Regulation of type III secretion system 1 gene expression in Vibrio parahaemolyticus is dependent on interactions between ExsA, ExsC and ExsD

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Vibrio parahaemolyticus ExsA is the transcriptional regulator for type III secretion system 1 (T3SS1) while ExsD blocks T3SS1 expression. Herein we show that deletion of exsc from V. parahaemolyticus blocked synthesis of T3SS1-dependent proteins under inducing conditions (contact with HeLa cells), while in trans complementation of the Δexsc strain with wild-type exsc restored protein synthesis. Under non-inducing conditions (Luria broth plus salt), in trans expression of exsc in a wild-type strain resulted in synthesis and secretion of T3SS1-dependent proteins. Deletion of exsc does not affect the synthesis of ExsA while expression of T3SS1 genes is independent of ExsC in the absence of ExsD. Co-expression of recombinant proteins with different antigenic tags demonstrated that ExsC binds ExsD and that the N-terminal amino acids of ExsC (positions 7 to 12) are required for binding. Co-expression and purification of antigenically tagged ExsA and ExsD demonstrated that ExsD directly binds ExsA and presumably prevents ExsA from binding promoter regions of T3SS1 genes. Collectively these data demonstrate that ExsD binds ExsA to block expression of T3SS1 genes, while ExsC binds ExsD to permit expression of T3SS1 genes. ExsA, ExsC and ExsD from V. parahaemolyticus appear to be functional orthologues of their Pseudomonas aeruginosa counterparts.

Introduction

Vibrio parahaemolyticus is a Gram-negative marine pathogen that can be transmitted to humans through consumption of contaminated fish and shellfish.¹⁻⁶ The main manifestation of V. parahaemolyticus infection is gastroenteritis with diarrhea, abdominal pain, vomiting, headache, fever and chills.⁶ V. parahaemolyticus-induced diarrhea is inflammatory with edema, congestion of blood vessels, hemorrhage and increased level of neutrophils in the lamina propria of the intestine.⁷ In contrast, V. cholerae causes non-inflammatory, secretory diarrhea resulting in severe and rapid dehydration and shock.⁸ In addition to gastroenteritis, V. parahaemolyticus also causes septicemia, particularly for individuals with preexisting liver disease.⁹¹⁰

Thermostable direct hemolysin (TDH) is a well-known exogenous toxin that is produced by clinical strains of V. parahaemolyticus.² Cell culture studies have shown that TDH is required to alter ion flux and to form pores in intestinal cell membranes.¹¹¹² Using a rabbit ileal loop model, Nishibuchi et al.¹³ demonstrated that fluid accumulation following infection with a tdh deletion strain was significantly reduced compared to the wild-type strain. Other studies showed that the pathogenicity of V. parahaemolyticus was also related to the adherence to human epithelial cells,¹⁴ production of TDH-related hemolysin (TRH)¹⁵ and vibrioferrin.¹⁶ Although our knowledge of V. parahaemolyticus pathogenesis continues to expand, a comprehensive understanding of the molecular mechanisms that are responsible for the inflammatory diarrhea has not been defined.

Type III secretion systems (T3SS) were first discovered in Yersinia¹⁷¹⁸ and it has been subsequently shown that many Gram-negative bacteria harbor T3Ss.¹⁹ A T3SS is composed of a basal body that spans the periplasmic space, a “needle” that extends from the bacterial membrane surface, and a translocator apparatus connecting the needle with the eukaryotic cell membrane.²⁰⁻²² This needle-like structure allows bacteria to inject effector proteins directly into host cells where they interfere with normal cell physiology leading to a variety cellular responses.²³⁻²⁵ The genome of V. parahaemolyticus encodes two T3Ss (T3SS1 and T3SS2).²⁶ T3SS1 is responsible for cytotoxicity while T3SS2 appears to contribute to enterotoxicity.²⁷⁻²⁸ Initial studies showed that T3SS1 of V. parahaemolyticus induced apoptosis,²⁹⁻³⁰ Later studies using the same strain showed that T3SS1 of V. parahaemolyticus induced autophagy³¹ but not apoptosis. Inhibition of autophagy does not block T3SS1-induced cytotoxicity against HeLa cells³² indicating that other mechanisms contribute to T3SS1-induced cell death. Using a different strain (NY-4),
The T3SS1 in *V. parahaemolyticus* may be regulated in a manner similar to the T3SS in *P. aeruginosa*. The T3SS1 in *V. parahaemolyticus* is activated by growing bacteria in Dulbecco’s modified Eagle’s medium (DMEM) or in contact with eukaryotic cells. The ExsA homologue in *V. parahaemolyticus* (Vp1699) is required for the positive regulation of T3SS1 by binding to a promoter motif that is different from Pseudomonas. In *W. parahaemolyticus* and *V. cholerae*, two T3SS systems are present. The T3SS1 in *V. parahaemolyticus* (Vp1656) is required for the synthesis of Vp1656 and Vp1659 upon V. *parahaemolyticus* infection of HeLa or Caco-2 cells. Wild-type (NY-4) (lanes 1 and 3) and ΔexsC (lanes 2 and 4) strains were used to infect HeLa (lanes 1 and 2) and Caco-2 (lanes 3 and 4) cells for 4 h. The whole-cell lysates of the infected samples were probed with polyclonal antibody against Vp1656 (upper), Vp1659 (middle) and for the DNA loading control DnaK (lower).

Figure 1. ExsC is required for the synthesis of Vp1656 and Vp1659 upon *V. parahaemolyticus* infection of HeLa or Caco-2 cells. Wild-type (NY-4) (lanes 1 and 3) and ΔexsC (lanes 2 and 4) strains were used to infect HeLa (lanes 1 and 2) and Caco-2 (lanes 3 and 4) cells for 4 h. The whole-cell lysates of the infected samples were probed with polyclonal antibody against Vp1656 (upper), Vp1659 (middle) and for the DNA loading control DnaK (lower).

