GUT MICROBES
2017, VOL. 8, NO. 4, 366–373
https://doi.org/10.1080/19490976.2017.1287655

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ADDENDUM

Biochemical basis for activation of virulence genes by bile salts in *Vibrio parahaemolyticus*

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ABSTRACT

Bile salts act as a stressor to bacteria that transit the intestinal tract. Enteric pathogens have hijacked bile as an intestinal signal to regulate virulence factors. We recently demonstrated that *Vibrio parahemolyticus* senses bile salts via a heterodimeric receptor formed by the periplasmic domains of inner-membrane proteins VtrA and VtrC. Crystal structures of the periplasmic complex reveal that VtrA and VtrC form a β-barrel that binds bile salts in its hydrophobic interior to activate the VtrA cytoplasmic DNA-binding domain. Proteins with the same domain arrangement as VtrA and VtrC are widespread in *Vibrio* and related bacteria, where they are involved in regulating virulence and other unknown functions. Here we discuss our findings and review current knowledge on VtrA and VtrC homologs. We propose that signaling by these membrane-bound transcription factors can be advantageous for the regulation of membrane and secretory proteins.

ARTICLE HISTORY
Received 7 November 2016
Revised 18 January 2017
Accepted 23 January 2017

KEYWORDS
bile; bile salts; intestinal pathogens; signaling; T3SS; T3SS2; type-three secretion; *Vibrio*; *Vibrio cholerae*; *Vibrio parahaemolyticus*

Bile as a stressor and a signal

Bile is a secretory mixture that plays a key role in digestion. It is synthesized in the liver, stored in the gallbladder, and secreted into the small intestine after a meal. Its principal components are bile salts, bilirubin, cholesterol, phospholipids, and inorganic salts.[1] Of these, bile salts play a major role in solubilizing lipids and fat-soluble vitamins to facilitate their absorption. Bile salts are surfactant molecules that are synthesized from cholesterol and conjugated to glycine or taurine to increase their solubility.[1] The detergent properties of bile salts render them antimicrobial, as they can disrupt cell membranes via their interaction with lipids and proteins, damage nucleic acids, and cause redox stress.[2] Commensal and pathogenic intestinal bacteria will inevitably come into contact with bile salts and must evolve strategies to cope with their damaging effects. Many intestinal bacteria have adapted to bile by decreasing membrane permeability, inducing efflux pumps, inducing biofilm formation, and upregulating redox and DNA damage repair genes.[2] Others are able to use bile salts as a cue for intestinal location to regulate virulence factors. These responses can be complex, vary among pathogens, and often depend on specific bile salts. For example, while deoxycholate has been shown to induce virulence gene expression in *Campylobater jejuni*,[3] this same bile acid represses invasion genes in *Salmonella*.[4] Although bile salt induced phenotypes in intestinal pathogens have been thoroughly documented,[5] the mechanisms used for sensing of bile salts, whether directly by binding to signaling proteins or indirectly by sensing cell damage, remain poorly characterized.

Virulence gene regulation by bile salts in Vibrio parahaemolyticus

V. parahaemolyticus is a halophilic bacterium that inhabits marine environments and enters the human body mainly through the consumption of contaminated water or undercooked seafood.[6] Pathogenic strains of *V. parahaemolyticus* are able to colonize and invade the digestive track, resulting in acute gastroenteritis.[7,8] Disease is primarily caused by a set of virulence determinants: pore-forming hemolysins and a

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Addendum to: Li P, Rivera-Cancel G, Kinch LN, Salomon D, Tomchick DR, Grishin NV, Orth K. Bile salt receptor complex activates a pathogenic type III secretion system. eLife 2016; 5.

