Altered expression of the ToxR-regulated porins OmpU and OmpT diminishes *Vibrio cholerae* bile resistance, virulence factor expression, and intestinal colonization

Daniele Provenzano and Karl E. Klose*

Department of Microbiology, University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78229-3900

Edited by John J. Mekalanos, Harvard Medical School, Boston, MA, and approved June 29, 2000 (received for review May 15, 2000)

The transmembrane transcriptional activators ToxR and TcpP modulate expression of *Vibrio cholerae* virulence factors by exerting control over *toxT*, which encodes the cytoplasmic transcriptional activator of the *ctx*, *tcp*, and *acf* virulence genes. However, ToxR, independently of TcpP and ToxT, activates and represses transcription of the genes encoding two outer-membrane porins, OmpU and OmpT. To determine the role of ToxR-dependent porin regulation in *V. cholerae* pathogenesis, the ToxR-activated *ompU* promoter was used to drive *ompT* transcription in a strain lacking OmpU. Likewise, the ToxR-repressed *ompT* promoter was used to drive *ompU* transcription in a strain lacking both ToxR and OmpT. This strategy allowed the generation of a *toxR*$^+$ strain that expresses OmpT in place of OmpU, and a *toxR*$^-$ strain that expresses OmpU in place of OmpT. Growth rates in the presence of bile salts and other anionic detergents were retarded for the *toxR*$^+$ *V. cholerae* expressing OmpT in place of OmpU, but increased in *toxR*$^-$ *V. cholerae* expressing OmpU in place of OmpT. Additionally, the *toxR*$^+$ *V. cholerae* expressing OmpT in place of OmpU expressed less cholera toxin and toxin-coregulated pilus, and this effect was shown to be caused by reduced *toxT* transcription in this strain. Finally, the *toxR*$^+$ *V. cholerae* expressing OmpT in place of OmpU was ≈100-fold reduced in its ability to colonize the infant-mouse intestine. Our results indicate that ToxR-dependent modulation of the outer membrane porins OmpU and OmpT is critical for *V. cholerae* bile resistance, virulence factor expression, and intestinal colonization.

Vibrio cholerae is a Gram-negative bacterium that causes the potentially fatal human disease cholera. The dramatic diarrheal dehydration of cholera is primarily caused by the effect of cholera toxin (CT), an ADP-ribosylating toxin that increases host cell cAMP levels (1). Within the small intestine, the bacteria synthesize CT and other virulence factors, including the toxin-coregulated pilus (TCP), which is required for intestinal colonization (2). Coordinate expression of virulence factors is mediated by the transcriptional activator ToxR, a transmembrane protein with a cytoplasmic DNA-binding domain (3). A second transmembrane transcriptional activator, TcpP, is required *in vitro* for ToxR-mediated transcription of the *toxT* gene, which encodes the cytoplasmic transcriptional activator of the genes encoding CT, TCP, and accessory colonization factors (4–6). Induction of this ToxR-dependent virulence cascade *in vitro* requires defined but artificial growth conditions, whereas induction within the host is presumed to involve yet unknown environmental signals present within the intestine (7).

ToxR, however, independently of the transcriptional activators TcpP and ToxT, modulates expression of two outer membrane porins, OmpU and OmpT (8–10). Transcription of *ompU* is induced by ToxR, whereas transcription of *ompT* is repressed by ToxR (11, 12). This results in virtually exclusive OmpU expression in *toxR*$^+$ strains and OmpT expression in *toxR*$^-$ strains, at least under laboratory growth conditions. Although genes encoding TcpP, ToxT, CT, and TCP are specifically associated with *V. cholerae*, ToxR is found in other *Vibrio* and *Photobacterium* species and regulates porin expression in these other bacteria as well (13, 14). Thus, ToxR-dependent modulation of porins apparently preceded, and possibly contributed to, the evolution of *V. cholerae* as a human pathogen, but little is known about the potential role of OmpU and OmpT in *V. cholerae* pathogenesis. It has been suggested that OmpU may act as an adhesin during intestinal colonization (15), although subsequent studies have disputed this (16). Our previous studies suggested a role for OmpU in bile resistance, because ToxR-dependent *ompU* transcription is stimulated by the presence of bile, and *toxR*$^-$ strains, which express low amounts of OmpU, are more bile sensitive (17).

The current study uncovers a role for ToxR-dependent porin modulation in *V. cholerae* virulence. *V. cholerae* strains were genetically manipulated to force a "porin swap," i.e., a *toxR*$^+$ strain that expresses OmpT in place of OmpU and a *toxR*$^-$ strain that expresses OmpU in place of OmpT. This strategy allowed us to dissect the role of each porin regardless of the presence/absence of ToxR. We found that porin modulation plays an important role in *V. cholerae* pathogenesis, including bile resistance, virulence factor expression, and intestinal colonization.