We have shown that T3SS1 induces cell-death in HeLa and U937 cells in a manner consistent with oncysis. The interaction is characterized by pore formation in the membrane, presence of active Poly ADP ribose polymerase (PARP) and the process is caspase-independent. More importantly, addition of osmoprotectants reduces cytotoxicity against both HeLa and U937 cells, indicating that oncysis is, at least partially, the outcome of T3SS1-induced cytotoxicity.

We infected HeLa cells or Caco-2 cells with *V. parahaemolyticus* strain NY-4 (wild-type) and a ΔexsC deletion mutant and 4 hr after infection we collected the whole-cell lysates to examine the synthesis of two T3SS1 substrates (Vp1656 and Vp1659). As expected, Vp1656 and Vp1659 were synthesized by the complemented ΔexsC strain (Fig. 1, lanes 1 and 3), but neither protein was synthesized by the ΔexsC strain (Fig. 1, lanes 2 and 4). A chaperone protein, DnaK, was included as a loading control (Fig. 1, lower). These results indicate that for cell culture infections *exsC* is required for the expression of T3SS1 genes in *V. parahaemolyticus*.

Complementation of *exsC* restores wild-type phenotype. To exclude the possibility of polar effects from the *exsC* deletion, we expressed *exsC* in trans from the pMMB207 shuttle plasmid and analyzed cell lysate using western blots. Upon infection with HeLa cells, both Vp1656 (Fig. 2, upper) and Vp1659 (Fig. 2, middle) were synthesized by the complemented ΔexsC strain (Fig. 2, lane 8). Overexpression of *exsC* also induced synthesis of Vp1656 and Vp1659 when cells were grown in LB-S (Fig. 2, lanes 1, 5 and 6). A western blot for DnaK verified
that differences in band intensities for different conditions were not due to unequal protein loading on the gels (Fig. 2, lower).

Expression of \textit{exsC} in trans in wild-type strain activates secretion of Vp1656 and Vp1659 under non-inducing condition. To further determine if ExsC can activate the entire T3SS1, we determined if Vp1656 and Vp1659 were secreted into the supernatant by the wild-type strain when transformed with the \textit{exsC} plasmid and cultured in LB-S. As expected, both Vp1656 (Fig. 3, lane 1, upper) and Vp1659 (Fig. 3, lane 1, middle) were not present in the supernatant of NY-4 strain grown in LB-S. Both Vp1656 and Vp1659 were present in the supernatant of NY-4 strain transformed with a plasmid carrying \textit{exsC} (Fig. 3, lane 2), but were not present in supernatant of NY-4 strain transformed with a plasmid carrying the \textit{bla} gene (Fig. 3, lane 3) under LB-S growth condition. As positive controls, both the NY-4 strain overexpressing \textit{exsA} (Fig. 3, lane 4) and the \textit{ΔexsD} strain (Fig. 3, lane 5) secreted Vp1656 and Vp1659 into the supernatant. To ensure that the absence of Vp1656 and Vp1659 in the supernatant of NY-4 and NY-4:\textit{pbla} strains was not due the improper protein precipitation, we examined the presence of DnaK in the supernatant (DnaK is secreted independent of T3SS1 expression; data not shown). The results showed that an equal amount of DnaK was present in the supernatant of these strains (Fig. 3, lower). These results indicate that in trans expression of \textit{exsC} can not only activate the synthesis of Vp1656 and Vp1659 (Fig. 2), but this permits expression of a functional T3SS1 secretion apparatus.

Deletion of \textit{exsC} does not reduce the expression of \textit{exsA} under inducing conditions. Deletion of \textit{exsC} inhibits expression of T3SS1 genes under inducing conditions (Fig. 1) and previous studies showed that ExsA is a transcriptional factor required for the expression of T3SS1 genes.\textsuperscript{47} Therefore, we examined \textit{exsA} expression in wild-type (NY-4) and \textit{ΔexsC} strains under both inducing (infection) and non-inducing (LB-S) conditions after adding an HA tag at the 3’ terminus of \textit{exsA} (chromosomal). As expected, ExsA-HA was not detectable in NY-4 strain (western blot, anti-HA) when bacteria were grown in LB-S condition (Fig. 4, lane 1), but ExsA-HA was detected from the NY-4 strain under inducing conditions (Fig. 4, lane 2). An identical expression pattern was evident for the \textit{ΔexsC} strain (Fig. 4, lanes 3 and 4). This experiment shows that under inducing conditions ExsC is not required for ExsA synthesis.

Expression of T3SS1 genes in a \textit{ΔexsD} strain is independent of ExsC. T3SS1 genes are constitutively expressed in a \textit{ΔexsD} strain even when grown under non-inducing conditions (LB-S) and this constitutive expression requires ExsA.\textsuperscript{47} When we examined T3SS1 gene expression in a \textit{ΔexsD} background, we confirmed that ExsC is not required for the expression of T3SS1 genes (Fig. 5A and B). This result verifies that ExsC
is not directly required for transcription of T3SS1 genes and that in the absence of ExsD, ExsC has no direct influence on T3SS1 transcription or expression.

Concurrent expression shows that ExsC binds ExsD in vivo. ExsC is required for the expression of T3SS1 genes in a wild-type strain (Fig. 1), but it is not required for T3SS1 gene expression in an exsD deletion strain (Fig. 5), suggesting that regulation of T3SS1 by ExsC occurs indirectly via interactions with ExsD. Because ExsC binds ExsD in Pseudomonas, we employed concurrent expression experiments to determine if a similar interaction occurs in *V. parahaemolyticus*. ExsC and ExsD were tagged with 6xHis and HA antigens, respectively, and were overexpressed by plasmids pDM31 and pMMB207, respectively, in the NY-4 strain. Western blots using antibody against the 6xHis tag or HA tag confirmed that both ExsC (Fig. 6, lane 1, lower) and ExsD (Fig. 6, lane 1, upper) were present in whole-cell lysate of *V. parahaemolyticus*. Whole-cell lysates were passed through a Ni⁺ column, washed extensively, and eluted twice. ExsC-6xHis and ExsD-HA were present in both elution fractions (Fig. 6, lanes 2 and 4, lower), indicating that N-terminus of ExsC is required to bind ExsD. Alternatively, deletion of N-terminus disrupted the proper protein folding, leading to the inability of truncated ExsC to bind ExsD.