Type-III Secretion System (T3SS2) that are encoded by a pathogenicity island (Vp-PAI) in chromosome II of *V. parahaemolyticus*.[9,10] The T3SS2 is a needle-like apparatus that spans the inner and outer bacterial membranes and translocates toxic effector proteins into eukaryotic cells. Several T3SS2 effectors have been shown to manipulate actin and hijack host signaling pathways; their functions are thoroughly reviewed by de Souza Santos et al.[11]

A regulatory network that is specifically responsive to bile salts restricts expression of Vp-PAI genes to when *V. parahaemolyticus* encounters the small intestine.[12,13] This network comprises 3 inner-membrane proteins: VtrA, VtrB, and VtrC.[13] VtrA and VtrB contain an N-terminal winged helix-turn-helix (wHTH) DNA-binding domain of the OmpR family that is attached to the inner membrane by a single transmembrane helix; VtrA also has a C-terminal periplasmic domain.[14] (Fig. 1A). We recently uncovered the third and essential protein component of this pathway, VtrC, which is encoded by a gene located downstream of and in an operon with *vtrA*.[13] VtrC is anchored to the inner membrane by a single transmembrane helix and contains a C-terminal periplasmic domain like VtrA, but lacks a cytoplasmic domain (Fig. 1A). We found that VtrA and VtrC form a 1:1 complex through their periplasmic domains to form a membrane-bound receptor that allows *V. parahaemolyticus* to sense bile salts.[13] X-ray structures of this complex reveal an obligate heterodimer where 8 β-strands from VtrC and a single β-strand from VtrA form a β-barrel that harbors a hydrophobic inner chamber with a bile salt binding pocket (Fig. 1B). Upon bile salt binding by this complex, VtrA is able to induce transcription from the *vtrB* promoter. Newly synthesized VtrB then binds to Vp-PAI promoters to induce the expression of T3SS2-related genes. [12,14]

Various *Vibrio* species possess VtrA and VtrC homologs and/or more diverse protein pairs with the same domain arrangement as VtrA and VtrC. These include VtrA/VtrC homologs of unknown function, as well as previously characterized regulators of virulence gene expression TcpP/TcpH and ToxR/ToxS.[15-17] Below, we summarize what is known about the function and mechanism of these proteins.

VtrA/VtrC homologs

Pathogenic non-O1/O139 *V. cholerae* that lack cholera toxin (CT) and toxin coregulated pilus (TCP), such as strain AM-19226, cause enterotoxicity via a T3SS pathogenicity island similar to Vp-PAI.[18] This pathogenicity island is controlled by VtrA and VtrB homologs $VttR_A$ and $VttR_B$, respectively.[18] A VtrC homolog present in *V. cholerae* AM-19226 is likely to be part of this regulatory pathway as well.[13,19] Variations in the genes in this pathogenicity island can be reduced to 2 lineages, T3SS2α and T3SS2β, both of which are distributed among *V. parahaemolyticus* and *V. cholerae* strains and were acquired through independent