Materials and Methods

Bacterial Strains. *Escherichia coli* DH5α was used for cloning (18) whereas strain SM10λpir (8) was used to transfer plasmids into *V. cholerae* by conjugation. *V. cholerae* strains used in this study were all isogenic with the classical 0395 Ogawa strain (19). *V. cholerae* strain KKV780 contains a 825-bp in-frame deletion within *ompU* (Δ*ompU*), strain KKV1089 contains Δ*ompU* and additionally *toxT*::*lacZ* (4), strain KKV804 contains a 819-bp in-frame deletion within *ompT* (Δ*ompT*), and additionally contains Δ *toxR1* (20), and strain KKV526 contains Δ*toxR1* and *toxT*::*lacZ*. These strains were constructed by using pCVD442 (21); characterization of these strains will be described in greater detail in an upcoming report.

Construction of Plasmids for Ectopic Expression of *ompU* and *ompT*. The promoters of *ompU* and *ompT* were amplified with PCR primers; the resultant fragments correspond to −674 to +149

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: CT, cholera toxin; TCP, toxin-coregulated pilus; DC, deoxycholate; Δ*ompU*, deletion within *ompU*; Up-U, *ompU* promoter-*ompU* ORF; Up-T, *ompU* promoter-*ompT* ORF; Tp-U, *omp-T* promoter-*ompU* ORF; Tp-T, *ompT* promoter-*ompT* ORF.

*To whom reprint requests should be addressed. E-mail: klose@uthscsa.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.170219997. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.170219997

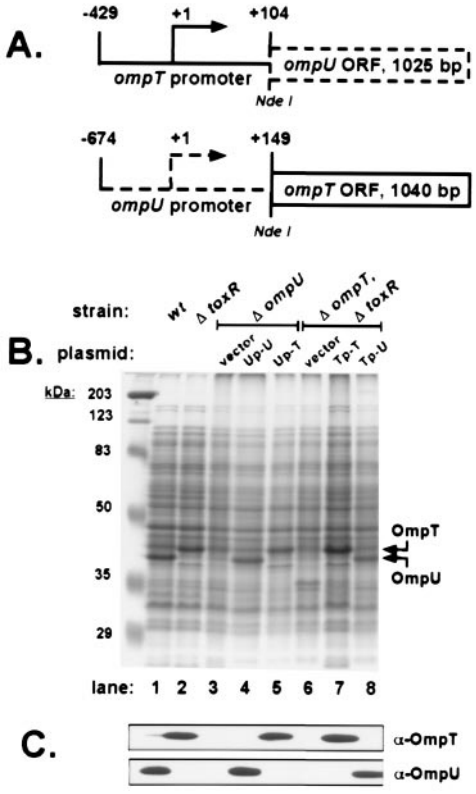

Fig. 1. Ectopic expression of OmpU and OmpT. (*A*) The *ompT* promoter fragment corresponding to −429 to +104 with respect to the transcription start site was fused to the *ompU* ORF to form Tp-U (pKEK256), which allows ectopic expression of OmpU in a *toxR*$^-$ strain. The *ompU* promoter fragment corresponding to −674 to +149 with respect to the transcription start site was fused to the *ompT* ORF to form Up-T (pKEK257), which allows ectopic expression of OmpT in a *toxR*$^+$ strain. Translational fusions were facilitated by the incorporation of an *Nde*I site (CAT*ATG*) at the initiating Met codon of each ORF. (*B*) *V. cholerae* strains O395 (*toxR*$^+$; lane 1); KKV61 (Δ*toxR*; lane 2); KKV780 (Δ*ompU*) carrying either plasmid pWSK30 (vector, lane 3); pKEK253 (Up-U, lane 4); or pKEK257 (Up-T, lane 5); and KKV 804 (Δ*ompT* Δ*toxR*) carrying either plasmid pWSK30 (vector, lane 6), pKEK255 (Tp-T, lane 7), or pKEK256 (Tp-U, lane 8). Whole-cell lysates were matched by OD_{600} and separated on 10% SDS/PAGE, then stained with Coomassie blue; left-hand lane contains molecular mass standards (in kDa), and OmpT and OmpU bands are designated by arrows. (*C*) The samples of lanes 1–8 were subjected to Western analysis by using rabbit polyclonal antisera against OmpT (α-OmpT, *Upper*) and OmpU (α-OmpU, *Lower*).