Binding of ExsC with ExsD is required for the expression of T3SS1 genes. Because N-terminal truncation of ExsC disrupted binding with ExsD, we determined if this produces a subsequent loss of T3SS1 expression (Fig. 2). As expected, both Vp1656 and Vp1659 were synthesized when GST-ExsC-His was produced in trans in NY-4 (LB-S; Fig. 8A, lane 2). In contrast, neither Vp1656 nor Vp1659 were synthesized when the truncated version of ExsC (GST_exsC_Δ5'-His) was produced (Fig. 8A, lane 1). A western blot using anti-His antibody confirmed that both full-length ExsC (GST_ExsC_His) and truncated ExsC (GST_exsC_Δ5'-His) fusions were produced in NY-4 strain (Fig. 8A, third). DnaK served as a loading control (fourth).

RT-PCR analysis showed that transcription of T3SS1 genes in the NY-4:pGST_exsC_His strain was greater than that in the NY-4:pGST_exsC_Δ5'-His when bacteria were grown in LB-S (Fig. 8B). SecY, a housekeeping gene, served as a positive control for the RT-PCR experiment.

Amino acids from 7 to 12 in ExsC are required for the interaction of ExsC with ExsD. To determine which part of N-terminus in ExsC is required to interact with ExsD, we constructed expression vectors for ExsC with only amino acid (Aa) 7 to Aa148 (GST_ExsC_7-148), Aa13 to Aa148 (GST_ExsC_13-148) or Aa27 to Aa148 (GST_ExsC_27-148). Each construct was expressed together with ExsD-HA in the NY-4 strain. All of the truncated ExsC proteins were present in the whole-cell lysates and elution fractions (Fig. 9, upper). ExsD was detected in the whole-cell lysate of all the strains (Fig. 9, lanes 1, 3, and 5). ExsD was only present in the elution of NY-4 strain with GST_ExsC_7-148 (Fig. 9, lane 2, lower), while absent in the elution of NY-4 strain with GST_ExsC_13-148 or GST_ExsC_27-148 (Fig. 9, lanes 4 and 6). Collectively, these results indicate that amino acids from position 7 to 12 are required for ExsC to bind ExsD.

GST-fused ExsC does not bind ExsA. Because expression of GST-fused ExsC permits transcription (Fig. 8B) and expression (Fig. 8A) of T3SS1 genes in LB-S, we needed to confirm that this does not involve binding between ExsC and ExsA. GST-fused ExsC (Fig. 10A, lower) and ExsA (Fig. 10A, upper) were present in the whole-cell lysate after growth in LB-S (Fig. 10A, lane 1), but only GST-fused ExsC was present in the elution (Fig. 10A, lane 2). These results indicate that GST-fused ExsC does not bind ExsA and therefore expression of T3SS1 genes does not involve a direct interaction between ExsC and ExsA.

ExsA binds ExsD. *Pseudomonas* ExsD interacts with ExsA to prevent its DNA binding activity. Previous efforts to test in vitro
binding between *V. parahaemolyticus* ExsD and ExsA were negative (data not shown) so for the present analysis we used co-expression and purification experiments to re-examine this potential interaction. His-tagged ExsA (Fig. 10B, lower) and HA-tagged ExsD (Fig. 10B, upper) were present in both the whole-cell lysate (Fig. 10B, lane 1) and elution fractions after passage over a nickel column (Fig. 10B, lane 2). These results indicate that ExsA binds ExsD in *V. parahaemolyticus* and presumably this prevents ExsA from binding T3SS1 promoter regions.

**Discussion**

In *Pseudomonas aeruginosa* ExsD negatively regulates expression of T3SS proteins by binding directly to ExsA\(^4\) and thereby blocking the ability of ExsA to bind the promoter regions of T3SS genes.\(^{45,48}\) Under limited calcium conditions ExsD is bound by another protein, ExsC, and this blocks the ExsD-ExsA interaction and permits transcription of T3SS genes.\(^{46}\) Although the conditions that are permissible for T3SS1 expression in *V. parahaemolyticus* probably do not mirror those required for T3SS expression in *P. aeruginosa*, the proximal means of controlling T3SS1 expression is very similar. At the broadest level, ExsA is a positive transcriptional regulator for T3SS1 genes\(^{47}\) (Fig. 11) and ExsA transcriptional activity is blocked when ExsD binds to ExsA\(^{48}\) (Fig. 11). This “anti-activator” function (\(\text{anti-activator}\)\(^{48}\) of ExsD is blocked when ExsC binds ExsD (Fig. 6).

Under non-inducing conditions, \(\text{exsD}\) is transcribed\(^{47}\) while \(\text{exsC}\) is not transcribed (data not shown), which would explain the lack of T3SS1 expression in LB-S, but this does not explain why T3SS1 genes are expressed under non-inducing conditions when \(\text{exsD}\) is deleted. It is possible that \(\text{exsA}\) is normally transcribed constitutively at a low level that is barely detectable by RT-PCR (Fig. 8B) and not detectable by western blot (Fig. 4), while expression is enhanced when other T3SS1 genes are expressed (although \(\text{exsA}\) is not autoregulated; Zhou et al. 2008). Under this scenario deletion of \(\text{exsD}\) is sufficient to permit upregulation of T3SS1 genes during growth in a non-inducing condition and enhanced transcription of \(\text{exsA}\) is promoted through a positive feedback with an unrecognized T3SS1 protein. One alternative is that exposure to LB-S media leads to a minimal increase in \(\text{exsA}\) transcription, but activity of newly synthesized ExsA is easily blocked by constitutive expression of ExsD. Another more complicated alternative would involve ExsD serving a dual function of blocking \(\text{exsA}\) transcription directly or indirectly while simultaneously binding ExsA and blocking ExsA promoter binding activity. We submit that the latter scenario is not plausible because ExsA is clearly expressed in the \(\text{exsC}\) deletion mutant (Fig. 4) where ExsD synthesis is presumably uninhibited but T3SS1 expression is blocked (Fig. 1).

