Vibrio cholerae OmpU and OmpT Porins Are Differentially Affected by Bile

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OmpT and OmpU are pore-forming proteins of the outer membrane of *Vibrio cholerae*, a pathogen that colonizes the intestine and produces cholera. Expression of the *ompU* and *ompT* genes is under the regulation of ToxR, a transmembrane transcriptional activator that also controls expression of virulence factors. It was recently shown that bile stimulates the ToxR-mediated transcription of *ompU* and that *ompU*-expressing strains are more resistant to bile and anionic detergents than *ompT*-expressing cells. In order to further understand the role of the OmpT and OmpU porins in the ability of *V. cholerae* to survive and colonize the host intestine, we examined the outer membrane permeability of cells expressing only *ompU* or only *ompT* or both genes in the absence and in the presence of bile. By comparing various strains in terms of the rate of degradation of the β -lactam antibiotic cephaloridine by the periplasmic β -lactamase, we found that the permeation of the antibiotic through the outer membrane of OmpU-containing cells was slower than the permeation in OmpT-containing cells. In addition, the OmpU-mediated outer membrane permeability was not affected by external bile, while the OmpT-mediated antibiotic flux was reduced by bile in a concentration-dependent manner. Our results confirm that OmpT and OmpU provide a passageway for hydrophilic solutes through the outer membrane and demonstrate that bile might interfere with this traffic in OmpT-producing cells by functionally inhibiting the OmpT pore. The insensitivity of OmpU to bile may be due to its small pore size and may provide an explanation for the resistance of OmpU-producing cells to bile in vivo.

Vibrio cholerae is a gram-negative bacterium that causes cholera. This bacterium colonizes the small intestine and, upon induction of virulence factors, stimulates diarrhea. Regulation of the virulence factors expressed by pathogenic strains of this bacterium has been the subject of many studies (for reviews, see references 7 and 24). Many of the virulence factor genes belong to the *toxR* regulon, which contains more than 20 genes. The transmembrane protein transcriptional activator, ToxR, and a second activator, TcpP, are required for transcription of the *toxT* gene, which regulates the expression of several virulence factors, including cholera toxin, which is primarily responsible for the diarrhea, and the toxin-coregulated pilus, an essential intestinal colonization organelle (6, 9, 12, 15, 27). Independent of *toxT* and *tcpP*, ToxR also regulates transcription of the genes encoding two putative outer membrane porins, *ompU* and *ompT*, inducing expression of *ompU* and repressing expression of *ompT* (3, 13, 14). The role that these proteins play in the pathogenicity of the organism remains unclear.

The porin-like character of OmpU and OmpT was initially shown by the association of these proteins with peptidoglycan and their temperature- and sodium dodecyl sulfate (SDS)-sensitive multimeric assembly (2), as well as their osmoregulated expression (2, 14). The ability of the multimeric forms

(but not the monomeric forms) to provide a passageway for solute flux when they were reconstituted into liposomes also strongly suggested that these proteins function as pores in the outer membrane (2). This was later confirmed for OmpU by an electrophysiological analysis of channel activity (1). OmpU has also been reported to act as an adhesin (25), and this property and porin activity are not necessarily mutually exclusive. Cloning and molecular characterization of the *ompU* and *ompT* genes have been carried out at the same time as functional characterization of the proteins (13, 26). These studies have revealed some homologies of OmpU and OmpT with porins from *Escherichia coli* and other species.

Expression of the virulence factors is coordinately regulated in response to certain environmental stimuli, including bile, pH, temperature, and osmolarity (8, 14, 24). This is also the case for regulation of porin expression (14). Recently, it has been demonstrated that the ToxR-dependent transcription of *ompU* is stimulated in the presence of bile (20). The increased synthesis of OmpU appears to confer resistance to bile, as the minimum bactericidal concentrations of bile and the bile component deoxycholate were lower for a Δ *toxR* strain (with an OmpU-deficient phenotype) than for the wild-type parent (20). The correlation between sensitivity to bile and the type of porin present in the *V. cholerae* outer membrane was convincingly confirmed in experiments in which cells were genetically manipulated to express *ompU* in place of *ompT* and vice versa, independently of *toxR* expression (21). Thus, cells with only OmpU in the outer membrane are more resistant to deoxycholate than cells with only OmpT. Cells that express *ompT*