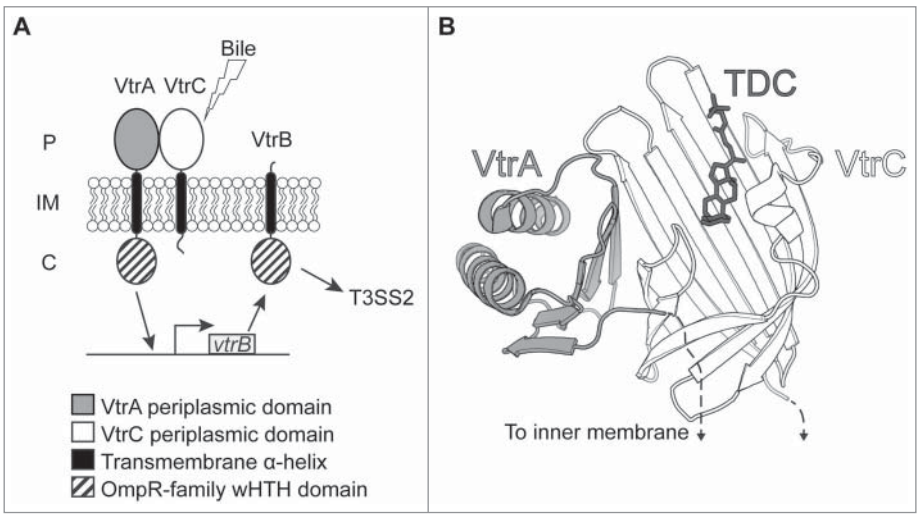

Figure 1. Bile salt sensing by VtrA/VtrC. (A) Schematic of bile salt signaling network formed by VtrA, VtrB and VtrC. The VtrA/VtrC complex senses bile salts in the periplasm, which activates the VtrA cytoplasmic DNA-binding domain to promote *vtrB* transcription, resulting in T3SS expression. P: periplasm; IM: inner membrane, C: cytoplasm. (B) Structure of the VtrA/VtrC periplasmic domain complex with the ligand taurodeoxycholate (TDC).

horizontal gene transfer events.[20] While strain AM-19226 and the *V. parahaemolyticus* strain used in our study (RIMD2210633) contain T3SS2α, we found genes encoding VtrA, VtrB, and VtrC homologs in strains with T3SS2β (*V. parahaemolyticus* TH3996 and *V. cholerae* strains 1587 and 623–39, unpublished). This suggests that the regulatory pathway controlling T3SS2s from both lineages has been conserved through evolution. There is evidence that genes outside of the *V. cholerae* strain AM-19226 T3SS pathogenicity island that are involved in flagellar biosynthesis, chemotaxis, metabolism, and type 6 secretion are influenced by $VttR_A$ and $VttR_B$,[21] supporting the idea that the this regulatory cascade goes beyond controlling T3SS.

VtrA, VtrB, and VtrC are also conserved in species with an incomplete set of T3SS genes, or no T3SS genes at all.[13] Remarkably, we found close VtrA/VtrC homologs in a group of species that lack a membrane-bound VtrB homolog.[13] These species have a gene encoding a predicted sphingomyelinase, an enzyme that hydrolyses sphingomyelin,[22] downstream of the *vtrA*/*vtrC* operon. Sphingomyelin is abundant in eukaryotic cell membranes and several bacterial pathogens produce sphingomyelinases that contribute to their virulence.[23-25] Some of the species that contain a *vtrA*/*vtrC* operon followed by a sphingomyelinase gene, like *Moritella marina* (formerly *V. marinus*), *V. campbellii, V. harveyi*, and *V. splendidus*, are pathogens of marine animals,[26-29] so it is possible that this enzyme could play a role in virulence toward aquatic organisms. More work is needed to determine if this sphingomyelinase is a virulence factor and whether VtrA/VtrC control its expression in response to an environmental or host-derived signal. The amino acid sequences of these VtrA/VtrC homologs diverge significantly from the *V. parahaemolyticus* VtrA/VtrC sequences and given that bacteria are exposed to diverse environments, we think it likely that these homologs will respond to signals other than bile salts. Further work will determine whether these homologs use sphingomyelin or a similar compound as a signal.

TcpP/TcpH

V. cholerae TcpP and TcpH adopt the same topology as VtrA and VtrC (Fig. 2), respectively. TcpP and TcpH are also encoded in a bicistronic operon as overlapping genes in the *Vibrio* pathogenicity island (VPI),[30] but lack homology to the periplasmic domains of VtrA and VtrC. They regulate the expression of cytoplasmic transcription factor ToxT, which activates the transcription of VPI genes encoding

