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The Virulence Regulatory Protein ToxR Mediates Enhanced Bile Resistance in *Vibrio cholerae* and Other Pathogenic *Vibrio* Species

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The transmembrane regulatory protein ToxR is required for expression of virulence factors in the human diarrheal pathogen *Vibrio cholerae*, including cholera toxin (CT) and the toxin coregulated pilus (TCP). ToxR is necessary for transcription of the gene encoding a second regulatory protein, ToxT, which is the direct transcriptional activator of CT and TCP genes. However, ToxR, independent of ToxT, directly activates and represses transcription of the outer membrane porins OmpU and OmpT, respectively. The genes encoding TCP and CT (and including ToxT) lie on horizontally acquired genetic elements, while the *toxR*, *ompU*, and *ompT* genes are apparently in the ancestral *Vibrio* chromosome. The contribution of ToxR-dependent modulation of outer membrane porins to cholera pathogenesis has remained unknown. We demonstrate that ToxR mediates enhanced bile resistance in a ToxT-independent manner. In both classical and El Tor biotypes of *V. cholerae*, a *toxR* mutant strain has a reduced minimum bactericidal concentration (MBC) of bile, the bile component deoxycholate (DC), and the anionic detergent sodium dodecyl sulfate (SDS) compared to both wild-type and *toxT* mutant strains. Classical and El Tor *toxR* mutant strains also exhibit reduced growth rates at subinhibitory concentrations of DC and SDS. Growth of either *V. cholerae* biotype in subinhibitory concentrations of bile or DC induces increased ToxR-dependent production of a major 38-kDa outer membrane protein, which was confirmed to be OmpU by Western blot. Measurement of transcription of a *ompU*p-*lacZ* fusion in both biotypes reveals stimulation (about two- to threefold) of ToxR-dependent *ompU* transcription by the presence of bile or DC, suggesting that ToxR may respond to the presence of bile. The *toxR* mutant strains of three additional human intestinal pathogenic *Vibrio* species, *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus*, display lower MBCs of bile, DC, and SDS and have altered outer membrane protein profiles compared to the parental wild-type strains. Our results demonstrate a conserved role for ToxR in the modulation of outer membrane proteins and bile resistance of pathogenic *Vibrio* species and suggest that these ToxR-dependent outer membrane proteins may mediate enhanced resistance to bile. We speculate that ToxR-mediated bile resistance was an early step in the evolution of *V. cholerae* as an intestinal pathogen.

The bacterium *Vibrio cholerae* causes the human diarrheal disease cholera. This organism colonizes the human small intestine, where it produces virulence factors that cause disease. Expression of a number of *V. cholerae* virulence factors, including the cholera toxin (CT) and the toxin coregulated pilus (TCP), is coordinately regulated by environmental signals resulting in high levels of expression within the host intestine but little to no expression outside the host (for a review, see reference 30). Coordinate expression of CT, TCP, and other virulence factors is controlled by a transmembrane DNA-binding protein, ToxR (27). ToxR requires another transmembrane transcriptional activator TcpP (13) to synergistically activate expression of *toxT* in response to specific laboratory conditions (14). ToxT is an AraC-like regulatory protein that directly activates transcription of several virulence genes, including *ctx* and *tcp* genes, which encode CT and TCP, respectively (8, 15).

ToxR, independent of TcpP and ToxT, also activates transcription of *ompU* (5), which encodes a major outer membrane porin (3) that has been suggested to be involved in adherence during pathogenesis (33). ToxR also represses the transcription of *ompT*, which encodes another outer membrane porin, in a TcpP- and ToxT-independent manner (V. DiRita, personal communication). These opposing activities of ToxR lead to virtually exclusive OmpU expression in wild-type strains and OmpT expression in *toxR* mutant strains, at least in vitro. Interestingly, both ToxR and OmpU homologues have been found in other *Vibrio* spp. (20, 29, 36), while *toxT* and the *tcp* and *ctx* genes are found on either a large pathogenicity island (17) or a lysogenic bacteriophage (35) only associated with epidemic *V. cholerae* strains. This suggests that *toxT*, *tcp*, and *ctx* genes were acquired relatively recently but *toxR* and *ompU* were present in the ancestral chromosome. The reasons for the ancestral regulatory protein ToxR gaining control over newly acquired virulence factor expression are unclear.

V. cholerae has the ability to cause global epidemics, or pandemics. It is believed that the first six cholera pandemics were caused by the classical *V. cholerae* biotype, while the seventh pandemic was caused by the El Tor biotype (1). These biotypes are differentiated in the laboratory by a number of characteristics, including different in vitro environmental signals which optimally induce virulence factor expression. Differential expression of virulence factors between the two biotypes of *V. cholerae* has been shown to be due to differential ToxR-dependent *toxT* expression (7). Presumably, ToxR-dependent transcription in both biotypes responds to common environmental signals within the host which have not yet been identified.

