *E. coli* contacting with the *hcp1 hcp2* mutant were significantly lower than that cultured alone, probably due to the faster growth rate of *V. alginolyticus* (Fig. 1b and S3), which restrains *E. coli* growth in the mixed cultures. In addition, the results showed that an *hcp1* mutant had a similar capacity to kill *E. coli* as the wt EPSG; in contrast, an *hcp2* mutant was as defective in killing as the *hcp1 hcp2* mutant (Fig. 1b). Furthermore, the defective killing capacity of the *hcp1 hcp2* mutant could be rescued by complementation with *hcp2* but not *hcp1* (Fig. 1b). Together, these data suggest that T6SS2, rather than T6SS1, is essential for EPSG to kill *E. coli* in the conditions used for these assays.

Bioinformatic analysis revealed that EPSG encodes three putative effector/immunity pairs bearing similarity to annotated pairs in other vibrio species and several putative orphan effector proteins (Supporting information Table S1). These 3 pairs are homologues of *V. alginolyticus* 12G01 RhsP1-RhsI1 (Hachani *et al.*, 2014; Salomon *et al.*, 2015) (EPGS_03540–03541), *V. cholerae* VasX-TsiV2 (Miyata *et al.*, 2013) (EPGS_01225–01226), and *V. parahaemolyticus* VPA1263-Vti2 (Salomon *et al.*, 2014) (EPGS_01602–01601) (Supporting information Table S1). These comparisons also suggest that the latter two pairs are associated with T6SS1-like gene clusters in other vibrios, while the former one is associated with T6SS2-like clusters (Miyata *et al.*, 2013; Salomon *et al.*, 2014). However, none of these putative EPSG effector/immunity loci are linked to the core T6SS2 genes located on chromosome II (Fig. 1c). EPSG/*E. coli* co-culture assays revealed that *rhsP1* and *rhsP1-rhsI1* mutants completely lacked the capacity to kill *E. coli*, whereas EPSG mutants missing the other two effectors or effector/immunity genes had little effect on EPSG's ability to kill *E. coli* (Fig. 1d). These observations suggest that the RhsP1 effector is required for T6SS2-mediated killing of *E. coli*.

QS regulates expression of EPSG's two T6SS in opposite fashions

In EPSG, QS is known to promote T6SS1 expression at low cell density, whereas at high density QS inhibits the expression of this T6SS (Sheng *et al.*, 2012). However, the role of QS in T6SS2 expression in this bacterium has not been defined. We compared the roles of QS in modulating expression of both T6SS1 and T6SS2 genes in EPSG. In these experiments, wt and *luxO^{D47E}* and *luxO^{D47A}* EPSG mutants were grown on LBS agar plates

and expression of T6SS2 and T6SS1 genes were assayed with qRT-PCR. LuxO^{D47E} is a LuxO~P mimic that constitutively activates expression of the *qrr* genes and thus the cells are “locked” in the low cell-density state; conversely, LuxO^{D47A} has a mutation that impaired in its ability to promote *qrr* expression and the cells mimic the high cell-density state (Vance *et al.*, 2003; Cao *et al.*, 2011; Rutherford *et al.*, 2011). We found that the expression T6SS1 genes (*ppkA1*, *icmF1*, *clpV1*, and *hcp1*) was significantly repressed in the *luxO*^{D47A} background, and de-repressed in the *luxO*^{D47E} background comparing to wt. In marked contrast, expression of T6SS2 genes (*ppkA2*, *pppA*, *icmF2*, *clpV2*, and *hcp2*) was repressed in the *luxO*^{D47E} background but was significantly elevated in *luxO*^{D47A} background relative to the wt (Fig. 2a). Mirroring these findings, immunoblots showed that production and secretion of Hcp2 in *luxO*^{D47E} cells was impaired, while that of Hcp1 was elevated in this background (Fig. 2b). Conversely, *luxO*^{D47A} cells produced and secreted low levels of Hcp1 and relatively high levels of Hcp2 (Fig. 2b). Furthermore, these gene expression phenotypes had functional significance in the EPGS/*E. coli* co-culture assay. The *luxO*^{D47E} mutant, which had deficient T6SS2 expression, no longer killed the *E. coli* prey, whereas the *luxO*^{D47A} mutant, which had elevated T6SS2 expression, was a more potent killer of *E. coli* than wt EPGS. LuxR is a critical activator of QS gene expression and similar to *luxO*^{D47E} mutant, the killing capacity of a *luxR* mutant was highly defective; the killing defect in this strain could be complemented by expression of *luxR* in trans (*luxR*+pLuxR, Fig. 2c). Collectively, these data indicate that QS modulates expression of *V. alginolyticus*’ T6SS1 and T6SS2 in distinct fashions. Similar observations of differential QS control of expression of *V. parahaemolyticus*’ two T6SS were previously reported (Gode-Potratz and McCarter, 2011). Furthermore, these findings reveal that *luxR*, a key QS regulator, is required for T6SS2 killing activity.

PpkA2 regulates T6SS2 secretion

The EPGS T6SS2 gene cluster encodes a serine-threonine kinase PpkA homolog, PpkA2 (Fig. 1c). We tested whether this gene product, which regulates T6SS function in other organisms (Mougous *et al.*, 2007; Fritsch *et al.*, 2013; Lin *et al.*, 2014), also modulates T6SS2 activity in EPGS by assaying the production and secretion of the hallmark Hcp2 protein in a *ppkA2* deletion mutant. Intriguingly, we found that Hcp2 was produced but not detectable in the medium of the *ppkA2* mutant, suggesting that PpkA2 modulates Hcp2 secretion (Fig. 3a). Hcp2 secretion is required for T6SS2 killing and we found that the *ppkA2* mutant was as defective as an *hcp2* mutant in killing *E. coli* in the co-culture assay (Fig. 3b). These secretion and killing defects could be restored by complementation of the *ppkA2* gene (Fig. 3a and b). Taken together, these observations reveal that T6SS2 secretion and killing is controlled by PpkA2.