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TABLE 1. *V. cholerae* strains and plasmid^a

Strain or plasmid	Genotype	Plasmid	Parent strain	Phenotype
Strains				
KKV1102	Wild type	pKEK340	O395	OmpU ⁺ OmpT ⁺
KKV1107	$\Delta ompT$		KKV809	OmpU ⁺ OmpT ⁻
KKV1115	$\Delta toxR \Delta ompT$		KKV986	OmpU ⁺ OmpT ⁻
KKV1103	$\Delta toxR$		KKV61	OmpU ⁻ OmpT ⁺
KKV1106	$\Delta ompU1$		KKV669	OmpU ⁻ OmpT ⁺
KKV1108	$\Delta ompU2$		KKV780	OmpU ⁻ OmpT ⁺
KKV1109	$\Delta toxR \Delta ompT$		KKV804	OmpU ⁻ OmpT ⁻
KKV1222	$\Delta ompU2 \Delta ompT$		KKV884	OmpU ⁻ OmpT ⁻
Plasmid				
pKEK340	Expresses <i>ompU</i> from <i>ompT</i> promoter			

^a All of the strains also contain the pBR322 plasmid for periplasmic β -lactamase secretion. The pKEK340 plasmid and the parent strains (i.e., strains without pBR322) have been described previously (23).

instead of *ompU* also have reduced virulence factor expression and are deficient in the ability to colonize the intestine (21).

In order to further understand the physiological role of these proteins, we examined the effect of bile on several strains of *V. cholerae* by using an antibiotic permeation assay. Our results demonstrate that the permeation properties of OmpU and OmpT are affected differently by bile: OmpU allows diffusion of the antibiotic to proceed in the presence of bile, while diffusion of the antibiotic through OmpT is slowed in the presence of bile.

MATERIALS AND METHODS

Cell lines. All of the *V. cholerae* strains and plasmids used in this study are shown in Table 1. Strains containing a pBR322 plasmid for β -lactamase production were constructed for this study.

Media and chemicals. Luria-Bertani (LB) broth contains 1% trypto-peptone (Difco), 0.5% yeast extract (Difco), and 1% NaCl (E.M. Science). Cephaloridine, bile (oxgall powder), and all other standard chemicals were obtained from Sigma.

Antibiotic permeation assay. The rate of permeation of the β -lactam antibiotic cephaloridine was determined as described previously (17). Briefly, a fresh culture was inoculated from an overnight culture grown from a single colony. Cells were grown at 37°C with shaking at 250 rpm in LB broth supplemented with 5 mM MgCl₂ and the appropriate antibiotics until the optical density at 650 nm (OD₆₅₀) was 0.6. The culture was collected by centrifugation and washed twice with permeability buffer (10 mM NaP_i, 5 mM MgCl₂; pH 6.0). The assay was performed by adding 100 μ l of a 5 mM cephaloridine stock solution to 100 μ l of cell suspension and 300 μ l of permeability buffer with or without bile. The change in OD₂₆₀ due to degradation of the antibiotic by the β -lactamase was monitored for 5 min with a Milton-Roy Spectronic spectrophotometer, and the rate was calculated with the associated kinetic software. Each strain was assayed at least three times. Measurements were also obtained for cell-free culture supernatants, which is the typical procedure for controlling for enzyme leakage due to lysis of cells. Each cell-free supernatant was obtained by spinning down a 1.5-ml aliquot of the cell suspension for 1 min with a microcentrifuge, and the assay was performed as described above by using 100 μ l of the cell-free supernatant in lieu of cells. The degradation rates obtained for cell supernatants were no more than 12% of the rate measured for intact cells. These values indicated that the enzyme leakage was minimal. This was confirmed by a Western blot analysis of 100 $\times$ -concentrated cell-free supernatants that were collected from each of the strains and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). The nitrocellulose blot was probed with a 1:500 dilution of antisera raised against β -lactamase (5Prime $\rightarrow$ 3Prime Inc.) and developed with an ECL kit (Amersham), which revealed no detectable bands (22). The effect of bile on β -lactamase activity was determined by sonicating cells that were producing the enzyme and measuring the rate of change in the resulting supernatant in the presence and in the absence of 0.6% bile.