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Bile is found at relatively high levels within the intestine, and resistance to bile is essential for enteric pathogens. Bile is composed primarily of bile salts, anionic detergents that not only aid in the digestion of fats but also are bacteriocidal due to their membrane solvent properties. The basic structure of the gram-negative bacteria provides some measure of resistance to bile by hiding the bile-sensitive cytoplasmic membrane beneath the relatively bile-resistant outer membrane (for a review, see reference 28). Lipopolysaccharide and outer membrane porins contribute to the resistance of *Escherichia coli* cells to bile (34). Additionally, efflux pumps have been identified which remove bile that reaches the cytoplasm of enteric bacteria (34). The inherent resistance of enteric bacteria to bile has been incorporated into their selective media, for example, thiosulfate-citrate-bile-sucrose (TCBS) for selection of *Vibrio* species. However, essentially nothing is known about *V. cholerae* resistance mechanisms to bile.

We demonstrate that ToxR mediates enhanced bile resistance in both biotypes of *V. cholerae* in a ToxT-independent manner in a way similar to the ToxR-dependent modulation of outer membrane porins. Moreover, ToxR modulates bile resistance and outer membrane protein expression in other pathogenic intestinal *Vibrio* species. Our results suggest that ToxR-dependent modulation of outer membrane proteins enhances bile resistance. Transcription of bile resistance gene(s) may have been one of the necessary prerequisites in the evolution of an ancestral transcriptional activator, ToxR, into the regulatory protein that controls virulence factor expression of an intestinal pathogen.

MATERIALS AND METHODS

Bacterial strains. *E. coli* DH5α (12) was used for cloning experiments, and strain SM10λ*pir* (26) was used to transfer plasmids to *Vibrio* strains by conjugation. All *V. cholerae* strains are isogenic with the classical strain O395 (23) or El Tor strain E7946 (24). *V. cholerae* KKV61, VJ740, and VJ739 have been described previously (4, 19). The Δ*toxR1* mutation was introduced into the chromosome of E7946 by plasmid pMD60, as previously described (19), to form strain KKV366. Strains O395, KKV61, VJ740, E7946, KKV366, and VJ739 were made phenotypically Lac$^-$ for β-galactosidase assays by the introduction of a chromosomal Δ*lacZ* mutation with plasmid pCG711, as described previously (9), to form strains KKV598, KKV62, KKV163, KKV557, KKV555, and KKV556, respectively.

The other *Vibrio* species used in these studies were ATCC strains 33809 (*V. fluvialis*), 33655 (*V. mimicus*), and 43996 (*V. parahaemolyticus*). Plasmids pKEK287, pKEK266, pKEK260, and pKEK273, containing internal "*toxR*" sequences from the various *Vibrio* species (see below), were mated into these strains to form the *toxR* mutant strains by plasmid cointegration (*toxR*::pGP704), as described previously (19). Correct insertion into the chromosomal *toxR* gene was confirmed by Southern blot.

Plasmid construction. Amplification and cloning of *toxR* fragments using PCR with degenerate oligonucleotides has been described previously (29). These fragments were digested with *Sal*I and *Eco*RI and then ligated into pGP704 (26) that had been similarly digested to form plasmids pKEK287 (*V. cholerae toxR*), pKEK266 (*V. fluvialis toxR*), pKEK260 (*V. mimicus toxR*), and pKEK273 (*V. parahaemolyticus toxR*). These plasmids were used to construct *toxR Vibrio* strains (see above).

The plasmids which express *toxR* from the arabinose-inducible promoter P_{BAD} were constructed by first amplifying the *toxR* genes from both O395 and E7946 chromosomal DNA by PCR using oligonucleotides TOXR1 (5′-TTCGGATTA GGACACAACTCA-3′) and TOXR2 (5′-GCTCTAGATCTATTTTGCATAG CAAGATC-3′) (the *Xba*I site is underlined). The resulting fragments were digested with *Xba*I and ligated into pBAD24 (11) that had been digested with *Nco*I, blunt ended with Klenow fragment of DNA polymerase, and then digested with *Xba*I. This resulted in the construction of pKEK86 and pKEK150, which fuse the second codon of ToxR from a classical strain and an El Tor strain, respectively, to the initiating methionine of a translational P_{BAD} fusion vector. There are four amino acid differences between the ToxR proteins of classical and El Tor strains (7).

The *ompU*p-*lacZ* transcriptional fusion plasmid pAL144, which contains the entire *ompU* promoter region from −675 to +22 with respect to the transcription startsite, has been described previously (5; the kind gift of V. DiRita).

Growth conditions and media. Minimum bactericidal concentration (MBC) and growth rate assays were performed by growth in Luria broth (LB) containing various concentrations of bile (sodium choleate; Sigma), deoxycholate (Amersham), sodium dodecyl sulfate (SDS; lauryl sulfate; Sigma), or Triton X-100 (Sigma). *V. cholerae* strains containing P_{BAD} vectors (e.g., in MBC and protein expression assays) were additionally grown in the presence of 0.05% arabinose and 50 μg of ampicillin per ml. *V. cholerae* strains were grown at 37°C, and the other *Vibrio* strains were grown at 30°C.