with respect to the *ompU* transcription start site (11) and −429 to +104 with respect to the *ompT* transcription start site (12). The entire ORFs of *ompU* and *ompT*, 1,025 and 1,034 bp, respectively (12, 22), were PCR amplified and cloned downstream of either promoter sequence in the low copy number vector pWSK30 (23). Use of a *Nde*I site in both promoter and ORF fragments corresponding with the initiating Met codon allowed for exact translational fusions of the promoter fragments to the respective porin ORFs (Fig. 1*A*). The four resulting plasmids carry the following fusions: pKEK253, *ompU* promoter-*ompU* ORF (Up-U); pKEK257, *ompU* promoter-*ompT* ORF (Up-T); pKEK255, *ompT* promoter-*ompT* ORF (Tp-T); and pKEK256, *ompT* promoter-*ompU* ORF (Tp-U).

Growth Conditions and Growth Kinetics. For all experiments, *V. cholerae* strains were grown in LB with appropriate antibiotics. Growth rate assays with anionic detergents were performed as described (17). Inducing conditions for virulence factor expression were 30°C, LB pH 6.5 and noninducing conditions were 30°C, LB pH 8.5.

Tissue Culture Adherence Assay. Microscopic and quantitative adherence assays with HEp-2 cells were performed as described (15), except Accustain formalin fixative (Sigma) was used to fix cells for microscopic analysis. Each well was seeded with ≈3 × 10^5 HEp-2 cells. Bacteria added and recovered were quantitated by plate counts, and adherence is expressed as the percent of bacteria adhered from the total added.

Detection of Protein Expression. *V. cholerae* total proteins were resolved by SDS/PAGE on 10% acrylamide gels and stained with Coomassie brilliant blue. Western blot was performed with rabbit polyclonal antisera against *V. cholerae* OmpU or OmpT (a kind gift of J. Peterson, University of Texas Medical Branch, Galveston) by using the enhanced chemiluminescence ECL detection system (Amersham Pharmacia). Culture supernatants were assayed for CT secretion by the ganglioside M_1 ELISA by using polyclonal mouse antiserum against the purified B subunit of CT (24). TCP expression was determined by transduction with CTXΦ-K_m (25). β-galactosidase assays were performed as described (26) by using strains O395 *toxT*::*lacZ* (4), KKV526, and KKV1089 carrying either pWSK30 (23), pKEK253, or pKEK257.

***In Vivo* Colonization Assay.** The infant mouse colonization assay has been described (27). Briefly, wild-type strain KKV598 (O395 Δ*lacZ*) was mixed with KKV780 (Δ*ompU*) carrying either pKEK253 (Up-U) or pKEK257 (Up-T); mixtures were given in a peroral inoculum ratio of approximately 10^5 mutant/10^5 wild type to 5-day-old CD-1 suckling mice. After a 22-h period of colonization, homogenates from the isolated small intestines were collected and the ratio of mutant/wild type was determined by plating on LB agar containing X-Gal. Tubes (5 ml) of LB were inoculated with the same mixtures and grown at 37°C for *in vitro* competition.

Results

Ectopic Expression of OmpU and OmpT. Because ToxR activates *ompU* transcription and represses *ompT* transcription, a *toxR*$^+$ *V. cholerae* strain expresses OmpU and a *toxR*$^-$ strain expresses OmpT (Fig. 1 *B* and *C*, lanes 1 and 2). The alteration of ToxR-dependent porin expression was accomplished in two steps. First, a large in-frame chromosomal deletion of *ompU* was constructed in a *toxR*$^+$ strain, resulting in no detectable expression of OmpU (Fig. 1 *B* and *C*, lane 3). Also, a large in-frame chromosomal deletion of *ompT* was constructed in a *toxR*$^-$ strain (Δ*ompT* Δ*toxR*), resulting in no detectable expression of OmpT (Fig. 1 *B* and *C*, lane 6).

Next, the *ompU* and *ompT* promoter sequences were fused to either the *ompU* or *ompT* coding sequence. This was accomplished by introducing a *Nde*I site at the initiating Met codon, allowing for an exact translational fusion of the *ompU* promoter to the *ompT* coding sequence (Up-T) and an exact translational fusion of the *ompT* promoter to the *ompU* coding sequence (Tp-U; Fig. 1*A*). Finally, the Up-T plasmid was transformed into the *toxR*$^+$ Δ*ompU* strain, which resulted in ectopic OmpT expression in place of OmpU (Fig. 1 *B* and *C*, lane 5). Likewise, the Tp-U plasmid transformed into the Δ*toxR* Δ*ompT* strain resulted in ectopic expression of OmpU in place of OmpT (Fig. 1 *B* and *C*, lane 8). Control plasmids containing the *ompU* promoter fused to the *ompU* ORF (Up-U) and the *ompT* promoter fused to the *ompT* ORF (Tp-T) fully complemented the Δ*ompU* and Δ*ompT* mutations with respect to protein expression, respectively (Fig. 1 *B* and *C*, lanes 4 and 7). OmpU and OmpT were localized in outer membrane fractions as expected (data not shown). These results demonstrate ectopic