Detection of protein expression. Whole-cell lysates were prepared from bacteria grown in LB broth at 37°C. Overnight cultures were matched on the basis of OD₆₀₀ and centrifuged at 13,000 rpm for 3 min. Cell pellets were resuspended directly in sample buffer, boiled for 5 min, resolved by SDS-10% PAGE, and stained with Coomassie blue. Protein gels were transferred to nitrocellulose for

Western blot analysis by using a transblotter (Bio-Rad). The blots were probed with rabbit polyclonal antisera against *V. cholerae* OmpT or OmpU and developed with the ECL detection system (Amersham).

RESULTS

Porin expression. Our goal was to determine whether the permeation properties of OmpU and OmpT are differentially affected by bile in live cells. To accomplish this goal, we selected several *Vibrio* strains that were readily available to us and that express both porins, only OmpU, only OmpT, or neither porin (Table 1). To obtain an OmpU⁺ OmpT⁻ phenotype, we used cells containing chromosomal deletions of *ompT* (KKV1107) or *toxR* and *ompT* in the presence of pKEK340, a plasmid expressing *ompU* from the *ompT* promoter (KKV1115). Since ToxR is a repressor of *ompT* (13) and an activator of *ompU* (3), strains deficient in *toxR* synthesize OmpT and not OmpU. In KKV1115, however, there is no synthesis of OmpT because of the chromosomal *ompT* deletion; the OmpU protein is nonetheless produced because the relief of repression of the *ompT* promoter by ToxR drives expression of *ompU* from the pKEK340 plasmid. The OmpU⁻ OmpT⁺ phenotype was obtained with cells with chromosomal deletions either in *toxR* (KKV1103) or in *ompU* (KKV1106, KKV1108). Finally, double mutants had chromosomal deletions either in both *ompU* and *ompT* (KKV1222) or in both *toxR* and *ompT* (KKV1109), in which the absence of ToxR prevented activation of *ompU*. All these strains also contained pBR322 for β -lactamase expression (see below). The parent strains without pBR322 have been described previously (23), and their designations are also given in Table 1.

To verify phenotypically the lack of OmpU or OmpT in the strains with genetically altered porin expression, we performed SDS-PAGE with whole-cell lysates from the different strains. The Western blot in Fig. 1B shows that the wild-type strain expressed both porin types and that the double mutants were completely devoid of OmpU or OmpT. KKV1107 and KKV1115, which were $\Delta ompT$, expressed OmpU exclusively. KKV1106 and KKV1108, which were $\Delta ompU$, as well as the $\Delta toxR$ strain KKV1103, expressed OmpT exclusively. The Western blot showed unambiguously that either OmpU or OmpT was not present in the relevant strains and thus confirmed that the strains had the expected porin phenotypes. Relative amounts of OmpU and OmpT could not be determined from the Western blots due to the use of different

FIG. 1. Protein profiles of *V. cholerae* strains. (A) Whole-cell lysates were separated from KKV1102 (OmpU⁺ OmpT⁺, wild type) (lane 1), KKV1107 (OmpU⁺ OmpT⁻ Δ ompT) (lane 2), KKV1115 (OmpU⁺ OmpT⁻ Δ toxR Δ ompT/pKEK340, which expresses *ompU* from the *ompT* promoter) (lane 3), KKV1103 (OmpU⁻ OmpT⁺ Δ toxR) (lane 4), KKV1106 (OmpU⁻ OmpT⁺ Δ ompU1) (lane 5), KKV1108 (OmpU⁻ OmpT⁺ Δ ompU2) (lane 6), KKV1109 (OmpU⁻ OmpT⁻ Δ toxR Δ ompT) (lane 7), and KKV1222 (OmpU⁻ OmpT⁻ Δ ompU2 Δ ompT) (lane 8). Samples were matched by using equivalent OD₆₀₀ units, separated by SDS-10% PAGE, and stained with Coomassie blue. The molecular masses (in kilodaltons) of the markers are indicated on the left. The arrows indicate the relative migration positions of OmpU (arrow U) and OmpT (arrow T). (B) Whole-cell lysates from the wild type and *omp* mutants (see above) were subjected to Western blot analysis (see text) by utilizing rabbit polyclonal antisera against OmpT (α -T) and OmpU (α -U).