Strains were first grown 6 h to overnight in a roller drum in 1 ml of LB in 11-mm-diameter culture tubes at the appropriate temperature. For MBC, protein expression, and transcription assays, cultures were then diluted 1:100 in 0.15 M NaCl, and then 10 μl was used to inoculate 5 ml of LB into 16-mm-diameter culture tubes and was grown in a roller drum at either 30 or 37°C. For MBC assays, cultures were then plated on LB to enumerate viable bacteria; the MBC is that at which no viable bacteria were recovered. Results from three experiments performed independently gave the same MBC values as those reported in the tables. Growth rate assays were performed by diluting overnight cultures 1:100 into 20 ml of LB in a 125-ml Ehrlenmeyer flask, followed by growth in a shaking water bath at 37°C; the cell density was then determined by measuring the optical density at 600 nm (OD_{600}). The relative growth rate was determined by measuring the slope of each exponential-phase growth curve and normalizing it to the exponential growth rate of the same strain grown in LB alone.

Transcription assays. *V. cholerae* strains containing plasmids pAL144 or pTL61T were grown (see above) to stationary phase in LB alone or supplemented with 0.4% bile or 0.1% DC; these conditions were chosen to match those used for the detection of protein expression. Media also contained 100 μg of ampicillin per ml for the retention of plasmid. Samples were permeabilized with chloroform and SDS and assayed for β-galactosidase activity by the method of Miller (25).

Detection of protein expression. Outer membrane fractions were prepared as described previously (22, 26). Proteins were separated by SDS–10% polyacrylamide gel electrophoresis (PAGE) prior to Western blotting with rabbit polyclonal antisera against *V. cholerae* OmpU (6; the kind gift of J. Peterson) utilizing an ECL detection system (Amersham).

RESULTS

***toxR V. cholerae* strains of both biotypes have reduced MBCs of bile.** The selective medium for *Vibrio* species, TCBS, contains a relatively high (0.8%) concentration of bile. We had observed a distinct growth defect of *toxR V. cholerae* strains relative to isogenic wild-type, *toxT*, or *tcpP* strains on this medium; the growth defect was not evident when the *toxR* strain was grown on the same medium lacking bile (data not shown). Based on this observation, experiments were designed to determine if ToxR plays a role in bile resistance of *V. cholerae*.

To determine the role of ToxR in bile resistance, wild-type *V. cholerae* strains of both classical and El Tor biotypes, and isogenic strains containing nonpolar chromosomal deletions of *toxR* (Δ*toxR*) or *toxT* (Δ*toxT*) were grown in various concentrations of bile, the individual bile component deoxycholate (DC), the anionic detergent SDS, or the nonionic detergent Triton X-100. For the wild-type and Δ*toxT* strains, the MBCs of bile, DC, and SDS were identical but were higher for the El Tor biotype than for the classical biotype (Table 1). However, for the Δ*toxR* mutant strains of both biotypes the MBCs of bile, DC, and SDS were lower than for the parental wild-type or Δ*toxT* strains. Expression of ToxR from the P_{BAD} promoter in the Δ*toxR* strains restored the wild-type MBCs of bile, DC, and SDS. These results demonstrate a ToxR-dependent mechanism for enhanced bile resistance that is independent of ToxT. For all strains of both biotypes, including the Δ*toxR* strains, the MBCs of the nonionic detergent Triton X-100 (45%) were identical, indicating that the ToxR-dependent resistance mechanism may be specific for anionic detergents.

***toxR* mutant *V. cholerae* strains of both biotypes have reduced growth rates in the presence of anionic detergents.** The MBCs for Δ*toxR* strains of the anionic detergents (see above), while lower than those for isogenic wild-type strains, are still relatively high for these compounds, but the concentrations of individual bile salts within the intestine is probably lower. The bile salt concentration varies depending on the nutrition status, but it is estimated to be approximately 20 mM (~1%) within the small intestine, where *V. cholerae* colonizes (16). In order to determine if Δ*toxR* strains exhibit defects at lower bile salt

TABLE 1. ToxR is required for increased MBCs of bile, DC, and SDS in *V. cholerae*

Biotype	Genotype[a]	Plasmid[b]	MBC		
			% Bile	% DC	% SDS
Classical (O395)	Wild type	pBAD24	7.50	5.00	0.30
		pKEK86 (*toxR*$^+$)	7.50	5.00	0.30
	Δ*toxR*	pBAD24	3.75	3.75	0.05
		pKEK86 (*toxR*$^+$)	7.50	5.00	0.20
	Δ*toxT*	pBAD24	7.50	5.00	0.30
		pKEK86 (*toxR*$^+$)	7.50	5.00	0.30
El Tor (E7946)	Wild type	pBAD24	15.00	7.50	0.50
		pKEK150 (*toxR*$^+$)	15.00	7.50	0.50
	Δ*toxR*	pBAD24	7.50	5.00	0.10
		pKEK150 (*toxR*$^+$)	15.00	7.50	0.40
	Δ*toxT*	pBAD24	15.00	7.50	0.50
		pKEK150 (*toxR*$^+$)	15.00	7.50	0.50

[a] Actual strains used (see Materials and Methods): classical biotype strains O395 (wild type), KKV61 (Δ*toxR*), and VJ740 (Δ*toxT*), and El Tor biotype strains E7946 (wild type), KKV366 (Δ*toxR*), and VJ739 (Δ*toxT*).