Autophosphorylation of PpkA2 is required for T6SS2 activation

PpkA kinase activity is known to be required for activation of T6S in other systems (Mougous *et al.*, 2007; Fritsch *et al.*, 2013; Lin *et al.*, 2014). We performed non-biased phosphoproteomics, by comparing the *in vivo* phosphoproteomes of wt and *ppkA2* strains, to identify PpkA2 phosphorylation substrates in EPGS (Supporting information Fig. S4a; Supporting information Table S2 and S3). To qualify as a candidate PpkA2 substrate in this analysis, threonine or serine phosphopeptides should be present in the EPGS sample, but

absent from the *ppkA2* mutant sample. In two independent experiments, ten phosphopeptides with specific threonine ($n=9$) or serine ($n=1$) residues belonging to 8 different EPGS proteins were identified (Table 1). Two of the 8 gene products, DotU2 and PpkA2 itself, are encoded within the T6SS2 cluster. The other 6 non-T6SS2 linked gene products were predicted to be related to diverse processes, including type II secretion (T2SS) and resistance to reactive oxygen species (Table 1; Supporting information Fig. S4b). These data suggest the possibility that PpkA2 modulates non-T6SS functions as well as T6SS2.

Mutants containing in-frame deletions of each of the 7 genes (except EPGS_01659) encoding these proteins were created and tested in the co-culture assay with *E. coli*. Three of the strains, those with deletions of *dotU2* and *ppkA2*, and a hypothetical protein EPGS_00471, were as defective in killing *E. coli* as the *hcp2* mutant (Fig. 4a; Supporting information Fig. S4c; Table 1), suggesting that they are critical for T6SS2 function.

Mass spectrometry (MS) also revealed a phosphorylated Thr 59 (pT59) located within PpkA2's N-terminal cytoplasmic kinase domain, suggesting that PpkA2 is autophosphorylated at this residue (Supporting information Fig. S5a). We created an EPGS mutant, where PpkA2 threonine-59 was replaced with alanine. This substitution impaired the capacity to kill *E. coli* almost as markedly as deletion of *ppkA* or *hcp2* in the co-culture assay (Fig. 4b), suggesting that phosphorylation of PpkA2 T59 is crucial for this kinase to enable T6SS2 function. Analysis of the production and secretion of Hcp2 in *ppkA2* related mutants, *ppkA2*, *ppkA2*^{T59A} and *ppkA2*^{D224AN229A}, a mutant with alanine substitutions in conserved Asp 224 (D224) and Asn 229 (N229) residues located within the kinase magnesium binding loop (Hsu *et al.*, 2009; Lin *et al.*, 2014), showed that like the *ppkA2* deletion mutant, the mutants with predicted defective phosphorylation (*ppkA2*^{T59A}) or kinase activity (*ppkA2*^{D224AN229A}) were critical for Hcp2 secretion, but not for its production (Fig. 4c). These data support the conclusion that PpkA2 autophosphorylation at residue Thr 59 is required for T6SS2 function.

DotU2 phosphorylation enables its interaction with Fha2 and is required for T6SS2 secretion

DotU (TssL) proteins localize to the inner membrane and serve as a critical component of the T6S anchor module (Durand *et al.*, 2012; Zoued *et al.*, 2016). *A. tumefaciens* TssL is phosphorylated solely at T14 by PpkA (Lin *et al.*, 2014) (Supporting information Fig. S7a). Our phosphoproteomic MS analysis showed that PpkA2 phosphorylated DotU2 at two threonine residues, T4 and T28 (Table 1; Supporting information Fig. S5b). This conclusion was supported by phosphate affinity SDS-PAGE (Phos-tag gel) experiments (Kinoshita *et al.*, 2006), analyzing the phosphorylation of Flag-tagged DotU2 or its derivatives in which T4 and T28 were individually or both replaced with alanine. The intensity of the band representing the *p*-DotU2-Flag protein was reduced in the individual DotU2 substitution mutants where T4 or T28 were replaced with alanine (Fig. 5a). Moreover, the band corresponding to the *p*-DotU2-Flag protein was completely eliminated on the Phos-tag gel when both the Thr 4 and Thr 28 residues were substituted with alanine (DotU2^{T4AT28A}) (Fig. 5a). The EPGS mutant harboring a deletion of *dotU2*, *dotU2*, was deficient in Hcp2 secretion, but not production, and this deficit could be complemented by expression of

dotU2 in trans from a plasmid (Fig. 5b). The single T4A and T28A DotU2 substitution variants reduced Hcp2 secretion, and the substitution of both of these threonine phosphorylation residues with alanine completely abolished Hcp2 secretion (Fig. 5b). Thus, phosphorylation of the DotU2 at both T4 and T28 residues is required for maximal T6SS2 secretion.

In *A. tumefaciens* phosphorylated TssL (DotU) interacts with Fha, a forkhead-associated domain-containing protein, and this phosphorylation dependent interaction is required for T6S activity (Mougous *et al.*, 2007; Lin *et al.*, 2014). Indeed, we found that EPGS mutants harboring either a deletion of *fha2* (*fha2*) or the substitution of conserved Fha2 residues implicated in phosphopeptide binding (Arg 36 and Ser 52) with alanine (R36A S52A) (Supporting information Fig. S7b) no longer secreted Hcp2, although the protein was still produced (Fig. 5b).

We tested whether DotU2's phosphorylation at either T4 or T28 or at both residues is required for its interaction with Fha2 using pull-down assays. In these experiments, purified Fha2-His6 or its derivative Fha2^{R36A S52A}-His6 were incubated with a cellular lysate derived from *dotU2* cells expressing Flag-tagged DotU2 or phosphorylation site defective variants of DotU2. Pull-down assays were then carried out to purify the His-tagged protein and any bound Flag-tagged DotU2 related proteins. We found that DotU2-Flag and T4A or T28A DotU2-Flag proteins were co-purified with Fha2-His6 (Fig. 5c). However, the T4A and T28A double substitution DotU2-Flag protein did not co-purify with Fha2, indicating that phosphorylation of at least one of these DotU2 residues is required for the interaction of DotU2 and Fha2. Similarly, mutation of the putative phosphopeptide-binding domain in Fha2 (Fha2^{R36A S52A}) disrupted the interaction between these two proteins (Fig. 5c).