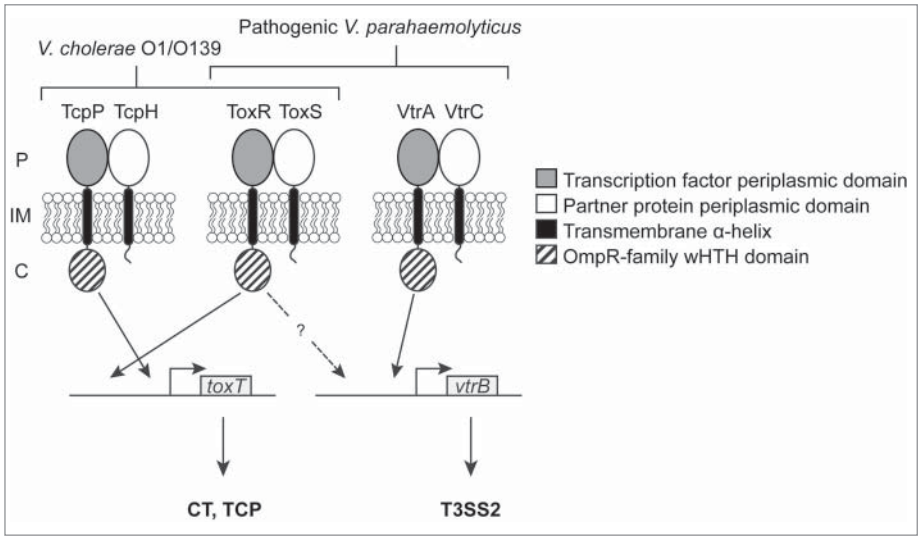

Figure 2. Proposed model for coordinate regulation of *V. cholerae* and *V. parahaemolyticus* virulence by inner-membrane proteins. In *V. cholerae* O1/O139 strains, TcpP, TcpH, ToxR and ToxS activate the transcription of *toxT*, whose gene product controls the expression of TCP and CT genes. In pathogenic *V. parahaemolyticus* strains, VtrA, VtrC, ToxR and ToxS control *vtrB* transcription. VtrB then activates the expression of T3SS genes. Known interactions between DNA-binding domains and promoters are indicated by solid arrows. A dashed arrow indicates that an interaction between ToxR and the *vtrB* promoter is pressumed but has not been experimentally confirmed. P: periplasm; IM: inner membrane, C: cytoplasm.

factors involved in colonization, such as TCP and CT genes.[31] The TcpP DNA-binding domain binds a site immediately upstream of the predicted RNA polymerase binding site in the *toxT* promoter, suggesting that it might interact with RNA polymerase to activate *toxT* transcription.[15,32] TcpP's interaction with TcpH, as well as the formation of a disulfide bond between the 2 cysteines in the TcpP periplasmic domain, protect it from degradation by regulated intramembrane proteolysis (RIP).[33-35]

While a signal that binds the TcpP or TcpH periplasmic domains has not been identified, the bile salt taurocholate has been reported to induce TcpP homodimerization and TcpP-dependent induction of *toxT* expression.[36] Recent data suggest that this effect is indirectly mediated by the thiol:disulfide interchange protein DsbA, rather than by bile salt binding by TcpP or TcpH.[37] *V. cholerae* DsbA was shown to bind taurocholate, which correlates with a shift in the redox equilibrium of this enzyme toward the reduced state in the presence of taurocholate.[37] This is proposed to interfere with its ability to catalyze intramolecular disulfide bond formation in TcpP, which favors the formation of active TcpP homodimers linked, instead, by intermolecular disulfide bonds.