To better characterize the role of ExsC in this process we first demonstrated that deletion of \(\text{exsC}\) blocks expression of two T3SS1 genes after contact with HeLa or Caco-2 cells (Fig. 1) indicating that ExsC is necessary for T3SS1 gene expression in addition to ExsA. Complementation with a wild-type \(\text{exsC}\) gene restored the ability of the \(\text{exsC}\) mutant to express T3SS1 genes after contact with HeLa cells indicating that deficiency in T3SS1 gene expression in the \(\text{exsC}\) mutant is not due to the polar effect of the gene deletion. In addition, expression of \(\text{exsC}\) in trans in wild-type or \(\text{exsC}\) mutant strains resulted in the constitutive expression of T3SS1 genes under inducing and non-inducing conditions (Fig. 2), indicating that the ability of ExsC to block
ExsD depends on the relative abundance of these two molecules. These results were similar to what is observed when ExsA is over-expressed under inducing or non-inducing conditions. ExsA is synthesized for both wild-type and ExsC mutant strains under inducing conditions, indicating that ExsC is not required for the synthesis of ExsA under inducing conditions (Fig. 4). These results indicate that downregulation of T3SS1 in the exsC deletion strain under inducing conditions is not caused directly by downregulation of transcriptional factor, ExsA. This is different from the observations for Pseudomonas, where deletion of ExsC significantly reduced the expression of ExsA. It is also possible that ExsC acts as a co-factor of ExsA and in the absence of ExsC, ExsA cannot activate the expression of T3SS1 genes under inducing condition. We did not, however, observe binding between ExsA and ExsC in V. parahaemolyticus (Fig. 10), implying that ExsC is not a direct partner protein of ExsA. We also showed that ExsC is not required for the transcription and expression of T3SS1 in the absence of ExsD (Fig. 5). ExsC is required for the expression of T3SS1 genes in wild-type strain, but not required for expression of T3SS1 gene in exsD mutant strain, indicating that ExsC regulates T3SS1 gene expression by blocking the inhibition mechanism of ExsD. Finally, in Pseudomonas, ExsC binds ExsD and we demonstrated that analogous binding occurs for these homologues in V. parahaemolyticus (Fig. 6).

ExsC has been demonstrated to bind ExsD in Pseudomonas, and this positively regulates T3SS1, but there is no experimental data describing which region of the protein is required for this interaction and if this interaction is required for the expression of T3SS. By substitution of charged residues with alanine or glycine, Lykken et al.46 identified several ExsC mutants that were not able to complement the phenotype of exsC mutant. Nevertheless, these mutants were still able to bind ExsD, suggesting that binding of ExsC with ExsD is sufficient for the induction of T3SS in Pseudomonas. These results were similar to what is observed when ExsA-ExsC-ExsD interaction is due to the interaction between ExsC and ExsD.

While our studies show that the ExsA-ExsC-ExsD interaction is very similar for V. parahaemolyticus and P. aeruginosa,
we also noted several distinctions. Dasgupta et al. observed diminished ExsA synthesis for their ∆exsC strain (Fig. 1E, lanes 7 and 8) whereas there is no evidence that deletion of exsC from V. parahaemolyticus influences ExsA synthesis (Fig. 4). McCaw et al. found that while deletion of exsD resulted in transcription of the T3SS regardless of culture conditions, secretion of TTSS proteins still required Ca2+ chelation. In contrast, deletion of exsD from V. parahaemolyticus permits both transcription and secretion of T3SS-dependent proteins regardless of the culture conditions tested (Fig. 3). Finally, ExsA in V. parahaemolyticus recognizes a promoter binding motif that is distinct from the one recognized by ExsA from P. aeruginosa. Dasgupta et al. suggested a regulatory model whereby ExsC is normally sequestered by another T3SS protein (ExsE) and thus not available for binding ExsD. Under this scenario, when the T3SS is activated ExsC acts as a chaperone to deliver the sequestering protein to the T3SS apparatus thereby becoming available to bind ExsD. We do not have any data to support or refute this model for V. parahaemolyticus, although a more parsimonious model would only require ExsC to be synthesized when appropriate environmental conditions are encountered.

Regardless of the differences noted above, it is quite remarkable that such phylogenetically distinct (different order, family and genus) and ecologically divergent (halophilic marine organism versus a generalist) species express barely recognizable homologous proteins that essentially retain conserved functions. Between V. parahaemolyticus and P. aeruginosa, the amino acid similarity for ExsA, ExsC and ExsD is only 45%, 34% and 22%, respectively. If these T3SSs were present in the original common ancestor then these functional traits have been conserved >500 million years for what might otherwise be considered an accessory pathogenicity island in these two facultative pathogens. That is a strong indication that evolutionary pressures have favored retention of the T3SS function within the diverse ecological niches that are occupied by these two organisms.

**Materials and Methods**

**Bacterial strains, plasmids and growth conditions.** V. parahaemolyticus was routinely grown in Luria-Bertani (LB) medium supplemented with 2.5% of NaCl (LB-S) at 37°C with shaking. Escherichia coli S17-1 lambda pir strain was used for gene cloning and construction of gene deletion mutants and was grown in LB at 37°C with shaking. Plasmid pMMB207 was used for the complementation and protein expression. Plasmid pDM4 was used for generate gene deletion mutants in V. parahaemolyticus. Plasmid pDM31 and pRY107 were used in the protein-protein interaction experiment. All of the wild-type and derivative strains of V. parahaemolyticus and E. coli are listed in Table 1. Antibiotics were used in the following concentration: 100 µg/ml ampicillin, 50 µg/ml kanamycin, 17 µg/ml chloramphenicol for E. coli, and 5 µg/ml chloramphenicol for V. parahaemolyticus. Generation of exsC deletion in wild-type and ∆exsD background strains. Deletion of exsC gene was achieved by homologous recombination as described in previous studies. Briefly, a 697 bp DNA fragment in the immediate upstream of exsC (vp1701) and a 700 bp DNA fragment in the immediate downstream