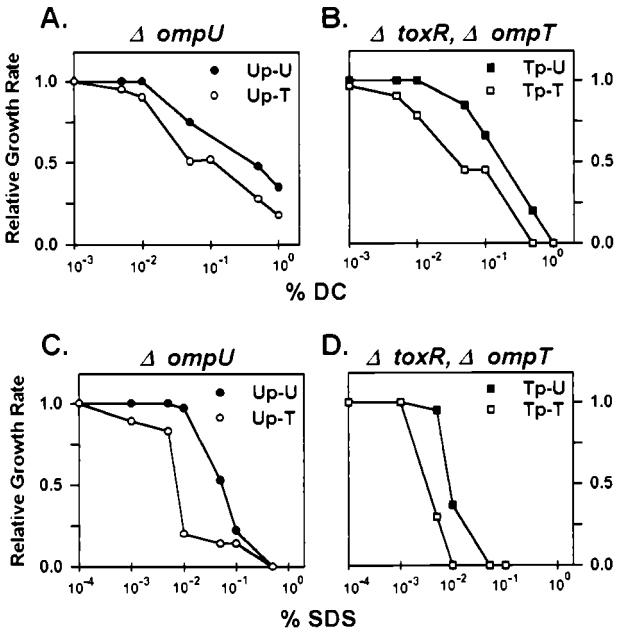

Fig. 2. *V. cholerae* strains expressing OmpT display retarded growth rates in anionic detergents compared with strains expressing OmpU, regardless of the presence/absence of ToxR. Growth rates are shown relative to the growth rate in the absence of DC or SDS (note logarithmic scale for DC and SDS concentrations). (*A*) *V. cholerae toxR*$^+$ strain KKV780 (Δ*ompU*) carrying either pKEK253 (Up-U, ●) or pKEK257 (Up-T, ○) was grown in LB medium at 37°C containing the DC concentrations indicated. (*B*) *V. cholerae* strain KKV804 (Δ*toxR* Δ*ompT*) carrying either pKEK256 (Tp-U, ■) or pKEK255 (Tp-T, □) were grown in LB medium at 37°C containing the DC concentrations indicated. (*C*) *V. cholerae toxR*$^+$ strain KKV780 (Δ*ompU*) carrying either pKEK253 (Up-U, ●) or pKEK257 (Up-T, ○) was grown in LB medium at 37°C containing the SDS concentrations indicated. (*D*) *V. cholerae* strain KKV804 (Δ*toxR* Δ*ompT*) carrying either pKEK256 (Tp-U, ■) or pKEK255 (Tp-T, □) was grown in LB medium at 37°C containing the DC concentrations indicated.

expression of OmpT in place of OmpU in a *toxR*$^+$ strain, and the ectopic expression of OmpU in place of OmpT in a *toxR*$^-$ strain.

Alteration of ToxR-Dependent Porin Regulation Alters Growth Kinetics of *V. cholerae* in the Presence of Anionic Detergents. Recent evidence has demonstrated that *toxR*$^-$ *V. cholerae* have lower minimum bactericidal concentrations and retarded growth kinetics compared with *toxR*$^+$ strains when grown in the presence of the anionic detergents bile, the bile salt deoxycholate (DC), or SDS (17). To determine whether the detergent-sensitive phenotype may be caused specifically by OmpT expression in the *toxR*$^-$ strain, we tested growth kinetics of the *toxR*$^+$ strain ectopically expressing OmpT in place of OmpU in SDS and DC. The *toxR*$^+$ strain expressing OmpT (Δ*ompU*/Up-T) had lower relative growth rates in both DC and SDS over a wide concentration range compared with the *toxR*$^+$ strain expressing OmpU (Δ*ompU*/Up-U; Fig. 2 *A* and *C*). Likewise, a *toxR*$^-$ strain expressing OmpU had higher relative growth rates in both DC and SDS compared with the *toxR*$^-$ strain expressing OmpT (Fig. 2 *B* and *D*). Notably, all strains grew at the same rates in the absence of DC or SDS. These experiments demonstrate that at least one of the ToxR-dependent factors modulating bile resistance are the porins OmpU and OmpT, as we hypothesized (17), and that OmpU has a protective role in *V. cholerae* resistance to anionic detergents.

