

1 **Bile acids activate the antibacterial T6SS1 in the gut pathogen *Vibrio***  
2 ***parahaemolyticus***

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9

10 **Abstract**

11 The marine bacterium *Vibrio parahaemolyticus* is a major cause of seafood-borne  
12 gastroenteritis in humans and of acute hepatopancreatic necrosis disease in shrimp. Bile acids,  
13 produced by the host and modified into secondary bile acids by commensal bacteria in the  
14 gastrointestinal tract, induce the virulence factors leading to disease in humans and shrimp.  
15 Here, we show that secondary bile acids also activate this pathogen's type VI secretion system  
16 1 (T6SS1), a toxin-delivery apparatus mediating interbacterial competition. This finding implies  
17 that *Vibrio parahaemolyticus* exploits secondary bile acids to activate its virulence factors and  
18 identify the presence of commensal bacteria that it needs to outcompete in order to colonize the  
19 host.

20

21 **Importance**

22 Bacterial pathogens often manipulate their host and cause disease by secreting toxic proteins.  
23 However, to successfully colonize a host, they must also remove commensal bacteria that  
24 reside in it and may compete with them over resources. Here, we find that the same host-  
25 derived molecules that activate the secreted virulence toxins in a gut bacterial pathogen, *Vibrio*  
26 *parahaemolyticus*, also activate an antibacterial toxin delivery system that targets such  
27 commensal bacteria. These findings suggest that a pathogen can use one cue to launch a  
28 coordinated, trans-kingdom attack that enables it to colonize a host.

29

30 **Keywords**

31 Regulation, deoxycholate, secondary bile acid, type VI secretion system, *Vibrio*  
32 *parahaemolyticus*

## 33 Observation

34 *Vibrio parahaemolyticus* (*Vpara*) is a Gram-negative bacterium common in marine and estuarine  
35 environments. It is a leading cause of seafood-borne gastroenteritis (1) and acute  
36 hepatopancreatic necrosis disease (AHPND), which globally affects the shrimp farming industry  
37 (2). *Vpara* virulence in mammals is dependent on a toxin delivery apparatus named type III  
38 secretion system 2 (T3SS2) (3, 4), which is encoded within a horizontally acquired genomic  
39 island, VPal-7 (5), in clinical isolates. AHPND in shrimp is caused by the PirAB binary toxin,  
40 encoded on a conjugative plasmid (6).

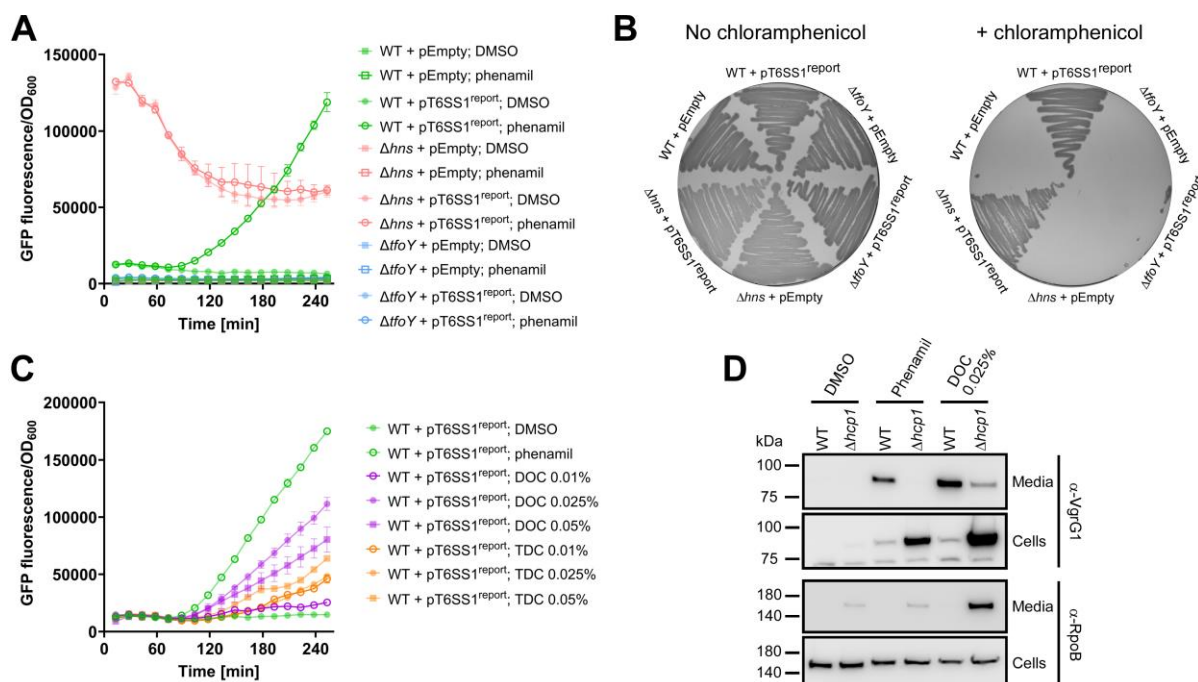
41 Although mutually exclusive in *Vpara* genomes (7), both T3SS2 and PirAB are induced by bile  
42 acids, an important component in the gastrointestinal tract of animals promoting lipid absorption,  
43 protein cleavage, and antimicrobial toxicity (8–11). Whereas bile-mediated activation of T3SS2  
44 occurs via the VtrA/C receptor, which is also encoded within VPal-7 (10, 11), AHPND-causing  
45 strains lack VtrA/C (7), and it is unclear how they sense bile acids to activate PirAB (9).