[b] Strains carry either the vector pBAD24 or plasmids pKEK86 and pKEK150, which express classical and El Tor biotype ToxR, respectively, from the P_{BAD} promoter of pBAD24.

concentrations, growth rates were determined for both wild-type and Δ*toxR* strains of both biotypes over a wide range of DC concentrations (Fig. 1A). The growth rate at each DC concentration relative to the growth rate in the absence of DC was plotted as a function of DC concentration. Although the Δ*toxR* and wild-type strains have identical growth rates in the absence of DC, at every concentration of DC supplemented to the medium of >2 orders of magnitude, the Δ*toxR* strains had slower growth rates than did the wild-type strains (Fig. 1A). While the Δ*toxR* strains of both biotypes exhibited reduced growth rates in the presence of a wide range of DC, isogenic Δ*toxT* strains had growth rates identical to those for the wild-type strains at all DC concentrations (data not shown).

The growth rates of Δ*toxR* strains of both biotypes were even more noticeably reduced compared to the wild-type strains when grown in the presence of the anionic detergent SDS (Fig. 1B). The reduced growth rates of Δ*toxR* strains were observed over a wide range (>2 orders of magnitude) of SDS concentrations. However, Δ*toxT* strains exhibited growth rates identical to those of the wild-type strains over this range of SDS concentrations (not shown). These results are consistent with a ToxR-dependent mechanism for enhanced growth in the presence of bile and anionic detergents.

Bile induces ToxR-dependent expression of OmpU in both biotypes. To identify ToxR-dependent factor(s) responsible for bile resistance that might be induced by the presence of bile or individual bile salts, wild-type and Δ*toxR* strains of both biotypes were grown both in the absence or presence of bile and DC. Resolution of the total cellular proteins by SDS-PAGE revealed overexpression of a prominent ~38-kDa protein in wild-type strains of either biotype grown in the presence of bile or DC (Fig. 2). Overexpression of this protein made an absolute determination of the molecular weight difficult, but further fractionation experiments (see below) revealed that this protein was the apparent size of the ToxR-dependent outer membrane porin OmpU. The ~38-kDa protein was absent in Δ*toxR* strains of either biotype grown either in the presence or in the absence of bile or DC; in these strains expression of the ToxR-repressed outer membrane porin OmpT (~40 kDa) is apparent. Total protein patterns of Δ*toxT* strains or Δ*toxR* strains carrying a plasmid expressing ToxR (pKEK86 or pKEK150) of either biotype were indistinguishable from those of the parental wild-type strains (not shown).

The only known ToxR-activated yet ToxT-independent factor is OmpU; therefore, we postulated that the ~38-kDa ToxR-dependent factor overexpressed in the presence of bile was in fact OmpU. The ~38-kDa protein was present in outer membrane fractions of wild-type cells of either biotype grown in bile but was absent from the outer membranes of Δ*toxR* strains grown in bile, which instead had a prominent ~40-kDa protein corresponding to the mobility of OmpT (Fig. 3A). Western blot with polyclonal antisera directed against OmpU confirmed that the ToxR-dependent ~38-kDa outer membrane protein overexpressed in the presence of bile or DC was OmpU (Fig. 3B).

ToxR-dependent transcription of *ompU* is stimulated by bile. Because OmpU is increased in the presence of bile, we tested whether this is due to increased ToxR-dependent *ompU* transcription. Wild-type, Δ*toxR*, and Δ*toxT* strains of both biotypes containing a plasmid with a *ompU* promoter-*lacZ* transcriptional fusion were measured for β-galactosidase activity in the absence or the presence of bile and DC (Fig. 4). Only very low levels of *ompU* transcription were detected in Δ*toxR* strains of either biotype under any growth condition, a finding consistent with the previous demonstration (5) that ToxR is required for high levels of *ompU* transcription. Relatively high levels of *ompU* transcription were detected in wild-type and Δ*toxT* strains of both biotypes grown in LB alone. *ompU* transcription