![Figure 8](image-url)
of exsC were amplified by PCR using primers pairs ExsC_1F/ExsC_1R and ExsC_2F/ExsC_2R, respectively (Table 2). The DNA fragment amplified by ExsC_1F/ExsC_1R was digested with restriction enzymes XhoI and XbaI. These two digested fragments were ligated with plasmid pDM4 that was digested with XhoI and XbaI resulting in the plasmid pDM4_exsC1 + 2. Plasmid pDM4_exsC1 + 2 was electroporated into E. coli S17 resulting in the strain S17_pDM4_exsC1 + 2. Plasmid pDM4_exsC1 + 2 was subsequently transferred from S17 into the wild-type strain of V. parahaemolyticus (NY-4) by conjugation. Chloramphenicol- and ampicillin-resistant transconjugants were selected and inoculated into LB-S broth supplemented with 10% sucrose to facilitate the excision of the plasmid from the chromosome of V. parahaemolyticus. V. parahaemolyticus in LB-S supplemented with sucrose was streaked onto LB-S plates with 10% sucrose and individual colonies were picked and spotted onto LB-S agar and LB-S agar with chloramphenicol. Chloramphenicol-sensitive colonies were picked and grown in LB-S broth for confirmation of exsC deletion by using PCR with primers ExsC_Foward and ExsC Reverse. One clone with a PCR confirmed exsC deletion was designated as ∆exsC. To generate the exsC gene deletion in ∆exsD strain, plasmid pDM4_exsC1 + 2 was transferred from the S17 strain into the ∆exsD strain by conjugation. Selection, screening and confirmation of exsC deletion were similar to that described above. Mutant strains were designated as ∆exsD exsC.

Complementation of ∆exsC strain with a wild-type exsC gene or an unrelated bla gene. Full-length exsC gene was amplified by PCR using ExsC_up and ExsC_down as primers and genomic DNA of NY-4 strain as template. PCR product was purified and digested with EcoRI and XbaI before ligation into pMMB207 that was digested with the same enzymes resulting in the plasmid pMMB207_exsC. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pMMB207_exsC. The plasmid was subsequently transferred from E. coli strain into NY-4 and ∆exsC strains of V. parahaemolyticus resulting in ∆exsC_pMMB207_exsC and NY-4_pMMB207_exsC, respectively. A partial bla gene (~680 bp) was amplified by using Blaup and Bladown as primers and pCX340,39 as template. PCR product for bla6xHis was purified and digested with XbaI and HindIII before being ligated into pMMB207 that was digested with the same enzymes, leading to the plasmid pMMB207_bla. This plasmid was electroporated into E. coli S17 (S17_pMMB207_bla) and subsequently transferred into NY-4 and ∆exsC strains of V. parahaemolyticus resulting in the ∆exsC_pMMB207_bla and NY-4_pMMB207_bla, respectively.

Generation of HA tagged ExsA in the chromosome. To add an HA tag at the 3′ end of the exsA gene in the chromosome, primers ExsA_HA_Insert_Fwd and ExsA_HA_Insertion_Rev were used to amplify the entire exsA gene with an HA tag at the C-terminus. PCR products were digested with XhoI and XbaI and ligated into the pDM4 plasmid that was digested with the same enzymes, resulting in the plasmid pDM4_exsA_HA_Insertion. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pDM4_exsA_HA_Insertion. Plasmid pDM4_exsA_HA_Insertion was conjugated into NY-4 and ∆exsC strains resulting in the strain NY-4_pDM4_exsA_HA_Insertion and ∆exsC_pDM4_exsA_HA_Insertion.

Western blot analysis to detect Vp1656 and Vp1659. For preparation of protein samples from V. parahaemolyticus grown in non-inducing conditions, overnight bacterial culture was diluted in LB-S (1:100) supplemented with antibiotics as needed. Diluted
culture was grown in 37°C with aeration for 4 h and pellets were collected by centrifugation for 10 min (4,000 xg). Pellets were resuspended in 1X Phosphate Buffered Saline (PBS) and sonicated until the bacterial resuspension was clear. For preparation of proteins from V. parahaemolyticus-infected host cells, monolayers of HeLa or Caco-2 cells were seeded in 6-well plates and inoculated with overnight bacterial culture to achieve an M.O.I of 100. Four hours after incubation (37°C with 5% of CO₂), adherent and non-adherent bacteria were collected by scraping off the HeLa or Caco-2 cells from the plate and centrifugation. Bacteria and HeLa or Caco-2 cells pellets were resuspended in 1X PBS and sonicated until the resuspension became clear. Protein samples were mixed with equal volume of 2X Laemmli buffer before being loaded onto 12% of SDS-PAGE gel. Electrophoresis was carried out for ~1 h with at 120 V. Separated proteins were transferred from the gel onto the nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% non-fat milk (Bio-Rad) in PBST (PBS containing 0.1% of Tween-20) for 1 h and then probed with primary polyclonal antibodies against Vp1656,⁴⁷ and Vp1659 (Cal et al, unpub data) in PBST (1:1,000) for 1 h. After being extensively washed, the membrane was probed with secondary goat anti-mouse IgG antibody conjugated to horseradish peroxidase (CalBiochem, San Diego, CA). The blots were developed as described previously.⁴⁷

RT-PCR analysis. RT-PCR analysis was performed as described previously.⁴⁷

Secretion of Vp1656 and Vp1659 by V. parahaemolyticus. Preparation of secreted proteins in the supernatant was described previously.⁴⁷ Briefly, supernatant of the bacterial culture was concentrated by addition of trichloroacetic acid (TCA) to achieve a final 10% concentration followed by centrifugation. Pellets were resuspended in acetone and centrifuged again to obtain protein samples. Protein samples were resuspended in 1X Laemmli buffer before being loaded onto SDS-PAGE gel. Presence of Vp1656 and Vp1659 in the supernatant of bacterial culture was determined by western blot as described above.