antisera and different development times. However, relative amounts could be estimated by direct measurement of the band intensities in the Coomassie-stained gel, which indicated that similar levels of OmpU and OmpT were present in the strains genetically manipulated to express *ompU* or *ompT* exclusively. A scanning densitometry analysis of the OmpU and OmpT bands was performed with the Scion Image software. This analysis revealed that the intensities of the OmpU bands in lanes 2 and 3 did not differ by more than 20% from the intensities of the OmpT bands in lanes 4 to 6.

Cephaloridine permeation in live *V. cholerae* cells. Permeation of β -lactam antibiotics, such as cephaloridine, is known to require the general diffusion porins (OmpF, OmpC, or PhoE) in *E. coli* (16). We hypothesized that this would be the case for the *Vibrio* porins as well and performed antibiotic flux assays similar to those performed with *E. coli*. These assays measured the rate of disappearance of external antibiotic. Since degradation of the antibiotic by the periplasmic β -lactamase is extremely fast, the measured decrease in the external antibiotic concentration is limited by the rate of permeation through the porins (17). Figure 2 shows that we observed rates greater than 0.01 OD₂₆₀ unit/min with all strains except the double mutant strains. These rates are comparable to the rates that we typically obtain for *E. coli* cells expressing only OmpF ($\sim$ 0.03 OD₂₆₀ unit/min) or only OmpC ($\sim$ 0.02 OD₂₆₀ unit/min). The rates obtained with cell-free supernatants were on the order of 6 to 12% of the rates obtained with cells, indicating that the latter were not due to leakage of the β -lactamase.

It is noteworthy that the two strains that synthesize only OmpU had flux rates that were significantly less than the flux rate of strains expressing only OmpT in the outer membrane. The wild-type strain had an intermediate flux value.

Effect of bile on flux rates. When the cephaloridine flux assay was performed in the presence of 0.6% bile, an average reduction of 70% in the absolute rate was observed in cells expressing only OmpT (Fig. 3). This reduction was observed for the Δ toxR (KKV1103) strain, as well as for both Δ ompU strains (KKV1106 and KKV1108). Since the assay was performed in 5 min, this inhibition did not reflect an effect of bile on porin expression. It is reasonable to interpret this result as due to rapid functional inhibition of OmpT by bile, as observed for polyamine inhibition of *E. coli* porins (4).

Permeation through OmpU was not affected by the presence of 0.6% bile in the assay mixture. The flux rate remained almost 100% of the control rate (in the absence of bile) regardless of the strain used (i.e., regardless of whether the gene was expressed from the chromosome [KKV1107] or the pKEK340 plasmid [KKV1115]). In wild-type cells (KKV1102), which contained both OmpT and OmpU, the flux decreased by $\sim$ 40%. This value was intermediate between the value obtained for strains expressing only *ompU* and the value obtained for strains expressing only *ompT* and most likely represented the composite effect of bile on the individual OmpT and OmpU porins present in the wild-type membrane.

We attempted to measure the effect of bile on the small residual flux observed in the double mutant strains, KKV1109 and KKV1222. In both cases, no inhibition of flux was observed, but rather there was some increase in the flux value, which was most likely due to release of the periplasmic β -lac-

FIG. 2. Antibiotic flux in *V. cholerae* strains. The absolute flux values are plotted on the ordinate as the changes in external cephaloridine concentration (expressed in OD₂₆₀) per minute. The strain designations are indicated at the bottom, and the different kinds of bars indicate different porin phenotypes. Measurements were made in triplicate in each experiment, and the experiments were repeated three to eight times. The error bars indicate standard errors of the means. WT, wild type; U, OmpU; T, OmpT.