46 We previously reported that pathogenic *Vpara* strains employ a toxin delivery apparatus, named  
47 type VI secretion system 1 (T6SS1), to outcompete rival bacteria (12, 13). We reasoned that  
48 T6SS1 plays an indirect role during host infection by enabling *Vpara* to combat gut commensal  
49 bacteria and colonize the host. Interestingly, another gastrointestinal pathogen, *Salmonella*  
50 Typhimurium, was shown to use the host bile acids as a cue to activate its antibacterial T6SS  
51 and establish a niche inside the host gut (14). Although environmental factors that regulate the  
52 *Vpara* T6SS1 have been investigated in several *Vpara* strains (12, 13), little is known about  
53 *Vpara* T6SS regulation by host factors. Nevertheless, a subset of *Vpara* T6SS1 genes was  
54 found to be transcriptionally induced during infection of an infant rabbit model (15), suggesting  
55 that host gut factors can regulate this system.

56 The abovementioned observations led us to hypothesize that *Vpara* uses bile sensing to induce  
57 its antibacterial T6SS1 in addition to its major virulence factors, thus gaining a competitive  
58 advantage during host infection. To test this hypothesis, we first set out to investigate the effect  
59 of bile acids on T6SS1 transcriptional regulation. Based on our previous delineation of the  
60 *Vpara* T6SS1 regulatory cascade in the type strain RIMD 2210633 (16), we constructed a  
61 reporter plasmid (pT6SS1<sup>report</sup>) in which the predicted promoter region of *vp1400*, the first gene  
62 in an operon encoding several conserved T6SS1 structural components, is fused to a  
63 promoterless *cat* and *gfp* reporter cassette. To verify that this reporter reliably represents  
64 T6SS1, we monitored GFP expression in *Vpara* strain RIMD 2210633 under conditions known  
65 to induce or repress T6SS1, in defined M9 media containing 3% NaCl (wt/vol) at 30°C. As  
66 expected, GFP fluorescence was detected when *hns*, encoding a histone-like nucleoid  
67 structuring protein previously shown to repress T6SS1 (17), was deleted (**Fig. 1A**).

68 Fluorescence was also detected upon the addition of phenamil, an inhibitor of the polar flagella  
69 motor shown to induce T6SS1 by mimicking surface sensing activation (12), to the media of  
70 wild-type *Vpara* (**Fig. 1A**). In contrast, phenamil addition did not induce GFP fluorescence in a  
71 strain in which we deleted the T6SS1 positive regulator, *tfoY* (18). Similar results were obtained  
72 when we monitored the expression of the *cat* reporter gene, providing chloramphenicol  
73 resistance, as growth on agar plates containing chloramphenicol (**Fig. 1B**). Therefore, we  
74 conclude that pT6SS1<sup>report</sup> reliably represents the status of T6SS1 activation.