Generation V. parahaemolyticus strains for co-expression of exsC, exsA and exsD. To generate a strain that co-expresses exsC and exsD, we cloned exsA and as an IPTG-inducible promoter P_tac into a plasmid, pDM31. The full-length exsA gene (447 bp) was amplified using ExsA_IP_F and ExsA_IP_R (Table 2) as primers and genomic DNA of NY-4 as template. The P_tac promoter was amplified using P_tac_F and P_tac_R (Table 2) as primers and plasmid pMMB207 as template. The DNA fragment of P_tac was digested with SacI and SmaI. The DNA fragment of exsA and P_tac were ligated into pDM31,⁴⁷ that was pre-digested with SacI and SmaI, resulting in the plasmid pDM31_P_tac_exsA_His. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pDM31_P_tac_exsC. The full-length exsD gene was amplified using ExsD_up and ExsD_HA_down as primers (Table 2) with genomic DNA of NY-4 as template. The amplified DNA fragment of exsD gene was digested with EcoRI and XbaI. Digested fragments were ligated into plasmid pMMB207 that was pre-digested with the same enzymes, resulting in the plasmid pMMB207_exsD_HA. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pMMB207_exsD_HA. The full-length exsA gene was amplified using ExsA_up and ExsA_HA_down as primers and genomic DNA of NY-4 strain as template. The amplified DNA fragment of exsA was digested with BamHI and XbaI. Digested fragments were ligated into plasmid pMMB207 that was pre-digested with the same enzymes, resulting in the plasmid pMMB207_exsA_HA. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pMMB207_exsA_HA. His-tagged ExsA was constructed by using primers ExsA_IP_F and ExsA_IP_R, resulting in the plasmid pDM31_P_tac_exsA_His. To determine the interaction between ExsC and ExsD, plasmids pDM31_P_tac_exsC_His and pMMB207_exsD_HA were transferred from corresponding E. coli S17 strains into NY-4 by conjugation, resulting in the strain NY-4: pexsD-HA pexsC-His. Plasmid pMMB207_exsD_HA was transferred into NY-4 by conjugation resulting in the strain NY-4: pexsD-HA.

To generate GST-fused ExsC, a Glutathione S-transferase (GST) gene as well as P_tac promoter was amplified using GST_FW and GST_RE as primers and plasmid pGEX-KG⁵⁵ as
Table 1. Strains and plasmids used in this study

| Strains and plasmids | Descriptions | Sources |
|----------------------|--------------|---------|
| **E. coli**          |              |         |
| S17-1Δpir            | thi pro hsdR λsdM recA RP4-2-Tc::Mu-Km-Tn7 λpir | 57     |
| S17_pDM4_exsC1 + 2   | S17 carrying pDM4_exsC1 + 2 | This study |
| S17_pMM207_exsC     | S17 carrying pMM207_exsC | This study |
| S17_pMM207_bla      | S17 carrying pMM207_bla | This study |
| S17_pDM4_exsA_HA_Insertion | S17 carrying pDM4_exsA_HA_Insertion | This study |
| S17_pDM31_Ptac_exsC | S17 carrying pDM31_Ptac_exsC_His | This study |
| S17_pMM207_exsD_HA  | S17 carrying pMM207_exsD_HA | This study |
| S17_pMM207_exsA_HA  | S17 carrying pMM207_exsA_HA | This study |
| S17_pGST_exsC_His   | S17 carrying pRY107_GST_exsC_His | This study |
| S17_pGST_exsC_Δ5'-His | S17 carrying pRY107_GST_exsC_Δ5'-His | This study |
| S17_pDM4_exsA_HA_Insertion | S17 carrying pDM4_exsA_HA_Insertion | This study |
| **V. parahaemolyticus** |              |         |
| NY-4                 | Clinical isolate O3:K6 | 47     |
| ΔexsC                | exsC (vp1701) deletion mutant | This study |
| ΔexsD exsC           | exsC and exsD double deletion mutant | This study |
| ΔexsD                | exsD deletion mutant | 47     |
| ΔexsC_pMM207_exsC    | ΔexsC strain with pMM207_exsC | This study |
| NY-4_pMM207_exsC    | NY-4 with pMM207_exsC | This study |
| ΔexsC_pMM207_bla    | ΔexsC strain with pMM207_bla | This study |
| NY-4_pMM207_bla     | NY-4 with pMM207_bla | This study |
| NY-4_pexsA          | NY-4 strain with pMM207_exsA | 47     |
| NY-4_pexsD-HA       | NY-4 with pDM31_Ptac_exsC_His and pMM207_exsD_HA | This study |
| NY-4_pexsD-HA       | NY-4 with pMM207_exsD_HA | This study |
| NY-4_pGST-exsC-His  | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pexsD-HA       | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsD-HA   | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsD-HA   | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsC(7-148)-His | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsC(13-148)-His | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsC(7-148)-His | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pGST-exsC(13-148)-His | NY-4 with pDM31_Ptac_exsA_His and pMM207_exsD_HA | This study |
| NY-4_pDM4_exsA_HA_Insertion | NY-4 with HA tagged exsA in the chromosome | This study |
| ΔexsC_pDM4_exsA_HA_Insertion | ΔexsC with HA tagged exsA in the chromosome | This study |