As expected, the *dotU2* and *fha2* deletion mutants did not kill the *E. coli* prey in the co-culture assay (Fig. 5d). Ectopic expression of DotU2 in trans in the *dotU2* strain rescued the killing deficiency of the *dotU2* strain (Fig. 5d). The killing capacity of the *dotU2* strain could be largely restored by the expression of DotU2^{T4A} or DotU2^{T28A} *in trans*, however, expression of DotU2^{T4AT28A} did not complement the *dotU2* strain's killing defect (Fig. 5d). Taken together, these findings strongly suggest that phosphorylation of DotU2's Thr4 and Thr28 residues enable its interaction with Fha2 and T6SS2 function, a manner similar to that reported in *A. tumefaciens* (Lin *et al.*, 2014),

VtsR phosphorylation is required for T6SS2 gene expression and function

MS analysis revealed that a hypothetical protein EPGS_00471 (hereafter termed VtsR) was phosphorylated at serine 61 in wt but not in the *ppkA2* strain (Supporting information Fig. S5c; Table 1). *vtsR* is not linked to either of EPGS' T6SS gene clusters, and is predicted to encode a 64 aa peptide that lacks a detectable conserved domain. VtsR homologues are found in most of the Harveyi clade of other *Vibrios*, where Ser 61 is highly conserved (Supporting information Fig. S8a–b), but not present in *V. cholerae* or in *P. aeruginosa*, *A. tumefaciens*, or *S. marcescens*; the latter three species share similar PpkA-mediated T6SS activation mechanisms (Supporting information Fig. S8a). The phosphorylation of VtsR S61 was corroborated using phosphate affinity SDS-PAGE. The phosphorylation of VtsR-Flag

was detected on a Phos-tag gel, whereas no phosphorylation of VtsR^{S61A}-Flag (containing an Ala substitution for Ser 61) was observed (Fig. 6a).

Since VtsR is likely a cytoplasmic protein (Supporting information Fig. S8c and below), we hypothesized that it promotes expression of T6SS2 genes to enable T6SS2 activity (Fig. 4a; Table 1). Consistent with this possibility, we found that deletion of *vtsR* in EPGS significantly diminished the expression of *hcp2* compared to the wt strain and this expression defect was complementable by expression of *vtsR* in trans (*vtsR*+pVtsR) (Fig. 6b). In contrast, when non-phosphorylatable *vtsR* mutants, *vtsR*^{S61A} or *vtsR*^{S61D} were used to complement the *vtsR* deletion, expression of *hcp2* remained compromised (Fig. 6b). However, complementation with a VtsR^{S61E} variant, which is predicted to mimic phosphorylated VtsR, yielded complementation of *hcp2* expression (Fig. 6b). Interestingly, the expression of Hcp1 in all the same strains above were opposite compared to wt (Fig. 6b). Together, these findings suggest that phosphorylation of VtsR promotes *hcp2* expression while reduces *hcp1* expression.

Given the reduction in *hcp2* expression in the *vtsR* mutant, it was not surprising to find that there was no detectable Hcp2 production or secretion in this strain (Fig. 6c). This defect was rescued by the introduction of a plasmid expressing VtsR (*vtsR*+pVtsR) or by creating a wt revertant (Fig. 6c and data not shown). Similarly, there was no Hcp2 production or secretion detected from the strains expressing the VtsR phosphorylation site variants (VtsR^{S61A} and VtsR^{S61D}); however, expression of the phosphorylation site mimic, VtsR^{S61E}, complemented the Hcp2 production and secretion defects of the *vtsR* strain as well as native VtsR (Fig. 6c). In contrast, the expression levels of Hcp1 in the same strains above were opposite to that of Hcp2 (Fig. 6c). These findings are consistent with the gene expression data and indicate that phosphorylation of VtsR at serine 61 promotes Hcp2 expression but decreases Hcp1 expression.

We also measured transcript levels of two additional T6SS2 genes, *icmF2*, *clpV2*, as well as 2 T6SS1 genes, *icmF1*, and *clpV1* in the *vtsR* background and in a *vtsR* strain expressing either VtsR^{S61A} or VtsR^{S61E}. IcmF and ClpV are core T6SS proteins, which function as a transmembrane-spanning scaffold protein and a cytoplasmic AAA+ ATPase, respectively (Silverman *et al.*, 2012). Expression of VtsR^{S61E} and native VtsR elevated transcript levels of the two T6SS2 genes, and reduced expression of the T6SS1 genes; in contrast, expression of the non-phosphorylatable VtsR^{S61A} had minimal effect on transcript levels of the T6SS2 or T6SS1 genes (Fig. 6d). Thus, *vtsR* appears to modulate expression of T6SS2 and T6SS1 genes in opposite fashion. As predicted, the defects in T6SS2 gene expression and Hcp2 production and secretion observed in the *vtsR* strain complemented with different versions of *vtsR* led to corresponding defects in the capacity of the mutant strains to kill *E. coli* prey in the co-culture assay. The *vtsR* strain, as well as the deletion strain expressing VtsR^{S61A} or VtsR^{S61D} exhibited no capacity to kill *E. coli*, whereas expression of VtsR^{S61E} fully complemented the killing defect (Fig. 6e). Rescue of *E. coli* killing was achieved by expression of *vtsR* in the *vtsR* mutant (*vtsR*+pVtsR), further confirming the necessity for *vtsR* in proper T6SS2 function (Fig. 6e).

VtsR activates T6SS2 activity by controlling luxR expression

To begin to assess how VtsR controls T6SS function, we first explored VtsR subcellular localization. An EPGS derivative expressing a functional GFP-labeled VtsR and a functional mCherry-ClpV2 fusion (Supporting information Fig. S8d) was created to test if VtsR co-localizes with the T6SS2 apparatus. As reported for other T6SS, the tagged ClpV2, which marks the T6SS sheath tube (Mougous *et al.*, 2007), was detected as discrete punctae in the cell; in contrast, VtsR-GFP was found diffusely throughout the cytoplasm (Fig. 7a), suggesting that VtsR does not form part of the T6SS apparatus. Phosphorylation of VtsR S61 does not appear to modify VtsR's localization, since a VtsR^{S61A}-GFP fusion was also diffusely distributed in the cell cytoplasm (Fig. 7a, lower panels). No ClpV2-mCherry punctae were observed in the strain expressing VtsR^{S61A}-GFP, likely because VtsR promotes expression of T6SS2 genes (Fig. 6d).