75



**Fig. 1. Bile acids activate *Vibrio parahaemolyticus* T6SS1.** **A, C)** Fluorescence intensity over time in the indicated *V. parahaemolyticus* RIMD 2210633 strains carrying an empty plasmid (pEmpty) or a plasmid containing a *gfp* and *cat* reporter cassette fused to the promoter of *vp1400* (pT6SS1<sup>report</sup>), measured as GFP fluorescence to OD<sub>600</sub> readings (arbitrary units). Bacteria were grown at 30°C in M9 media supplemented with 3% (wt/vol) NaCl and kanamycin (250  $\mu$ g/mL), to maintain the plasmids. Where indicated, the media were supplemented with phenamil (20  $\mu$ M), DMSO (20% [vol/vol], added to the media as a control at the same volume of the phenamil solution), or the indicated concentrations of DOC or TDC (wt/vol). Data are shown as the mean  $\pm$  SD, n = 3 independent replicates. **B)** Growth of the indicated *V. parahaemolyticus* RIMD 2210633 strains at 30°C on MLB (LB with 3% [wt/vol] NaCl) agar plates containing kanamycin (250  $\mu$ g/mL) to maintain the plasmids, with or without chloramphenicol (10  $\mu$ g/mL) to monitor *cat* expression. **D)** Expression (cells) and secretion (media) of VgrG1 from the indicated *V. parahaemolyticus* RIMD 2210633 strains grown for 4 hours in M9 media supplemented with 3% (wt/vol) NaCl and either phenamil, DMSO (as detailed for A,C), or DOC at 30°C. RNA polymerase  $\beta$  subunit (RpoB) was used as a loading and lysis control. In A-D, results from a representative experiment out of at least three independent experiments are shown. WT, wild-type.

76 To investigate the effect of bile acids on T6SS1 transcription, we chose two secondary bile  
 77 acids, which are bile acids modified in the host gut by commensal bacteria (8):  
 78 taurodeoxycholate (TDC) and deoxycholate (DOC), which are strong and intermediate inducers  
 79 of the *Vpara* T3SS2, respectively (19). Remarkably, the addition of DOC to the growth media at  
 80 concentrations previously shown to activate T3SS2 (i.e., 0.025-0.05% [wt/vol]) (19) resulted in  
 81 induction of GFP fluorescence from the T6SS1 reporter to levels comparable to those of the  
 82 known inducer, phenamil (Fig. 1C). Surprisingly, TDC, which is a strong activator of T3SS2, had  
 83 only a mild effect on the T6SS1 reporter (Fig. 1C). Neither bile acid hampered *Vpara* growth at  
 84 these concentrations (Fig. S1). Moreover, neither DOC nor phenamil induced expression from  
 85 the promoter of a T6SS1-unrelated gene, *vpa1270*, thus indicating that the observed

86 phenotypes do not result from global effects on *Vpara* transcription or translation (Fig. S2).  
87 Taken together, these results suggest that bile acids induce T6SS1.

88 Next, we sought to determine whether the observed bile acid-induced T6SS1 transcriptional  
89 activation translates to the assembly of a functional T6SS1 apparatus. To this end, we  
90 investigated the effect of DOC on T6SS1 activity by monitoring the expression and secretion of  
91 the hallmark T6SS structural secreted protein, VgrG1 (20). In agreement with the results  
92 obtained for the transcriptional reporter, DOC induced VgrG1 expression and secretion to a  
93 level comparable to that of phenamil (Fig. 1D). Moreover, intracellular accumulation of VgrG1  
94 was evident upon inactivation of T6SS1 by deleting the conserved structural component Hcp1  
95 ( $\Delta hcp1$ ) (21). Notably, although inactivation of T6SS1 hampered the DOC-induced secretion of  
96 VgrG1 compared to the wild-type strain, a low level of VgrG1 signal was detected in the "media"  
97 fraction of the DOC-treated  $\Delta hcp1$  strain; this observation can be explained by cell lysis  
98 apparent in the  $\Delta hcp1$  strains (see the  $\alpha$ -RpoB panel in Fig. 1D), which was exacerbated in  
99 presence of DOC. These results demonstrate that bile acids activate *Vpara* T6SS1 in the clinical  
100 strain RIMD 2210633.

101

## 102 Conclusions

103 Our results suggest that *Vpara* use host-derived molecules, indicative of the presence of  
104 commensal bacteria, as a signal that activates virulence factors and antibacterial  
105 determinants playing a role in interbacterial competition. Therefore, bile acids may be  
106 exploited by *Vpara* to establish a niche inside the host. Future in-vivo competition assays  
107 using animal models are required to test this prediction. Notably, the bile acid receptor that  
108 regulates T6SS1 remains to be identified. Moreover, secondary bile acids were shown to  
109 repress the T6SS in another gut pathogen, *V. cholerae* (22), indicating that exploiting  
110 commensal bacteria-made molecules to activate antibacterial determinants in the host gut  
111 may not be a universal bacterial strategy.

112

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