A *V. cholerae toxR*$^+$ Strain Expressing OmpT in Place of OmpU Has Diminished CT and TCP Expression. *V. cholerae* wild-type O395 grown under inducing laboratory conditions expresses CT and TCP, which can be measured by ganglioside M_1-ELISA and CTXϕ transduction, respectively (Table 1; *Materials and Methods*). High levels of TCP expression under these conditions also result in a visible agglutination (Agg$^+$) phenotype. In contrast, a *toxR*$^-$ strain has no detectable CT or TCP expression and an Agg$^-$ phenotype (Table 1). The *toxR*$^+$ strain expressing OmpT in place of OmpU (Δ*ompU*/Up-T) grown under inducing conditions displayed an Agg$^-$ phenotype, and expressed ≈100-fold less CT than the wild-type strain or the *toxR*$^+$ strain expressing OmpU (Δ*ompU*/Up-U). There were also ≈100-fold less CTXϕ transductants of the *toxR*$^+$ strain expressing OmpT in place of OmpU, indicating less TCP expression in this strain compared with the wild-type strain or the *toxR*$^+$ strain expressing OmpU. These reductions in CT and TCP expression are caused by the presence of OmpT rather than by the absence of OmpU, because the Δ*ompU* strain expresses ≈ wild-type levels of CT and TCP. These results indicate that ToxR-dependent regulation of the outer membrane porins is important for CT and TCP expression.

A *V. cholerae toxR*$^+$ Strain Expressing OmpT in Place of OmpU Has Diminished *toxT* Transcription. Because the *ctx* and *tcp* genes are coordinately transcribed by ToxT, and the *toxR*$^+$ strain expressing OmpT in place of OmpU expresses diminished levels of CT and TCP, we hypothesized this may be caused by either (*i*) decreased *toxT* transcription or (*ii*) decreased ToxT transcriptional activity. We favor the former possibility, because expression of *toxT* from a ToxR-independent *lac* promoter (pKEK162; ref. 28) in the *toxR*$^+$ strain expressing OmpT in place of OmpU resulted in high levels of CT and TCP expression and an Agg$^+$ phenotype (data not shown), indicating a probable reduction of *toxT* transcription rather than lack of ToxT activity in this strain.

Table 1. Ectopic expression of OmpT in place of OmpU diminishes *V. cholerae toxT* transcription and CT/TCP expression

Relevant genotype*	Plasmid†	Agg	TCP‡	CT§	*toxT*::*lacZ*¶	% HEp-2‖ adherence
Wild type (*toxR*$^+$)	pWSK30	+	8.4×10^4	1010	96 ± 5	9.1 (4.3)
Δ*toxR*	pWSK30	−	0	<1	15 ± 1	3.4 (2.7)
Δ*ompU* (*toxR*$^+$)	pWSK30	+	8.6×10^4	880	115 ± 6	10.0 (4.3)
Δ*ompU* (*toxR*$^+$)	Up-U	+	4.4×10^4	935	111 ± 5	2.5 (2.9)
Δ*ompU* (*toxR*$^+$)	Up-T	−	7.7×10^2	10	62 ± 6	4.3 (6.1)

*Actual strains used (see *Materials and Methods*) O395, KKV61, KKV780, O395 *toxT*::*lacZ*, KKV526, and KKV1089.
†Plasmids used (see *Materials and Methods*): Up-U, pKEK253; and Up-T, pKEK257.
‡CTXΦKan transductants/ml/OD_{600}.
§ng/ml/OD_{600}.
¶Miller units based on triplicate samples.
‖% adherent bacteria of total added; shown in parentheses are results of a second independent experiment.

Table 1 shows transcriptional activation of a *toxT*::*lacZ* chromosomal transcriptional fusion in the strains grown under inducing conditions (pH 6.5). The *toxR*$^+$ strain expressing OmpT in place of OmpU (Δ*ompU*/Up-T) displayed lower levels of *toxT* transcription compared with the same strain expressing OmpU (Δ*ompU*/Up-U) or a wild-type strain. Notably, the decrease in *toxT* transcription in the Δ*ompU*/Up-T strain is caused by the presence of OmpT rather than by the absence of OmpU, because the Δ*ompU* strain itself transcribed approximately wild-type levels of *toxT*. The same strains grown under noninducing conditions (pH 8.5) all transcribed low levels of *toxT* (15–20 Miller units; data not shown). We hypothesize that the relatively small observed changes in ToxR-dependent *toxT* transcription would result in the larger observed differences in CT and TCP expression due to ToxT driving its own transcription from the *tcpA* promoter (29), thus resulting in an amplification of ToxT levels. These results demonstrate that expression of OmpT in place of OmpU has a negative effect on *toxT* transcriptional activation, which appears to result in decreased virulence factor expression *in vitro*.