Phosphorylated VtsR led to elevated expression of T6SS2 genes and reduced expression of T6SS1 genes, a pattern similar to that observed with the constitutive activation of QS, in the mutant expressing LuxO^{D47E} (Fig. 2). This similarity suggested the possibility of a connection between VtsR phosphorylation and QS regulatory pathways. To begin to test if *vtsR* modulates the QS signaling pathway, we used qRT-PCR to measure transcript level of *luxR*, the master QS regulator in *V. alginolyticus* (Gu *et al.*, 2016) in mutants lacking VtsR or expressing different VtsR variants. In the *vtsR* mutant, *luxR* transcript levels were significantly reduced and this defect was complementable by expression of *vtsR* (Fig. 7b). Similar defects in *luxR* transcript levels were observed in strains expressing VtsR^{S61A} or VtsR^{S61D}, but not in the strain expressing VtsR^{S61E} (Fig. 7b), suggesting that phosphorylated VtsR promotes *luxR* expression. LuxR levels were also reduced in the *vtsR* mutant and strains expressing VtsR^{S61A} or VtsR^{S61D} (Fig. 7c). We also tested whether VtsR phosphorylation modulated production of Asp, the exotoxin regulated by LuxR and QS in *V. alginolyticus* (Gu *et al.*, 2016). Consistent with *luxR* transcript levels, the *vtsR* mutant and the strains expressing the phosphorylation site variants VtsR^{S61A} and VtsR^{S61D} had marked reductions in Asp production; whereas Asp levels were similar in the wt, VtsR^{S61E} and *vtsR*+pVtsR strains (Fig. 7c).

The regulation of LuxR and Asp production by phosphorylated VtsR suggested that *p*-VtsR might reside upstream of LuxR in the regulatory cascade and raised the possibility that we could bypass the defect in T6SS2 expression and activity in the *vtsR* mutant by ectopic expression of *luxR*. Indeed, ectopic expression of LuxR rescued Hcp2 production and secretion in the *vtsR* mutant and in the strain producing VtsR^{S61A} (Fig. 7d). Furthermore, ectopic expression of *luxR* rescued the killing defects observed in these two strains in the co-culture assays, when they were used as the predator strains (Fig. 7e).

Next, we investigated whether *vtsR*-mediated *luxR* and *asp* expression requires LuxO, which ordinarily represses expression of both these QS-controlled genes (Gu *et al.*, 2016). Levels of LuxR and Asp in the *vtsR luxO* double mutant were similar to those observed in the *luxO* mutant (Fig. 7f), consistent with the possibility that (phosphorylated) VtsR regulates LuxR expression in a LuxO-dependent fashion. Furthermore, overexpression of VtsR, LuxO, and LuxO^{D47A} in the *vtsR luxO* strain did not significantly alter the expression of LuxR or Asp, but overexpression of the phosphomimetic LuxO^{D47E} markedly decreased

their levels (Fig. 7f). Together, these observations suggest that VtsR modulates LuxR production by acting on or upstream of LuxO in the QS cascade, but additional experiments are required to define exactly how phosphorylated VtsR controls QS regulatory pathways. Regardless of the precise mechanism by which VtsR modulates QS signaling, these findings reveal that by elevating *luxR* expression, phosphorylated VtsR modifies how QS controls T6SS gene expression.

We also investigated whether VtsR modulation of LuxR expression requires the assembly of a functional T6SS2. To address this issue, the production of LuxR and Hcp2 secretion was monitored in the following strain backgrounds: *dotU2*, *DotU2^{T4AT28A}*, and *ppkA2* which are unable to assemble a functional T6SS2 and thus defective at T6SS2 secretion (Fig. 3), and *hcp2* without the secretory tube, that produce either VtsR or VtsR^{S61A} or VtsR^{S61E}. Expression of VtsR or VtsR^{S61E} was sufficient to enable LuxR production in all the mutant backgrounds; in contrast, no LuxR was detectable in any of these backgrounds when VtsR^{S61A} was expressed (Fig. 7g). Together, these observations are consistent with the idea that T6SS2 production/assembly or secretory activity is not required for VtsR to promote LuxR expression; instead phosphorylation of VtsR Ser 61 by PpkA2 appears to be sufficient for VtsR to enable LuxR production. The observation that the phosphomimetic VtsR^{S61E} variant bypassed the PpkA2 phosphorylation cascade and enabled LuxR production supported this hypothesis (Fig. 7g). Since *vtsR* can support expression of *luxR* even in the absence of *ppkA2* (Fig. 7g), we speculate that VtsR may be the substrate for an additional as yet unidentified kinase(s) in addition to PpkA2. Furthermore, none of the strains tested, including the *ppkA2* mutant expressing the constitutively active VtsR variant, secreted Hcp2 (Fig. 7g). Collectively, these data reveal that both VtsR activation as well as PpkA2 phosphorylation pathways are essential for T6SS2 function.

Discussion

Here, by using a phosphoproteomic screen to define the substrates of the PpkA2 kinase, a known regulator of T6SS, we uncovered a new regulatory circuit that entwines QS and T6SS in the marine pathogen *V. alginolyticus*. Our findings suggest that the phosphorylation cascade mediated by PpkA2 controls the firing of T6SS2 by phosphorylation of DotU2, thereby enabling its interaction with Fha2, a similar fashion as described in *A. tumefaciens* (Lin *et al.*, 2014). In addition, in *V. alginolyticus*, we found that this cascade also regulates expression of LuxR, a key QS transcription factor governing expression of both T6SS1 and T6SS2 as well QS-related genes (Fig. 8). Thus, stimuli that trigger PpkA2 autophosphorylation likely trigger both T6SS2 secretion as well as expression of genes encoding this secretion system. PpkA2-dependent phosphorylation of VtsR, the product of EPGS_00471 (which previously had no known function), serves as the bridge linking QS and T6SS expression in *V. alginolyticus* EPGS. This circuit establishes the basis for regulatory cross-talk in the control of T6S and QS. Presumably, this regulatory scheme also applies to the other vibrios that encode VtsR homologues (Supporting information Fig. S8a).