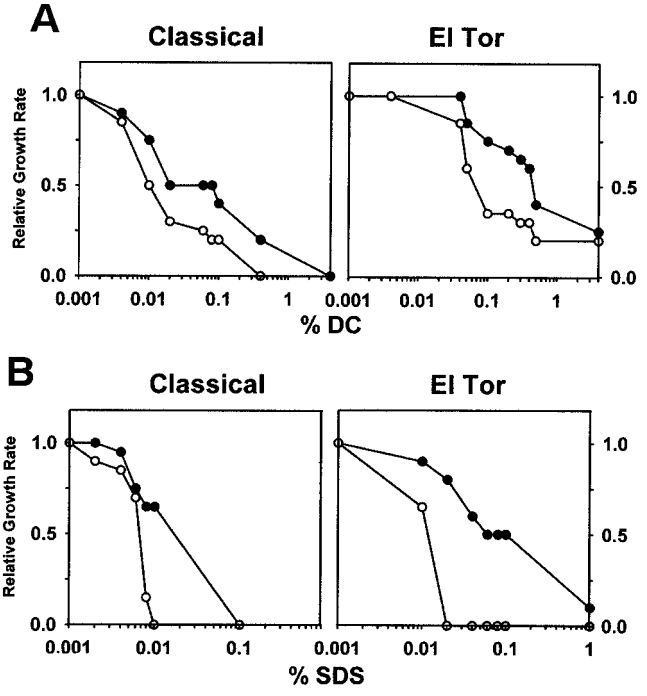

FIG. 1. Δ*toxR* mutant strains of classical and El Tor *V. cholerae* biotypes display slower growth rates in DC and SDS. (A) Relative growth rates of classical and El Tor *V. cholerae* strains in the presence of various concentrations of DC. Classical *V. cholerae* strains O395 (wt, ●) and KKV61 (Δ*toxR*, ○) and El Tor *V. cholerae* strains E7946 (wt, ●) and KKV366 (Δ*toxR*, ○) were grown in LB containing the DC concentrations indicated at 37°C (note the logarithmic scale for DC concentrations). Growth rates are shown relative to the growth rate in the absence of DC. (B) Relative growth rates of classical and El Tor *V. cholerae* strains in the presence of various concentrations of SDS. *V. cholerae* classical strains O395 (wt, ●) and KKV61 (Δ*toxR*, ○) and El Tor strains E7946 (wt, ●) and KKV366 (Δ*toxR*, ○) were grown in LB containing the SDS concentrations indicated at 37°C (note the logarithmic scale for SDS concentrations). Growth rates are shown relative to the growth rate in the absence of SDS.

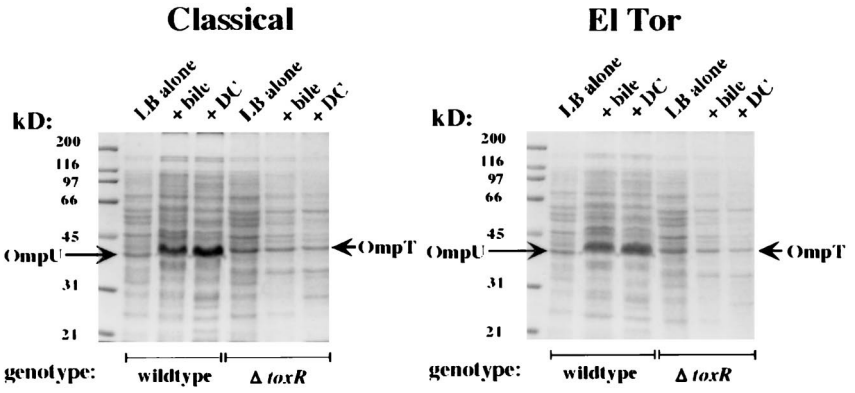

FIG. 2. A ToxR-dependent ~38-kDa protein is overexpressed during growth of classical and El Tor *V. cholerae* strains in bile and DC. Whole-cell lysates of *V. cholerae* classical strains (left panel) O395 (wild type) and KKV61 (Δ*toxR*) and El Tor strains E7946 (wild type) and KKV366 (Δ*toxR*) grown in LB alone, LB plus 0.4% bile, or LB plus 0.1% DC, as indicated above lanes. Samples were matched by equivalent OD_{600} units, separated by SDS–10% PAGE, and stained with Coomassie blue. The first lane of each panel has molecular mass markers, which are noted in kilodaltons to the left. The known mobilities of OmpU and OmpT are indicated by arrows.

increased approximately two- to threefold when these strains were grown in the presence of either bile or DC. These results indicate that the overexpression of OmpU evident in *toxR*$^+$ strains grown in bile or DC is apparently due at least in part to increased ToxR-dependent *ompU* transcription, suggesting that ToxR responds to bile.

ToxR modulates outer membrane proteins and enhanced bile resistance in *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus*. Because the *toxR* gene appears to be an ancestral *Vibrio* gene, we investigated whether ToxR-mediated outer membrane protein modulation and bile resistance were conserved among other *Vibrio* species and specifically in those that are intestinal pathogens. We previously identified *toxR* genes in two human intestinal pathogenic *Vibrio* species, *V. mimicus* and *V. fluvialis* (29), and *toxR* had been additionally identified in the intestinal pathogen *V. parahaemolyticus* (20). Insertional *toxR* mutant strains of *V. mimicus* and *V. fluvialis*, as well as of *V. parahaemolyticus* and *V. cholerae* were constructed as described (see Materials and Methods). The MBCs of bile, DC, and SDS were determined for wild-type and *toxR* mutant strains of *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus*. The *toxR* mutant strains of all three pathogenic *Vibrio* species exhibited lower MBCs for bile, DC, and SDS than the isogenic wild-type strains (Table 2).