Adherence of *V. cholerae* to Cultured Epithelial Cells Is Not Affected by the Presence or Absence of OmpU or OmpT. Previous reports suggested a role for OmpU in *V. cholerae* adherence to cultured epithelial cells, because polyclonal antisera against OmpU could block adherence (15). By using the same experimental procedures, we tested the various *V. cholerae* strains expressing OmpU, OmpT, or neither, for adherence to HEp-2 cells (Table 1). Similar levels of adherence were seen, regardless of the presence or absence of either OmpU or OmpT, in both *toxR*$^+$ and *toxR*$^-$ strains. Microscopic evaluation of Giemsa-stained HEp-2 cells (not shown) confirmed the results obtained from the quantitative experiments.

Ectopic Expression of OmpT in Place of OmpU Reduces *V. cholerae* Intestinal Colonization. To determine the role of ToxR-dependent porin modulation in intestinal colonization, an *in vivo* competition experiment was performed in suckling mice. The *toxR*$^+$ strain expressing OmpU (Δ*ompU*/Up-U) colonizes like a wild-type strain, as expected, and has a competitive index similar to a wild-type strain [(competitive index) c.i. 2.3 ± 1.3; Fig. 3]. However, the *toxR*$^+$ strain expressing OmpT in place of OmpU (Δ*ompU*/Up-T) demonstrated a significant colonization defect ($P < 0.01$), being outcompeted ≈100-fold *in vivo* by the wild-type strain (c.i. 0.008 ± 0.006). The competitive index of this strain *in vitro* is similar to wild type (c.i. 0.591), indicating that the *in vivo* colonization defect is not caused by a general growth defect. These results suggest that expression of OmpT in place of OmpU is deleterious to *V. cholerae* intestinal colonization, and thus ToxR-dependent regulation of porins is critical for *V. cholerae* pathogenesis.

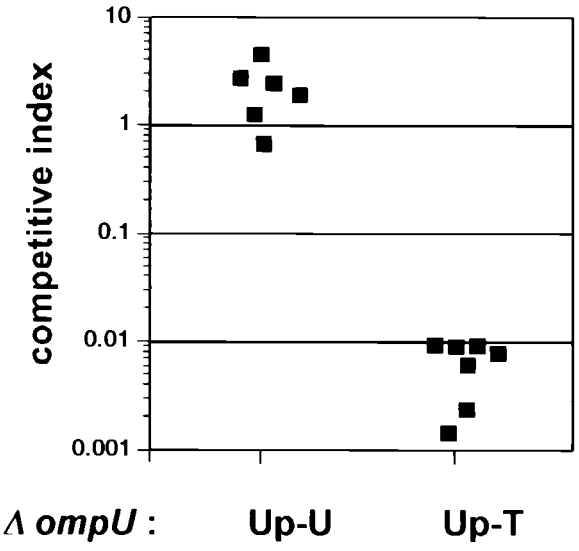

Fig. 3. The *V. cholerae toxR*$^+$ strain expressing OmpT in place of OmpU demonstrates a colonization defect. Assay was performed as described in *Materials and Methods*. Strain KKV780 (Δ*ompU*) carrying either plasmid pKEK253 (Up-U) or pKEK257 (Up-T) was coinoculated with isogenic strain KKV598 (O395 Δ*lacZ*). The competitive index is the ratio of output mutant/wild type (recovered from small intestine) divided by the ratio of input mutant/wild type (inoculated into mouse); thus, if a mutant strain has no colonization defect, we expect a competitive index close to 1. Data points represent individual mice. Value for colonization of KKV780/Up-T had a P value < 0.01 when compared with the value for colonization of KKV780/Up-U by using the Student's two-tailed *t* test.

Discussion

In the current study, the construction of a *toxR*$^+$ *V. cholerae* strain that expresses the ToxR-repressed porin OmpT in place of the ToxR-activated porin OmpU has allowed us to dissect the role these porins play in *V. cholerae* pathogenesis. Amazingly, merely substituting one outer membrane porin for another resulted in a number of attenuated pathogenic properties, including bile resistance, virulence factor expression, and intestinal colonization. ToxR-dependent regulation of outer membrane porins appears to have preceded the evolution of *V. cholerae*, because ToxR performs similar functions in other members of the *Vibrionaceae* (14, 17). However, these other bacteria do not have the additional regulatory factors TcpP and ToxT whose genes are located on the *V. cholerae*-specific pathogenicity island VPI (30). The recruitment of the ancestral ToxR protein into the virulence regulatory cascade involving recently acquired elements ties porin regulation to pathogenesis. We suggest that the contribution of porin regulation to bile resistance is one of the factors that led to the evolution of *V. cholerae* as an intestinal pathogen and the recruitment of ToxR into the virulence cascade.