There is accumulating evidence that even closely related organisms can have distinct regulatory schemes governing their T6SSs. For example, Salomon *et al.* (2015) showed that *V. alginolyticus* and *V. parahaemolyticus*, two related vibrios that share similar habitats,

regulate their T6SSs (which are similar in terms of gene content and organization) in distinct fashions. Similarly, our work reveals that *V. alginolyticus* EPGS and 12G01 (Salomon *et al.*, 2015) activate their respective T6SSs differently. Both T6SS1 and T6SS2 appear to be active in high salt conditions (Salomon *et al.*, 2015) in 12G01, whereas only T6SS2 but not T6SS1 is active in high salt condition (LBS agar) in EPGS. Moreover, our observations revealed that the regulation of EPGS' two T6SS by QS differs; T6SS1 was repressed by QS while T6SS2 expression was activated by QS (Fig. 2). Presumably, the opposite effects of QS input on expression of T6SS1 and T6SS2 enable the bacterium to use its two T6SSs to adapt and thrive in different conditions, such as in high cell density biofilms or low cell density planktonic conditions. Another potential advantage of deploying T6SS1 and T6SS2 at different times is to avoid unnecessary crosstalk between the two T6SSs.

V. alginolyticus T6SS1/2 are parts of a subset of T6SS-encoding bacterial species that utilize a PpkA serine threonine kinase (Mougous *et al.*, 2007; Boyer *et al.*, 2009; Lin *et al.*, 2014) to relay complex environmental cues into a signaling cascade leading to activation of T6S. *V. alginolyticus* appears to lack counterparts of the TagQRST gene products that are thought to act upstream of PpkA in *P. aeruginosa*, to detect the cues that trigger PpkA autophosphorylation and T6S activation (Hsu *et al.*, 2009; Silverman *et al.*, 2011; Casabona *et al.*, 2013). Whether *V. alginolyticus* encodes functional analogues of these upstream components of the signaling cascade requires additional investigation. The first target of PpkA2 phosphorylation is itself in EPGS. Interestingly, T59 is not the site of autophosphorylation in *P. aeruginosa* PpkA, where residues T158 and T161 are phosphophorylated (Hsu *et al.*, 2009) (Supporting information Fig. S6), raising the possibility that PpkA2 might not function in the same fashion as other PpkA homologs (Mougous *et al.*, 2007; Hsu *et al.*, 2009; Lin *et al.*, 2014). The downstream T6SS target of phosphorylated PpkA2 in *V. alginolyticus*, DotU2, is the same as seen in *A. tumefaciens*, and as in *A. tumefaciens* phosphorylation of DotU2 enables its interaction with Fha (Lin *et al.*, 2014). Intriguingly, our unbiased phosphoproteomics also led to the identification of 6 additional proteins that are not the products of T6SS2 genes (Table 1) and are likely to be directly or indirectly phosphorylated by PpkA2. One of these gene products, VtsR proved to be critical for control of T6SS2 expression and killing (Fig. 6), while the other 5 have no obvious connection to T6SS2 regulation or function. It is possible that PpkA2 phosphorylation of these gene products coordinates their activity with T6S activity; alternatively, their potential regulation by PpkA2 phosphorylation is not related to T6S. Further investigation of the physiological consequences of PpkA2 phosphorylation of these substrates is warranted. Overall, it is fascinating to consider the conservation/divergence of the inputs that govern PpkA phosphorylation, its substrates and the pathways it controls, in the diverse bacteria that encode this regulatory kinase.

It is important to note that although our phosphoproteomic screen revealed polypeptides whose phosphorylation is dependent on PpkA2, it does not establish that these polypeptides are directly phosphorylated by PpkA2. Since PpkA2 and DotU2 homologues are known to be directly phosphorylated by PpkA in *P. aeruginosa* and *A. tumefaciens* (Hsu *et al.*, 2009; Lin *et al.*, 2014), it is likely that these two proteins are direct targets of PpkA2; however, the other polypeptide targets of PpkA2 phosphorylation we identified might not be directly phosphorylated by this kinase. For example, there could be an intermediate PpkA2 substrate

that phosphorylates VtsR. Arguing against this possibility is our finding that individual deletion of the five additional PpkA2 substrates did not diminish T6SS2 killing (Fig. 4a). VtsR phosphorylation seems to be independent of PpkA2 phosphorylation of DotU2 since deletion of *dotU2*, or its substitution with a phosphorylation-deficient variant *dotU2*^{T4AT28A} did not prevent VtsR regulation of LuxR (Fig. 7g). Additional studies are also required to define the mechanism(s) by which VtsR promotes expression of *luxR*; e.g. potentially by interacting with and inhibit LuxO activity (Fig. 7f).

Collectively, our data provides new insights into the complexity of T6SS regulation and its coordination with other cell processes. Multiple levels of inter-connected regulatory mechanisms, including QS and PpkA2 phosphorylation, control the activation of T6SS2. Phosphoproteomics proved a powerful tool to uncover new aspects of T6SS regulation and led to the discovery of the regulatory interplay controlling both QS and T6SS and suggesting the possibility of the coordination between T6S and non-T6SS processes. Ultimately, the complex coordination of QS and T6SS presumably enhances the overall competitive fitness of vibrios for thriving in marine environments (Weber *et al.*, 2009; Borgeaud *et al.*, 2015; Majerczyk *et al.*, 2016). Finally, by additional understanding of the regulation of T6SS activity in *V. alginolyticus*, we may be able to develop strategies, such as using chemicals that affect T6SS activity or interfere with QS, to reduce the competitive fitness of the bacterium and effectively control the pathogen which plagues the aquaculture industry.