Outer membrane proteins from wild-type and *toxR* mutant strains of *V. cholerae*, *V. fluvialis*, *V. mimicus*, and *V. parahaemolyticus* grown in the presence of 0.4% bile were separated by SDS-PAGE (Fig. 5A). Differences in major outer membrane proteins were evident between the wild-type and *toxR* strains of *V. fluvialis*, *V. mimicus*, and *V. parahaemolyticus*, as had already been established for *V. cholerae* (lanes 1 and 2). *V. fluvialis* has a prominent outer membrane protein of ca. 36-kDa that is expressed at a lower level or possibly missing from the *toxR V. fluvialis* strain (and replaced by a protein of slightly higher molecular weight; lanes 3 and 4). *V. mimicus*, the most closely related *Vibrio* species to *V. cholerae*, expresses high levels of an outer membrane protein of the approximate molecular size of *V. cholerae* OmpU (~38 kDa) that is reduced or missing in the *toxR V. mimicus* strain (lanes 5 and 6). The *toxR V. mimicus* strain expresses an outer membrane protein of the approximate size of *V. cholerae* OmpT (~40 kDa) that is reduced or absent from the wild-type *V. mimicus* strain. Finally, *V. parahaemolyticus* expresses an outer membrane protein of ca. 36 kDa that is reduced or absent in the *toxR V. parahaemolyticus* strain (lanes 7 and 8).

The outer membrane fractions of these *Vibrio* strains were subjected to Western blot analysis with polyclonal antisera against *V. cholerae* OmpU (Fig. 5B). The high levels of OmpU present in the outer membrane of the *V. cholerae* wild-type strain were detected with the OmpU antisera, and very little OmpU could be detected in the *V. cholerae toxR* strain outer membrane (lanes 1 and 2; this antisera cross-reacts with a ~35-kDa *V. cholerae* outer membrane protein that is not ToxR regulated). The *V. fluvialis* ~36-kDa outer membrane protein positively regulated by ToxR cross-reacts with OmpU antisera and cannot be detected in the *V. fluvialis toxR* mutant strain

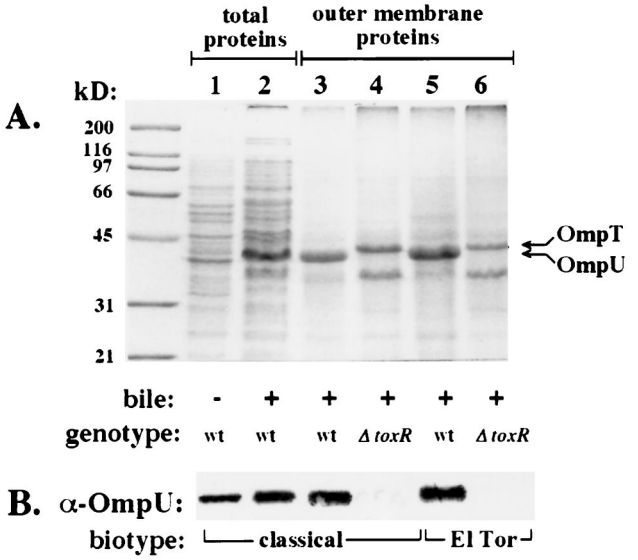

FIG. 3. The ToxR-dependent ~38-kDa protein localized in the outer membrane is OmpU. (A) Outer membrane fractions were prepared (22) of *V. cholerae* classical strains O395 (wt, lane 3) and KKV61 (Δ*toxR*, lane 4) and El Tor strains E7946 (wt, lane 5) and KKV366 (Δ*toxR*, lane 6) grown in LB with 0.4% bile (+bile); also shown are whole-cell lysates (total proteins) of strain O395 grown in LB alone (−bile, lane 1) or LB plus 0.4% bile (+bile, lane 2). Samples were matched by equivalent OD_{600} units and were separated by SDS–10% PAGE and stained with Coomassie blue. The left lane has molecular mass markers, which are noted in kilodaltons. The known mobilities of OmpU and OmpT are indicated by arrows. The identity of the ~35-kDa outer membrane protein most apparent in Δ*toxR* outer membranes is unknown. (B) The whole-cell and outer membrane protein samples above were subjected to Western analysis (see Materials and Methods) utilizing rabbit polyclonal antisera against OmpU.