Our results demonstrate that ToxR-dependent regulation of OmpU and OmpT clearly affects bile resistance, with OmpU playing a protective role relative to OmpT. The difference in bile sensitivity between *V. cholerae* containing OmpU or OmpT is likely caused by the selectivity of the porins themselves. The *E. coli* outer membrane porins OmpF and OmpC also affect bile resistance, with OmpC playing a protective role compared with OmpF (31). Both OmpF and OmpC are cation selective, but OmpC is more cation selective than OmpF and thus would be predicted to allow less passage of negatively charged molecules, such as bile salts or other anionic detergents (32). Our results are consistent with *V. cholerae* OmpU having similar ion selectivity to *E. coli* OmpC and *V. cholerae* OmpT having similar ion selectivity to *E. coli* OmpF. Presumably, the facilitated passage of anionic detergents through OmpT would lead to disruption of the bile-sensitive cytoplasmic membrane and lower growth rates at sublethal concentrations.

The presence of OmpT in place of OmpU in a *toxR*$^+$ strain leads to decreased *toxT* transcription, resulting in decreased CT and TCP expression. Thus, the ToxR-dependent regulation of the outer membrane porins directly affects virulence factor expression. pH is the inducing signal used to stimulate *toxT* transcription in the *in vitro* studies performed here, but the true environmental signals that induce *toxT* transcription within the intestine have not been identified (33). The ion selectivity of the porins likely affects the passage of charged inducing (or repressing) signals across the outer membrane, such as pH induction, that influence ToxR- and TcpP-dependent transcription of *toxT*. We have shown that ToxR-dependent transcription of *ompU* is

increased in the presence of bile (17). On arrival in the intestine, therefore, the presence of bile should induce increased ToxR-dependent OmpU expression (and simultaneous repression of OmpT), resulting in the correct synthesis of outer membrane porins necessary to allow maximal induction of virulence factor expression. Thus, one function of the ToxR-dependent and ToxT-independent *omp* regulatory branch (9) may be to facilitate sensing of the inducing signals that stimulate the ToxR- and ToxT-dependent *ctx* and *tcp* regulatory branch.

The *toxR*$^+$ strain expressing OmpT in place of OmpU is diminished in its ability to colonize the infant mouse intestine, demonstrating the importance of porin regulation in *V. cholerae* pathogenesis. Although we have not ruled out a role for OmpU as an adhesin *in vivo*, we were unable to demonstrate any decrease in adherence in strains lacking *ompU in vitro*. Rather, we speculate that the decreased bile resistance and virulence factor expression seen in this strain *in vitro* contribute to its decreased colonization *in vivo*. In *Bordetella* pathogenesis, ectopic expression of the Vir-repressed flagellar regulon reduces colonization (34). Our results demonstrate that the ectopic expression of the ToxR-repressed porin OmpT is deleterious to *V. cholerae* colonization, consistent with an important role for ToxR not only in the activation of specific genes, including *ompU* and *toxT*, but also repression of other gene(s), including *ompT*, during *V. cholerae* intestinal colonization. Previous reports have shown reduced virulence when certain pathogenic bacteria, including *Salmonella typhimurium* (35) and *Shigella flexneri* (36), contained knockout mutations in major outer membrane porins, which may have altered outer membrane structure and/or integrity. We have demonstrated virulence defects when one porin is substituted for another, suggesting a relationship between porin function and pathogenesis.

We thank J. Peterson for providing antisera, M. Waldor for providing CTXΦKan, C. Hase for providing the *toxT*::*lacZ* strain, and M. Blaylock for assistance with adherence assays. This work was supported by National Institutes of Health Training Grant AI07271–15 to D.P. and an institutional new faculty award of the Howard Hughes Medical Institute to K.E.K.