Experimental procedures

Bacterial stains, plasmids and culture conditions

The strains and plasmids used in this study were listed in Supporting information Table S4. The *V. alginolyticus* strains were cultured in LBS broth (Luria-Bertani broth containing 3% sodium chloride) at 30°C. *E. coli* and *Edwardsiella* bacteria were cultured in LB broth (Luria-Bertani broth containing 1% sodium chloride) at 37°C. When necessary, media was supplemented with carbenicillin (100 µg ml⁻¹), chloramphenicol (25 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹), kanamycin (50 µg ml⁻¹), or L-arabinose (0.2 mg ml⁻¹). Overlap PCR was used to generate DNA fragments for creation of in-frame deletions or amino acid substitutions; these fragments were then cloned into the R6K-based suicide vector pDM4. The in-frame deletion mutants or site-directed mutants were generated as previously described (Sheng *et al.*, 2013). Complementation was achieved by amplifying the desired open reading frame (ORF) and cloning it into a mobilizable version of pBAD33 (Gu *et al.*, 2016), which was then conjugated into the desired strain. To construct over-expression strains for protein purification, fragments containing ORF regions were cloned into pET28a, and then transferred into BL21 (DE3). Proteins purification was as previously described (Gu *et al.*, 2016).

Fluorescence microscopy

Cells were harvested from LBS agar plates and washed with phosphate-buffered saline (pH 7.2) containing 3% NaCl. Cell suspensions were then spotted on the agarose pads (1.5% agarose with 10% LBS) with Gene Frame (Thermo Scientific). Images were acquired with a Nikon Eclipse Ti microscope equipped with 100× objective.

Bacterial killing assay

Overnight cultures were used for the interbacterial killing assays. Cultures were first normalized to $OD_{600}=0.5$, and then predator and prey cells were mixed at a ratio of 4:1. Twenty five μ l of these mixtures were spotted on LBS agar plates and then co-cultured at 30°C for 4 h. CFU of the strains in the mixtures spotted on LBS agar plates at $t = 0$ were determined by plating 10-fold serial dilutions on appropriate plates. After 4 h, bacterial spots were collected from LBS agar plates and the CFU of the surviving predator and prey were determined. An empty pBAD33 or pRK415 plasmid was used to render *E. coli* and *V. alginolyticus* resistant to chloramphenicol or tetracycline respectively.

Quantitative real-time reverse transcription PCR (qRT-PCR)

V. alginolyticus strains were harvested from LBS agar plates after 4 h of growth, and total RNA was isolated and then treated with DNaseI (Promega, Madison, WI, USA). One μ g total RNA was used to generate cDNA with reverse transcriptase (Toyobo, Tsuruga, Japan) and 6-mer random primers. qRT-PCR assays were performed on at least three independent experiments, each in triplicate by Applied Biosystems 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA), and transcript levels were normalized to *gyrB* in each sample using the C_T method as previously described (Gu *et al.*, 2016). The primers for qRT-PCR (Supporting information Table S5) were designed using the NCBI primer pick tool with predicted product sizes ranging between 100–200 bp.

Immunoblot analysis

Bacteria were harvested from LBS agar plates and suspended in ddH₂O to normalize cell densities. Then, the samples were added to loading buffer and boiled for 10 min. A volume of 15 μ l of each sample was loaded onto 12% denaturing polyacrylamide gels. The proteins were resolved by electrophoresis and then transferred to PVDF membranes (Millipore). The membranes were blocked in 5% milk solution, then incubated with a 1:2,000 dilution of rabbit antibodies raised against Hcp1, Hcp2, LuxR, Asp, or VtsR (GL Peptide Ltd., Shanghai, China or GenScript Nanjing, China). After washing with phosphate-buffered saline-0.5% Tween 20 (PBST), the membranes were incubated with a 1:2,000 dilution of a horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The same samples were also incubated with a 1:5,000 dilution of mouse monoclonal anti-RNA polymerase alpha subunit antibodies (RNAP alpha) (4RA2 antibody; Santa Cruz Biotechnology), followed by incubation with a 1:2,000 dilution of anti-mouse peroxidase-conjugated IgG secondary antibody (Sigma). Finally, the blots were visualized using ECL reagent (Thermo Fisher Scientific Inc., Waltham, MA USA).

Mass spectrometry analysis of the phosphoproteomes

The phosphoproteomic analyses were carried out by the Beijing Genomics Institute (BGI Shenzhen, Guangdong, China). The wt and *ppkA2* strains were grown on LBS agar plates for 4 h at 30°C. Bacterial cells were harvested from the LBS plates by washing with LBS and then collected by centrifugation at 5,000 *g* at 4 °C for 20 min. The cell suspensions were washed with lysis buffer containing 1 mM PMSF, 2 mM EDTA and 10 mM DTT. Bacterial cells were sonicated for 15 min on ice and lysates were cleared by centrifugation at 25,000 *g*

at 4 °C for 20 min. The lysates were treated with 10 mM DTT at 56 °C for 60 min and then alkylated by addition of 55 mM IAM for 45 min in the darkroom and then precipitated by cold acetone. The precipitates were dissolved in TEAB by ultrasonication for 15 min, and then cleared by centrifugation at 25,000 *g* at 4 °C for 20 min. The protein concentrations were determined using a Bradford kit (Pierce) and treated with trypsin. The digest was desalted using Strata × C₁₈ column and then vacuum-dried. One milligram of dry peptides were dissolved in 65% ACN, 2% TFA solution, and saturated by glutamic acid (20 mg ml⁻¹, pH 2.0–2.5). Phosphopeptides were enriched using TiO₂ chromatography (GL Science, Saitama, Japan). The phosphopeptides were subjected to the LC-20AD (Shimadzu, Japan) followed by mass spectrometry with a Triple TOF 5600 (AB SCIEX, Concord, ON) equipped with an ion source (AB SCIEX, Concord, ON) and radiator (New Objectives, Woburn, MA). The raw data were processed using the search engine MASCOT (version 2.3.02) with the following parameters: peptide mass tolerance, 0.05 Da; MS/MS ion mass tolerance, 0.1 Da; Gln->pyro-Glu (N-term Q), Oxidation (M) and Phospho (S/T/Y) was set as variable modifications. Carbamidomethyl (C) as fixed modifications, and two missed cleavages were allowed. Hits were considered confident if they had a localization probability of > 0.75 and their peptide ion scores exceeded the value of 30 (the threshold score suggested by MASCOT for confident matching of a single peptide sequence at *P* < 0.001).