**Plasmids**

| Plasmids      | Descriptions | Sources |
|---------------|--------------|---------|
| pDM4          | A suicide vector with ori R6K sacB; Cm' | 58     |
| pMM207        | RSF1010 derivative, IncQ lacI Cm' Ptac oriT | 59     |
| pDM31         | TcR; p15A origin, pACYC184 derivative that lacks cm' and includes mob | 54     |
| pRY107        | KmR, pW5K29 MCS oriT mob | 56     |
| pDM4_exsC1 + 2| Flanking region sequences of exsC cloned into pDM4 | This study |
| pMM207_exsC   | exsC coding sequences with sequences for 6 His amino acids at the C-terminus cloned into pMM207 | This study |
| pMM207_bla    | Partial bla, cloned into pMM207 | This study |
| pDM31_Ptac_exsC_His | exsC coding sequence with sequence for 6 His amino acids at the C-terminus and Ptac promoter sequence at the N-terminus cloned into pDM31 | This study |
| pMM207_exsD_HA | exsD sequence with HA sequence at the C-terminus cloned into pMM207 | This study |
| pMM207_exsA_HA | exsA sequence with HA sequence at the C-terminus cloned into pMM207 | This study |
| pDM31_Ptac_exsA_His | exsA sequence with sequence for 6 His amino acids at the C-terminus and Ptac promoter sequence at the N-terminus cloned into pDM31 | This study |
| Table 1. Strains and plasmids used in this study |
|-----------------------------------------------|
| **pRY107_GST_exsC_His** | exsC sequence with GST sequence at the N-terminus and 6 His sequence at the C-terminus cloned into pRY107 | This study |
| **pRY107_GST_exsC\(\_\Delta S\)^5-His** | N terminal 102 bp truncated exsC sequence with GST sequence at the N-terminus and 6 His sequence at the C-terminus cloned into pRY107 | This study |
| **pRY107_GST_exsC(7-148)-His** | N terminal 18 bp truncated exsC sequence with GST sequence at the N-terminus and 6 His sequence at the C-terminus cloned into pRY107 | This study |
| **pRY107_GST_exsC(13-148)-His** | N terminal 36 bp truncated exsC sequence with GST sequence at the N-terminus and 6 His sequence at the C-terminus cloned into pRY107 | This study |
| **pRY107_GST_exsC(27-148)-His** | N terminal 78 bp truncated exsC sequences with GST sequence at the N-terminus and 6 His sequence at the C-terminus cloned into pRY107 | This study |
| **pDM4_exsA_HA\_Insertion** | exsA with HA sequence at the C-terminus cloned into pDM4 | This study |

| Table 2. Primers used in this study |
|------------------------------------|
| **Primer name** | **Sequences (5'-3')** |
| ExsC\_1F | AGG ATA AAC TCG AGA TAG AGT TCT CCT ATC ACT TTC |
| ExsC\_1R | AGT TAG TCT AGA AGA AAC AGT CCT TTT GAG AAT TT |
| ExsC\_2F | AGT TAG TCT AGA GTG TCT TAT GTC TAA TGA CAT C |
| ExsC\_2R | AGT TAG AGA TCT GCG GAA GCA CTG GAA ATT G |
| ExsC\_Forward | CCG GCC AAA ATA TAA TAA CTA AC |
| ExsC\_Reverse | CGA CCT TGA AAC GTT CTT TG |
| ExsC\_up | AGG ATA GAA TTC TAA GGA GGT AGG ATA ATA ATG TCA GCA CGCCAA ACT ATC |
| ExsC\_down | AGT TAG TCT AGA TTA ATG GTG TGT ATG GTG AAC TCT CAG ATC TAA ACT TGG AG |
| Blaup | AGG ATA TCT AGA CCG ATC CTC GAG CAC CCA GAA ACG CGT GTG AAA G |
| Bladown | AGT TAG AAG CTT TTA CCA ATG CTT AAT CAG TGA GGC |
| ExsC\_IP\_F | AGG ATA CCC GGG ATG CAC CAT CAC CAT CAC CAT TCA GCA CGCCAA ACT ATC |
| ExsC\_IP\_R | AGT TAG ACT AGT TTA ATG GTG ATG ATG ATG ATG AAC TCT CAG ATC TAA ACT TGG AG |
| Ptac\_F | AGG ATA GAG CTC CAG ACT GGA GGT GGC AAC |
| Ptac\_R | AGT TAG CCC GGG TGT TCT CGT GAA ATT GTT ACT |
| ExsD\_HA\_down | AGT TAG TCT AGA TTA AGC GTA ATC TGG TAC GTC GTA TGG GAA AAT CTA GCT GAG ATG GTT ACA AG |
| ExsD\_up | AGG ATA GAA TTC TAA GGA GGT AGG ATA ATA ATG CCG AGA AGA ACA ATG |
| ExsA\_HA\_down | AGT TAG TCT AGA TTA AGC GTA ATC TGG TAC GTC GTA TGG GTA ATT CGC GAT GGC GAC TGG |
| ExsA\_up | AGG ATA GAA TTC TAA GGA GGT AGG ATA ATA ATG GAT GTG TCA GGC CAA CT |
| ExsA\_IP\_F | AGG ATA CCC GGG ATG CAC CAT CAC CAT CAC CAT CAG TGA TCA GCCCAA CTA AAC |
| ExsA\_IP\_R | AGT TAG ACT AGT TTA ATG GTG ATG ATG ATG ATG ATT CGC GAT GGC GAC TGG C |
| GST\_FW | AGG ATA GAG CTC GGC AAA TAT TCT GAA ATG AGC TG |
| GST\_RE | AGT TAG CCC GGG ATC CGA TTT TGG AGG ATG GTC |
| GST\_ExsC\_FW | AGG ATA CCC GGG TCA GCA CGC CAA ACT ATC |
| GST\_ExsC\_RE | AGT TAG ACT AGT TTA TCA ATG GTG ATG ATG ATG AAC TCT CAG ATC TAA ACT TGG AG |
| GST\_ExsC\_C\_FW | AGG ATA CCC GGG GAT GAT CAC TGG AAG GTT CAT TCT |
| GST\_ExsC\_C\_1F\_FW | AGG ATA CCC GGG ATG GAT GGT TTA CAG AAG |
| GST\_ExsC\_C\_2\_FW | AGG ATA CCC GGG AGG AAT TCT CAC GTG TTA CAA AGA |
| GST\_ExsC\_C\_4\_FW | AGG ATA CCC GGG GAC AAT GAA GTA AGC TTA AGC TTA GCT TTT G |
| ExsA\_HA\_Insert\_Fwd | AGG ATA CTC GAG ATG GAT GTG TCA GGC CAA CT |
| ExsA\_HA\_Insert\_Rev | AGT TAG TCT AGA TTA AGC GTA ATC TGG TAC GTC GTA TGG GTA ATT CGC GAT GGC GAC TGG |
The full-length exsC gene was amplified using GST_ExsC_FW and GST_ExsC_RE as primers and genomic DNA of NY-4 as template. The amplified GST fragment was digested with SacI and SmaI. The DNA fragment of exsC was digested with SmaI and SpeI. Digested fragments of GST and exsC were ligated into pRY107,56 that was pre-digested with SacI and SpeI, resulting in the plasmid pRY107_GST_exsC. This plasmid was electroporated into E. coli S17 resulting in the strain S17_pGST_exsC. To determine the protein interaction between GST-fused ExsC and ExsD, plasmids pRY107_GST_exsC and pMMB207_exsD_HA were transferred from corresponding E. coli S17 strains into NY-4 strain by conjugation resulting in the strain NY-4:pGST-exsC-His pexsD-HA. To determine the protein interaction between GST-fused ExsC and ExsA, plasmids pRY107_GST_exsC and pMMB207_exsA_HA were transferred from corresponding E. coli S17 strains into NY-4 strain by conjugation resulting in the strain NY-4:pGST-exsC-His pexsA-HA. To determine the protein interaction between truncated ExsC and ExsD, the GST gene was amplified and digested as described above. A truncated exsC fragment with deletion of 102 bp at the N-terminus was amplified using ExsC_IP_C_FW and ExsC_IP_C_RE as primers and genomic DNA from NY-4 as template. Amplified truncated exsC gene was digested with SacI and SpeI. Digested GST and truncated exsC were ligated into plasmid pRY107, leading to the plasmid pRY107_GST_exsC_A5'-His. This plasmid was subsequently electroporated into E. coli S17, leading to the strain S17_pGST_exsC_A5'-His. To determine the protein interaction between N-terminal truncated ExsC with ExsD, plasmids pRY107_GST_exsC_A5'-His and pMMB207_exsD_HA were transferred into NY-4 by conjugation resulting in the strain NY-4:pGST-exsC_A5'-His-pexsD-HA. To generate a serial truncation of ExsC fused with GST, the exsC gene from Aa7 to Aa148 was amplified using GST_ExsC_C1_FW and GST_ExsC_RE as primers and genomic DNA of NY-4 as template. The amplified GST fragment was digested with SacI and SmaI. The DNA fragment of exsC from Aa7 to Aa148 was digested with SmaI and SpeI. Digested fragments of GST and exsC were ligated into pRY107 that was pre-digested with SacI and SpeI resulting in the plasmid pRY107_GST_exsC (7-148)_His. pRY107_GST_exsC (7-148) and pMMB207_exsD_HA were transformed into NY-4, resulting in the strain NY-4:pRY107_GST_exsC (7-148)_pexsD_HA. pRY107_GST_exsC (13-148) and pMMB207_exsD_HA were transformed into NY-4, resulting in the strain NY-4:pRY107_GST_exsC (13-148)_pexsD_HA. pRY107_GST_exsC (27-148) and pMMB207_exsD_HA were transformed into NY-4 resulting in the strain NY-4: pRY107_GST_exsC (27-148)_pexsD_HA.