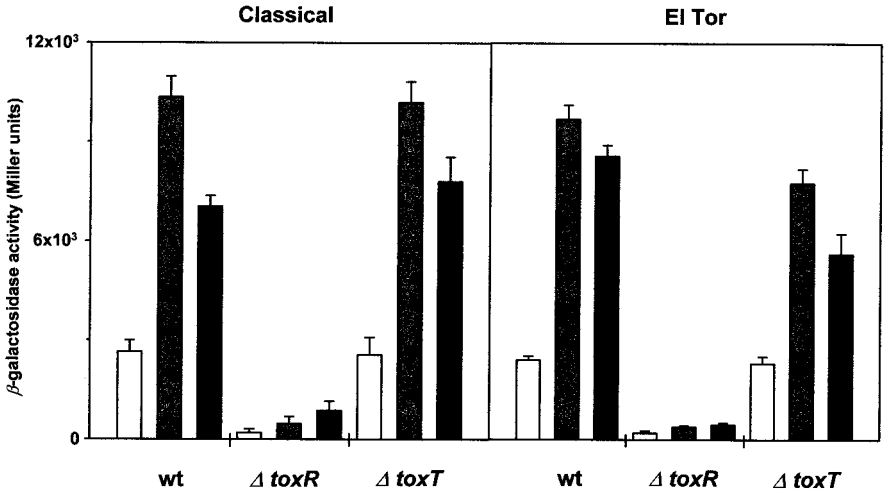

FIG. 4. ToxR-dependent *ompU* transcription is increased in the presence of bile or DC. *V. cholerae* classical biotype strains KKV598 (wild type [wt]), KKV62 (Δ*toxR*), and KKV163 (Δ*toxT*), and El Tor biotype strains KKV557 (wt), KKV555 (Δ*toxR*), and KKV556 (Δ*toxT*) carrying the *ompU*p-*lacZ* transcriptional fusion plasmid pAL144 were grown in LB (open bars) alone or supplemented with 0.4% bile (shaded bars) or 0.1% DC (solid bars) and then assayed for β-galactosidase as described in the text. Media also contained 100 μg of ampicillin per ml. Results are the average of three samples. The β-galactosidase activity of each strain harboring the vector pTL61T (21) alone grown under these conditions (ca. 500 Miller U), which can be considered background activity, has been subtracted out.

(lanes 3 and 4). The *V. mimicus* ~38-kDa outer membrane protein cross-reacted with the OmpU antisera, and the *toxR V. mimicus* strain clearly expressed less of this protein, or possibly a distinct but antigenically related protein of higher mobility (lanes 5 and 6). Interestingly, another outer membrane protein of ~35 kDa that cross-reacts with the OmpU antisera is apparent in the *toxR V. mimicus* strain but not in the wild-type strain, suggesting that this protein is negatively regulated by ToxR. A *V. parahaemolyticus* ~38-kDa outer membrane protein cross-reacted with the OmpU antisera but appears to be expressed at the same level in both wild-type and *toxR V. parahaemolyticus* strains. Our results demonstrate that ToxR modulates bile resistance and outer membrane proteins in other pathogenic *Vibrio* species. Furthermore, it appears that an OmpU homologue is positively regulated by ToxR in *V. fluvialis* and *V. mimicus*. The outer membrane protein of *V. parahaemolyticus* that is positively regulated by ToxR (~36 kDa) does not cross-react with the *V. cholerae* OmpU antisera and may therefore not be an OmpU homologue.

DISCUSSION

The transmembrane protein ToxR is the master regulator of *V. cholerae* pathogenesis. ToxR is required for expression of the major virulence factors CT and TCP (27). However, ToxR is not the direct activator of TCP genes and apparently also not the direct activator of CT genes (4). Instead, ToxR, together with another transmembrane protein, TcpP, activates the *toxT* gene under inducing environmental conditions (8, 13). ToxT, an AraC-like transcriptional activator, then directly activates the genes encoding CT and TCP (4). The environmental signals that stimulate *toxT* transcription were originally thought to be sensed and responded to by ToxR (7) but now appear to be inducing conditions for the expression of *tcpP* (2, 31). Thus, TcpP, once made, appears to "coerce" ToxR into activating *toxT* transcription, something ToxR apparently does not normally do in the absence of TcpP (13). The *tcp* genes (including *tcpP* and *toxT*) are on a large pathogenicity island that may in

TABLE 2. ToxR is required for increased MBCs of bile, DC, and SDS in other *Vibrio* species

Species	Genotype[a]	MBC		
		% Bile	% DC	% SDS
V. fluvialis	Wild type	25.00	7.50	0.50
	toxR	7.50	5.00	0.30
V. mimicus	Wild type	15.00	0.15	0.50
	toxR	10.00	0.05	0.30
V. parahaemolyticus	Wild type	10.00	0.25	0.30
	toxR	7.50	0.05	0.10

[a] Actual strains used (see Materials and Methods): *V. fluvialis* strains ATCC 33809 (wild type) and 33809*toxR*, *V. mimicus* strains ATCC 33655 (wild type) and 33655*toxR*, and *V. parahaemolyticus* strains ATCC 43966 (wild type) and 43966*toxR*.

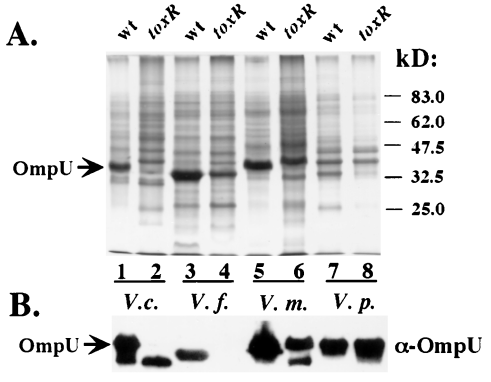

FIG. 5. Outer membrane proteins of *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus* are modulated by ToxR. (A) Outer membrane fractions were prepared as described earlier (26) of *V. cholerae* (*V.c.*) strains O395 (wild type [wt], lane 1) and O395*toxR* (*toxR*, lane 2), *V. fluvialis* (*V.f.*) strains 33809 (wt, lane 3) and 33809*toxR* (*toxR*, lane 4), *V. mimicus* (*V.m.*) strains 33655 (wt, lane 5) and 33655*toxR* (*toxR*, lane 6), and *V. parahaemolyticus* (*V.p.*) strains 43996 (wt, lane 7) and 43996*toxR* (*toxR*, lane 8). Strains were grown in LB supplemented with 0.4% bile. Samples were matched by equivalent OD_{600} units and separated by SDS–10% PAGE and stained with Coomassie blue. The mobility of molecular mass markers are noted in kilodaltons to the right. The known mobility of OmpU is indicated by arrow. (B) The outer membrane protein samples from wild-type and *toxR* mutant *Vibrio* strains (above) were subjected to Western analysis (see Materials and Methods) utilizing rabbit polyclonal antisera against OmpU; *V. cholerae* OmpU is indicated by an arrow.