Phos-tag SDS-PAGE analysis

The Phos-tag SDS-PAGE analysis was performed according to the user manual for Phos-tag Acrylamide AAL-107 (Wako Pure Chemical Industries, Osaka, Japan) (Kinoshita *et al.*, 2006). Protein samples were separated on 8% polyacrylamide gels containing 375 mM Tris-HCl (pH 8.8), 100 mM Phos-tag Acrylamide AAL-107, and 200 mM MnCl₂. After electrophoresis, Phos-tag gels were washed with transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) containing 1 mM EDTA for 15 min with gentle shaking followed by a second wash in transfer buffer without EDTA for 15 min. The gels were then transferred to PVDF membranes with a submarine blotting apparatus.

Pull-down assays

V. alginolyticus cells were collected from LBS agar plates in LBS, followed by washing three times in lysis buffer (20 mM Tris-HCl, pH 7.4; 150 mM NaCl) and resuspended in the same buffer with 1% TritonX-100, 100 µg ml⁻¹ PMSF and Phos-stop (Roche). Then, the suspensions were sonicated on ice and then centrifuged twice at 12,000 *g* for 20 min at 4°C, and the soluble fraction were held on ice, until being added to resin-bound purified proteins. The purified Fha2-His6 or Fha2^{R36AS52A}-His6 protein were added to Dynabeads for His-Tag (Life Technologies). After 4 h of incubation at 4°C, the resins were washed 3 times with both washing buffer II (50 mM Tris-HCl, pH 7.4; 150 mM NaCl) and washing buffer III (100 mM Tris-HCl, pH 7.4; 750 mM NaCl) containing 1% TritonX-100, 100 µg ml⁻¹ PMSF and Phos-stop. Then the soluble fractions (described above) were added and incubated at 4°C overnight; then, the beads were washed 3 times with both washing buffer II and washing buffer III containing 1% TritonX-100, 100 µg ml⁻¹ PMSF and Phos-stop. The Dynabeads-bound proteins were then analyzed using western blots.

Statistical analysis

The GraphPad Prism (version 5) was used to perform the statistical analyses. To compare gene expression or CFU between the groups, a two-tail Student's unpaired *t*-test was used. A value of $P < 0.01$ was considered significant for competition assays and qRT-PCR analysis. *, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$; NS, non-significant ($P > 0.01$).

V. alginolyticus genome accession number

The draft genome sequence of EPGS has been deposit at GenBank under accession number of GCA_001273715.1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Originality-Significance Statement

All the data and related materials are our original research, and have not been previously published and have not been submitted for publication elsewhere while under consideration.

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Fig. 2. QS regulates EPGS' two T6SS systems in distinct fashions.

(a) qRT-PCR analysis of transcript levels of indicated T6SS1 or T6SS2 related genes in wt and $luxO^{D47E}$ and $luxO^{D47A}$ mutant strains, which simulate states that are locked into low or high cell densities, respectively. Expression in the mutants is shown relative to that observed in the wt, which is set to 1. The experiments were repeated three times and the results show the mean value of the triplicates.

(b) Western blot analysis of Hcp1 and Hcp2 expression and secretion in EPGS, and the $luxO^{D47A}$ and $luxO^{D47E}$ mutants. RNAP was used as the loading control for the cell pellets.

(c) CFU counts of *E. coli* before ($t=0$) and after 4 h ($t=4$) co-culture *E. coli* with wt, and $luxR$ or $luxO$ EPGS mutants.

Fig. 3. PpkA2 regulates T6SS2 secretion and killing activity.

(a) Western blot analysis of Hcp2 production and secretion in EPGS, *hcp2*, and *ppkA2* deletion mutants, and in a *ppkA2*+pPpkA2 strain complementing the *ppkA2* deletion. RNAP was used as the loading control for the cell pellets.

(b) CFU counts of *E. coli* before ($t = 0$) and after 4 h ($t = 4$) co-culture with the indicated *V. alginolyticus* predator strains.

Fig. 4. Phosphorylation of PpkA2 threonine-59 (pT59) is critical for T6SS2 activity.

(a) CFU of *E. coli* before ($t = 0$) and after 4 h ($t = 4$) co-culture with the indicated *V. alginolyticus* predator strains.

(b) CFU counts of *E. coli* before ($t = 0$) and after 4 h ($t = 4$) co-culture with the indicated *V. alginolyticus* predator strains.

(c) Western blot analysis of Hcp2 expression and secretion in the EPGS wt and the indicated *ppkA2* mutants; *ppkA2*^{D224AN229A} carries D224A and N229A substitutions within the kinase's putative magnesium binding loop. RNAP was used as the loading control for the cell pellets.

Fig. 5. DotU2 phosphorylation at Thr4 and Thr28 enable its binding to Fha2 and T6SS2 activity.

(a) Detection of the phosphorylation of DotU2-Flag or the related phosphorylation site mutants on a Phos-tag gel. Immunoblots were used to analyze the same lysate volumes with a Flag specific antibody. SDS-PAGE without Phos-tag is shown as a control.

(b) Western blot analyses of Hcp2 expression and secretion in EPGS, *dotU2*, *fha2* and complement strains. The *dotU2* mutant expressing DotU2 or the DotU2 T4A, T28A, or T4A T28A substitution variants, and the *fha2* mutant expressing Fha2, as well as a strain containing a chromosomal *fha2* R36A S52A substitution mutant are also included. RNAP was used as the loading control for the cell pellets.

(c) Pull-down analyses of DotU2- Fha2 interaction using purified Fha2-His6 protein and *p*-DotU2-Flag from cell lysates. The Fha2-His6 protein was first mixed with the His-beads and then cell lysates containing *p*-DotU2-Flag (or the mutant DotU2-Flag) proteins were added. After elution, the presence of DotU2-Flag (or the mutant DotU2-Flag proteins) and Fha2-His6 were detected with specific antibodies.