ToxR/ToxS

ToxR and ToxS adopt the same domain topology as VtrA and VtrC (Fig. 2) respectively, and are expressed from a bicistronic operon that is part of the *V. cholerae* ancestral genome, but is also widespread among *Vibrio* species. In *V. cholerae*, ToxR controls the expression of the outer membrane proteins important for surviving in the small intestine [38,39] and is also involved in the regulation of *toxT* by binding to a site upstream of the TcpP binding site on the *toxT* promoter.[32] Overexpressed TcpP can activate *toxT* transcription in the absence of ToxR,[15] suggesting that TcpP is the main player in activation whereas ToxR's role is to enhance TcpP activity by recruiting it to the *toxT* promoter.[32] The involvement of ToxS in ToxR-dependent regulation is not completely clear, however, studies have shown that ToxS enhances ToxR function as a transcriptional activator[16] and that it is able to dimerize with ToxR.[40] ToxS also decreases ToxR degradation through RIP under starvation conditions and after alkaline pH shock.[41] ToxR has been reported to respond to a variety of stimuli, such as pH, osmolarity, presence of amino acids, bile, and cyclo(Phe-Pro),[16,17,42-44] but it remains to be determined whether these signals act on ToxR (and/or ToxS) directly or indirectly via additional factors. The sequence similarity between the periplasmic domains of ToxR/ToxS and VtrA/VtrC is limited (< 25%), hindering inferences about ligand binding based on the VtrA/VtrC structure. Additional biochemical and biophysical studies are needed to determine what, if any, compounds bind ToxR/ToxS.

ToxR and ToxS have adopted alternative roles in *Vibrio* species that lack TCP and CT, like non-O1/O139 *V. cholerae* strains and *V. parahaemolyticus*. Studies with *lacZ* reporter fusions indicate that ToxR moderately affects T3SS expression in *V. cholerae* strain AM-19226.[19] The *V. parahaemolyticus* ToxR homolog, Vp-ToxR, has recently been shown to be necessary for *vtrB* expression, after it was identified in a genetic screen for factors contributing to colonization of the mammalian intestine.[45] This evidence suggests that ToxR/ToxS works with VtrA/VtrC (or its homologs) to control *vtrB* promoter expression in *V. parahaemolyticus* and *V. cholerae* non-O1/O139 strains, analogous to what has been proposed for ToxR/ToxS and TcpP/TcpH to control *toxT* expression.[32] Further evidence that $VttR_A$ or VtrA overexpression in the absence of ToxR restores T3SS and T3SS2 expression, respectively,[45,46] supports a scenario where ToxR plays a secondary role by enhancing VtrA's transcription factor activity. A possible mechanism that could explain this role is that ToxR binding to the *vtrB* promoter recruits VtrA to the promoter or affects VtrA's ability to activate transcription by other mechanism. Future studies will determine if ToxR binds the *vtrB* promoter and whether protein-protein interactions between ToxR and VtrA are involved in this process.

Homology beyond Vibrio spp.

Although the VtrA and VtrC periplasmic domains lack sequence homology with proteins of known structure, their tertiary structure bears striking structural similarity the calycin superfamily β-barrel fold.[47] Calycins have diverse biologic functions and are found in both prokaryotes and eukaryotes. Many members of this superfamily bind small hydrophobic molecules such as fatty acids, retinol, and biotin inside their characteristic β-barrel.[47] Thus, it is not surprising that the structure of VtrA/VtrC in complex with taurodeoxycholate reveals that bile salts bind inside this

β-barrel, displacing a disordered loop that covers the binding site in the apo structure.[13] Interestingly, the VtrA/VtrC complex is the first example of a calycin that forms an obligate heterodimer and can transmit a signal.

Our finding raises the question of how bile salt binding to the VtrA/VtrC complex in the periplasm activates VtrA's function as a transcription factor in the cytoplasm. While VtrA's domain topology with a DNA-binding domain anchored to the inner membrane is atypical – only 3% of prokaryotic transcriptional regulators incorporating a sensing domain and a HTH domain have transmembrane segments [48] – it is also found in other proteins with diverse periplasmic sensing domains. Examples of these are the CadC pH-responsive regulator found in *Salmonella enterica* serovar Typhimurium,[49] *Escherichia coli*,[50] and *V. cholerae*,[51] *Yersinia pestis* PsaE, a regulator for the production of the pH6 antigen,[52] *V. cholerae* TfoS, a regulator for compentence in response to chitin,[53,54] and the *Pseudoalteromonas tunicata* WmpR, a regulator for antifouling compound production.[55] Further work to determine how signals are transduced across the membrane by VtrA/VtrC might reveal mechanistic similarities between these distinct systems.