fact be a filamentous bacteriophage (18), like bacteriophage CTXϕ, which encodes the *ctx* genes (35). These horizontally transferable elements are found only in epidemic strains of *V. cholerae*, but *toxR* has been found in other bacteria within the genera *Vibrio* and *Photobacterium* (20, 29, 36). This leads to the question of what the original role of ToxR was in *V. cholerae* prior to acquisition of the cholera-specific virulence genes.

The original role of ToxR appears to be as a regulator of outer membrane proteins. In *V. cholerae*, ToxR, independent of ToxT and TcpP, activates transcription of *ompU*, which encodes a major outer membrane porin (5), and also represses transcription of *ompT*, which encodes another major outer membrane porin (V. DiRita, personal communication). In *Photobacterium profundum* ToxR likewise activates expression of a "porin-like" outer membrane protein OmpL while repressing expression of another outer membrane protein, OmpH (36). In this study we have demonstrated that ToxR modulates the expression of outer membrane proteins in *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus*, some of which are OmpU homologues (and therefore likely porins). These results suggest that the ancestral role of ToxR was as a modulator of outer membrane proteins, but then why would this protein be usurped as the regulator of virulence factor expression in *V. cholerae*?

The present study has uncovered a previously unknown role for ToxR as a modulator of enhanced bile resistance. ToxR, independent of ToxT, is required for enhanced survival and the growth of both *V. cholerae* biotypes in the presence of bile salts and anionic detergents. Moreover, ToxR is required for enhanced survival of the intestinal pathogens *V. mimicus*, *V. fluvialis*, and *V. parahaemolyticus* in the presence of bile salts and anionic detergents. All of these *Vibrio* species must be resistant to bile in order to persist within the intestine and cause disease. Perhaps this ancestral role in bile resistance led to the evolution of ToxR as the regulator of recently acquired virulence genes in *V. cholerae* that are expressed within the intestine.

Several lines of evidence suggest that the ToxR-regulated outer membrane proteins are involved in bile resistance. (i) OmpU and OmpT are the only known ToxR-dependent but ToxT- and TcpP-independent factors in *V. cholerae*, as with ToxR-dependent bile resistance. (ii) ToxR modulates both bile resistance and outer membrane proteins in other *Vibrio* species. (iii) OmpU is overexpressed when *V. cholerae* is grown in the presence of bile, apparently in part a result of increased ToxR-dependent *ompU* transcription. A previous study by Gupta and Chowdhury (10) failed to identify OmpU overexpression during *V. cholerae* growth in bile; in that study only outer membrane preparations were compared, rather than whole-cell lysates and *ompU* transcription, which may explain this discrepancy. One mechanism for ToxR-mediated enhanced bile resistance in *V. cholerae* would be inhibited influx of anionic detergents through the OmpU porin channel in comparison to the OmpT porin channel. This mechanism of bile resistance is seen in *E. coli*, where a strain expressing only the OmpF porin exhibits slower growth kinetics in the presence of DC compared to a strain expressing only the OmpC porin (34). However, no direct proof of OmpU and OmpT involvement in bile resistance exists yet in *V. cholerae*. An *ompU* mutant strain would be predicted to be more sensitive to bile, if OmpU has a protective role in the presence of bile, but we have not yet succeeded in our attempts to create such a mutant strain. Other laboratories have also noted failure in attempts to create a *ompU V. cholerae* strain, and this has been attributed to a possible essential role for OmpU (32).

ToxR transcribes high levels of *ompU* even in the absence of bile or DC, but in their presence *ompU* transcription increases, suggesting that ToxR transcriptional activity may be modulated by the presence of bile salts. The transcriptional activity of ToxR from *P. profundum* is modulated by pressure and also by local anesthetics such as procaine (36). Welch and Bartlett (36) postulate that both pressure and anesthetics change the membrane structure and that ToxR, which resides within the cytoplasmic membrane, actually responds to these membrane changes. Procaine has also been shown to modulate expression of the ToxR regulon in *V. cholerae* (13). Our studies indicate that ToxR may respond to another class of membrane-disruptive agents, namely, bile salts. A conserved mechanism of ToxR sensing and responding to membrane disruption in *Vibrio* species is an attractive hypothesis that awaits verification. The presence of bile salts in the environment signifies entry into the intestinal tract and would be a possible signal to stimulate ToxR-dependent transcription not only of bile resistance mechanisms but also of the ToxT-dependent virulence cascade.

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