(d) CFU counts of *E. coli* before (*t* = 0) and after 4 h (*t* = 4) co-culture with the indicated *V. alginolyticus* predator strains.

Fig. 6. Phosphorylated VtsR promotes T6SS2 gene expression.

(a) Detection of the phosphorylation of VtsR-Flag or its phosphorylation site mutants on a Phos-tag gel. Immunoblots were used to analyze the same lysate volumes with a Flag specific antibody. SDS-PAGE without Phos-tag is shown as a control.

(b) qRT-PCR analysis of *hcp1* and *hcp2* transcript levels in the *vtsR*, *vtsR^{S61A}*, *vtsR^{S61D}* and *vtsR^{S61E}* and complemented strain (*vtsR+pVtsR*) backgrounds; transcript levels are normalized to those found in wt EPGS.

(c) Western blot analysis of Hcp1 and Hcp2 production and secretion in the wt, *vtsR*, *vtsR^{S61A}*, *vtsR^{S61D}*, *vtsR^{S61E}* and *vtsR+pVtsR* strains. RNAP was used as the loading control for the cell pellets.

- (d) qRT-PCR analysis of transcript levels of *icmF1*, *clpV1*, *icmF2*, *clpV2* in *vtSR* *vtSR*^{S61A}, *vtSR*^{S61E}, *vtSR*+pVtsR strain backgrounds; an unpaired two-tailed *t*-test was used to compare the transcript levels of the indicated genes to levels found in the *vtSR* strain.
- (e) CFU counts of *E. coli* before ($t = 0$) and after 4 h ($t = 4$) co-culture with the indicated *V. alginolyticus* predator strains.

Fig. 7. VtsR controls T6SS2 activity by regulating QS.

(a) Detection of VtsR-GFP (green) and ClpV2-mCherry (red) by fluorescence microscopy in indicated *V. alginolyticus* genetic backgrounds. The strains were cultured at a *V. alginolyticus* to *E. coli* of 4:1 and spotted onto LBS plate for 4 h at 30 °C. Cells were visualized after 4 h coincubation. The scale bar represents 5 μm .

(b) qRT-PCR analysis of transcript levels of *luxR* in wt, *vtsR* or *vtsR* with substitutions of phosphorylation site (*vtsR^{S61A}*, *vtsR^{S61D}* and *vtsR^{S61E}*) and complemented strain *vtsR⁺*.

(c) Western blot analysis of LuxR and Asp production in the same strains as in (b). RNAP was used as the loading control for the cell pellets.

- (d) Western blot analysis of Hcp2 production and secretion in the *vtsR* and *vtsR*^{S61A} strains, harboring a plasmid-borne arabinose inducible *luxR* or the empty vector. RNAP was used as the loading control for the cell pellets.
- (e) CFU counts of *E. coli* before ($t = 0$) and after 4 h ($t = 4$) co-culture with the indicated *V. alginolyticus* predator strains with (+) or without (–) the presence of arabinose, pLuxR or the empty vector.
- (f) Western blot analysis of LuxR and Asp production in the indicated strains grown on LBS agar. RNAP was used as the loading control for the cell pellets.
- (g) Western blot analysis of LuxR, Hcp2 and VtsR production in the indicated strain backgrounds with expression of VtsR or VtsR^{S61A}, VtsR^{S61D}. RNAP was used as the loading control for the cell pellets.

Fig. 8. Model outlining the interplay of QS and T6SS mediated by the PpkA2 phosphorylation cascade.

Environmental cues, such as contact with other bacteria trigger PpkA2 autophosphorylation. *p*-PpkA2 transfers its phosphate group to DotU2, enabling it to interact with Fha2; this complex can activate RhsP1 effector secretion, mediating T6SS2 killing of target bacteria. *p*-PpkA2 can also lead to the phosphorylation of VtsR and *p*-VtsR promotes expression of T6SS2 genes by stimulating *luxR* expression, potentially through inhibition of LuxO activity. *p*-PpkA2 also phosphorylates additional proteins, which have no known association with T6SS, possibly regulating additional phenotypes. QS signaling, phosphorylation activation of T6S, as well as non-T6SS functions related pathways are boxed with dotted lines. Activation of target gene expression or phosphorylation cascades is depicted with arrowed lines and the repression of gene expression with bar-ended lines.

Table 1

Protein phosphorylations present in the EPGS phosphoproteome but absent in that of the *ppkA2* mutant

prot_accession	P-value	m/z	Score	Sequence	Function	Killing ability ^a
EPGS_02037	5.10E-06	498.5762	61.5	MEQT(ph)IVKPTPGGR	T6SS protein DotU2	Absent
	3.90E-08	578.2853	83.06	SLDNT(ph)VVISK		
EPGS_02041	1.30E-06	484.2467	67.19	LSQKENT(ph)PLVVK	T6SS protein PpkA2	Absent
EPGS_00313	4.60E-04	686.314	41.68	MTSENKKT(ph)VTPA	Phasin protein	Intact
	4.00E-04	516.1992	38.55	MYT(ph)DFEK		
EPGS_01337	4.90E-08	658.3173	82.67	ADPT(ph)KQPVVIEHQSDTK	Hypothetical protein	Intact
EPGS_01659	4.50E-04	477.2555	62.32	AT(ph)VTAALVK	Elongation factor Ts	Undetermined
EPGS_02312	1.90E-06	526.2613	82.68	ANVTKT(ph)DHQLEKL	Oxygen tolerance protein	Intact
EPGS_02626	1.70E-04	526.7774	46.28	LLQTT(ph)QLR	T2SS associated protein	Intact
EPGS_00471	1.30E-06	577.2957	68.1	ISQIGLS(ph)LDK	Hypothetical protein	Absent

^aDeletion mutants of the corresponding gene were tested in the co-culture assay with *E. coli* as prey. When killing capacity of the mutants was similar to the *hcp2* mutant, killing is called 'Absent'; when killing capacity was similar to wt EPGS, killing is called 'Intact'. See also Figs. 4A and S4C.