1. Kaper, J. B., Fasano, A. & Trucksis, M. (1994) in *Vibrio cholerae and Cholera: Molecular to Global Perspectives*, eds. Wachsmuth, I. K., Blake, P. A. & Olsvik, O. (Am. Soc. Microbiol., Washington, DC), pp. 145–176.
2. Taylor, R. K., Miller, V. L., Furlong, D. B. & Mekalanos, J. J. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 2833–2837.
3. Miller, V. L., Taylor, R. K. & Mekalanos, J. J. (1987) *Cell* **48,** 271–279.
4. Hase, C. C. & Mekalanos, J. J. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 730–734.
5. DiRita, V. J., Parsot, C., Jander, G. & Mekalanos, J. J. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 5403–5407.
6. Higgins, D. E., Nazareno, E. & DiRita, V. J. (1992) *J. Bacteriol.* **174,** 6974–6980.
7. DiRita, V. J., Neely, M., Taylor, R. K. & Bruss, P. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 7991–7995.
8. Miller, V. L. & Mekalanos, J. J. (1988) *J. Bacteriol.* **170,** 2575–2583.
9. Champion, G. A., Neely, M. N., Brennan, M. A. & DiRita, V. J. (1997) *Mol. Microbiol.* **23,** 323–331.
10. Chakrabarti, S. R., Chaudhuri, K., Sen, K. & Das, J. (1996) *J. Bacteriol.* **178,** 524–530.
11. Crawford, J. A., Kaper, J. B. & DiRita, V. J. (1998) *Mol. Microbiol.* **29,** 235–246.
12. Li, C. C., Crawford, J. A., DiRita, V. J. & Kaper, J. B. (2000) *Mol. Microbiol.* **35,** 189–203.
13. Osorio, C. R. & Klose, K. E. (2000) *J. Bacteriol.* **182,** 526–528.
14. Welch, T. J. & Bartlett, D. H. (1998) *Mol. Microbiol.* **27,** 977–985.
15. Sperandio, V., Giron, J. A., Silveira, W. D. & Kaper, J. B. (1995) *Infect. Immun.* **63,** 4433–4438.
16. Nakasone, N. & Iwanaga, M. (1998) *Infect. Immun.* **66,** 4726–4728.
17. Provenzano, D., Schuhmacher, D. A., Barker, J. L. & Klose, K. E. (2000) *Infect. Immun.* **68,** 1491–1497.
18. Hanahan, D. (1983) *J. Mol. Biol.* **166,** 577–580.
19. Mekalanos, J. J., Swartz, D. J., Pearson, G. D., Harford, N., Groyne, F. & de Wilde, M. (1983) *Nature (London)* **306,** 551–557.
20. Klose, K. E. & Mekalanos, J. J. (1998) *J. Bacteriol.* **180,** 303–316.
21. Donnenberg, M. S. & Kaper, J. B. (1991) *Infect. Immun.* **59,** 4310–4317.
22. Sperandio, V., Bailey, C., Giron, J. A., DiRita, V. J., Silveira, W. D., Vettore, A. L. & Kaper, J. B. (1996) *Infect. Immun.* **64,** 5406–5409.
23. Wang, R. F. & Kushner, S. (1991) *Gene* **100,** 195–199.
24. Svennerholm, A. M. & Holmgren, J. (1978) *Curr. Microbiol.* **1,** 19–23.
25. Waldor, M. K. & Mekalanos, J. J. (1996) *Science* **272,** 1910–1914.
26. Miller, J. H. (1992) *A Short Course in Bacterial Genetics* (Cold Spring Harbor Lab. Press, Plainview, NY).
27. Gardel, C. L. & Mekalanos, J. J. (1996) *Infect. Immun.* **64,** 2246–2255.
28. Schuhmacher, D. A. & Klose, K. E. (1999) *J. Bacteriol.* **181,** 1508–1514.
29. Medrano, A. I., DiRita, V. J., Castillo, G. & Sanchez, J. (1999) *Infect. Immun.* **67,** 2178–2183.
30. Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B. & Reeves, P. R. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3134–3139.
31. Thanassi, D. G., Cheng, L. W. & Nikaido, H. (1997) *J. Bacteriol.* **179,** 2512–2518.
32. Hancock, R. E. W., Schmidt, A., Bauer, K. & Benz, R. (1986) *Biochim. Biophys. Acta* **860,** 263–267.
33. Skorupski, K. & Taylor, R. K. (1997) *Mol. Microbiol.* **25,** 1003–1009.
34. Akerley, B. J., Cotter, P. A. & Miller, J. F. (1995) *Cell* **80,** 611–620.
35. Chatfield, S. N., Dorman, C. J., Hayward, C. & Dougan, G. (1991) *Infect. Immun.* **59,** 449–452.
36. Bernardini, M. L., Sanna, M. G., Fontaine, A. & Sansonetti, P. J. (1993) *Infect. Immun.* **61,** 3625–3635.