Conclusion

The prominent roles of inner-membrane bound transcription factors and their partner proteins in *Vibrio* spp suggest that this arrangement has a functional advantage over other systems that couple sensing of the external environment to gene regulation, such as 2-component systems. Both membrane-bound transcription factors and 2-component systems, the latter of which are composed of a histidine kinase and a response regulator, allow for transduction of signals received from the environment. We speculate that membrane-bound transcription factors have the additional feature in that they localize DNA transcription to the cytoplasmic membrane by a mechanism reminiscent of, but distinct from, transertion (simultaneous transcription, translation and membrane insertion, resulting in DNA attachment to the membrane; for a review, see ref. 56). Membrane-anchored transcription factors promote the enrichment of mRNAs encoding membrane-bound and secreted proteins at the membrane, potentially facilitating co-translational insertion and assembly of T3SS apparatus components, as well as effector secretion. This is analogous to the idea that certain mRNAs encoding membrane, polar and septal proteins are targeted to their site of function in the prokaryotic cell via information contained within the untranslated region of mRNA transcripts.[57] Studies suggest that the 3'-untranslated region of some mRNA transcripts of flagellar and pathogenic T3SS proteins in *E. coli* and *Salmonella*, respectively, are important for membrane protein localization.[58,59] Evidence of mRNA localization goes beyond transcripts encoding flagellar or T3SS proteins and does not always involve the unstranslated region of mRNAs. An example of this is the *bglF* mRNA transcript from *E. coli*, encoding a membrane-bound β-glucoside permease, that was shown to localize to the membrane by way of a signal within its coding sequence, independently of translation.[60] It is important to note that not all mRNAs encoding membrane-bound or secreted proteins localize to the membrane. Nonetheless, membrane localization of some mRNAs may have evolved to minimize non-specific interactions between newly translated proteins thereby facilitating the assembly of multiprotein complexes.[57] Thus, it is conceivable that a similar approach of restricting T3SS gene transcription to the cytoplasmic membrane is used in *Vibrio* spp to expedite the assembly of the needle apparatus and effector secretion.

Despite significant advancements in our understanding of how environmental signals control virulence in *Vibrios*, key questions remain to be answered. Our recent results demonstrate that *V. parahemolyticus* senses bile salts via the periplasmic domains of VtrA and VtrC. This pair of inner-membrane proteins form a signaling unit that regulates virulence in the mammalian gut. To fully understand this system, we need to determine how the binding of bile salt to their periplasmic domains affects the VtrA DNA-binding domain. Since DNA recognition sites for OmpR DNA-binding domains tend to be direct repeats,[61] one possibility is that ligand binding promotes VtrA dimerization. Testing this hypothesis will require further work to identify the VtrA recognition site and to determine if dimerization is part of the mechanism by which VtrA activates transcription from the *vtrB* promoter. Nevertheless, mechanisms that do not involve a monomer to dimer transition are also possible.

Another aspect that needs further study is the crosstalk between ToxR/S and TcpP/H in *V. cholerae* and VtrA/C and ToxR/S in *V. parahaemolyticus*.

While it has been established that these receptor pairs influence the same virulence pathways, the actual mechanism of how this happens remains elusive. Last but not least, it would be interesting to know if the periplasmic domains of ToxR/S, TcpP/H and VtrA/C homologs from species that lack T3SS bind small molecules. The identity such signals, if any, would shed light on how these receptors are stimulated and could open an avenue for therapeutic development.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank the Orth Lab for helpful discussions and Dr. Nicole De Nisco for critical reading of the manuscript.

Funding

This work was supported by NIH under Grant R01-AI087808; Welch Research Foundation under Grant I-1561; and Once Upon A Time…

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