**Protein interaction assayed by co-purification.** To determine protein interaction between ExsC and ExsD, overnight culture of *V. parahaemolyticus* strain NY-4:pexsD-HA pexsC-His was diluted (1:100) into LB-S and grown in 37°C for ~2 h until the OD reached ~0.5. IPTG was added to reach a final concentration of 1 mM and protein expression was induced for an additional 6 h. Bacterial culture was centrifuged for 10 min (4,000 x g) to collect pellets that were resuspended in 1X purification buffer (50 mM NaH2PO4, pH 8.0, 0.5 M NaCl) for sonication. A portion of sonicated protein sample was collected as whole-cell lysate and the rest of the sample was loaded onto an Ni2+ resin column (Invitrogen, Carlsbad, CA). After binding overnight, the column was washed with washing buffer (1X purification buffer supplemented with 30 mM imidazole) eight times. Proteins were subsequently eluted with 0.5 ml of elution buffer (1X purification buffer supplemented with 300 mM imidazole) twice and subsequently designated as Elution 1 and Elution 2, respectively. Western blot analysis was performed to detect the presence of His- and HA-tagged protein in the whole-cell lysates and in the elutions using monoclonal anti-His antibody (Invitrogen) and polyclonal anti-HA antibody. To exclude the possibility that HA-tagged proteins bound the resin column, control strain NY-4:pexsD-HA was grown and protein samples were prepared as described above. Whole-cell lysate and elutions were examined for the presence of His- and HA-tagged proteins. NY-4:pGST-exsC-His pexsD-HA was used to determine the interaction between GST-fused ExsC and ExsD. NY-4:pGST-exsA-His pexsD-HA was used to determine the interaction between GST-fused ExsC and ExsA. NY-4:pexsD-HA-pexsA-His was used to determine the interaction between ExsA and ExsD. NY-4:pGST_exsC_A5'-His pexsD-HA was used to determine the interaction between N-terminal truncated ExsC and ExsD. NY-4:pGST-exsC_A5'-His pexsD-HA was used to determine the interaction between GST-fused ExsC and ExsA. NY-4:pexsD-HA-pexsA-His was used to determine the interaction between ExsA and ExsD. NY-4:pGST_exsC_A5'-His pexsD-HA was used to determine the interaction between N-terminal truncated ExsC and ExsD. Strain NY-4:pGST-exsC (7-148)_His pexsD-HA was used to determine the interaction between GST-fused ExsC and ExsD. Strain NY-4:pGST-exsC (13-148)_His pexsD-HA was used to determine the interaction between GST-fused ExsC (13-148) and ExsD. Strain NY-4:pGST-exsC (27-148)_His pexsD-HA was used to determine the interaction between GST-fused ExsC (27-148) and ExsD.

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