FIG. 3. Effect of bile on the antibiotic flux in *V. cholerae* strains. The ordinate indicates the ratio of the rate of antibiotic degradation in the presence of 0.6% external bile to the rate of antibiotic degradation in the absence of bile, determined with the same cell suspension for each strain. The strain designations are indicated at the bottom, and the different kinds of bars indicate the different porin phenotypes. Measurements were made in triplicate in each experiment, and the experiments were repeated three to seven times. The error bars indicate standard errors of the means. WT, wild type; U, OmpU; T, OmpT.

tamase in the presence of bile. This is not surprising, as these porin-deficient strains are likely to have a somewhat more fragile outer membrane. Finally, we determined that 0.6% external bile caused a 35% reduction in the activity of β -lactamase released by sonication.

The reduction in the rate of cephaloridine uptake in OmpT-expressing cells (KKV1108) was dependent on the concentration of external bile during the assay, as shown in Fig. 4. Flux inhibition became evident at bile concentrations greater than 0.01%, and the level of inhibition plateaued at ~80% in the presence of 1.2% bile. Further addition of bile resulted in skewed values, possibly due to the effect of bile on the membrane, and release of the periplasmic enzyme. On the other hand, the flux rate of cells expressing OmpU (KKV1107) did not change over the entire range of bile concentrations tested.

DISCUSSION

It has been suggested that the outer membrane proteins OmpU and OmpT of *V. cholerae* are porins based on results of liposome swelling assays and protein sequence homology to the *E. coli* porins OmpF and OmpC (2, 13, 26). Previous studies have demonstrated that permeation of β -lactam antibiotics in *E. coli* occurs primarily through the porins OmpC and OmpF (16, 17). Using an assay that measures the rate of diffusion of such an antibiotic through the outer membrane of intact *Vibrio* cells, we demonstrated here that both OmpU and OmpT are capable of providing a pathway for molecules through the outer membrane. In *V. cholerae* strains constructed so that both OmpU and OmpT were deleted from the outer membrane (KKV1109 and KKV1222), the rate of diffusion was decreased by more than 90%. The small residual flux rate may have been due to the appearance of an OmpA-like protein that is overexpressed in the double mutant strains

KKV804 ($\Delta toxR \Delta ompT$) and KKV884 ($\Delta ompU2 \Delta ompT$) (23). Although whether OmpA has channel properties in *E. coli* (5) is still debated and nothing is known about the OmpA homolog in *Vibrio*, it is possible that this protein is responsible for the low (but measurable) flux in strains lacking both OmpU and OmpT. Using Western blot analysis, Provenzano et al. (23) showed that this protein is essentially absent in strains expressing OmpU and/or OmpT. Therefore, the antibiotic flux measured here for strains expressing OmpU or OmpT or both essentially reflects the behavior of the dominant porin (i.e., OmpU or OmpT). Thus, this minor porin is unlikely to compromise our interpretation of the data for these strains.

The antibiotic flux rate is about two- to threefold lower in strains producing only OmpU than in strains producing only OmpT. The rate of antibiotic permeation depends on several factors, including the number of open porins, pore size, and interactions between the drug and the channel wall. The Coomassie blue-stained gel suggested that the OmpU and OmpT levels are comparable, and thus OmpU might form smaller pores, but this needs to be confirmed by more precise measurements, such as electrophysiological measurements of single-pore conductance. A difference in pore size also exists between OmpC and OmpF of *E. coli* (4, 5, 18). Chakrabarti and colleagues (2) have proposed that OmpU forms larger pores than OmpT forms. Their results were based on the rate of diffusion of arabinose and stachyose in proteoliposomes containing purified OmpU or OmpT or outer membrane fragments. As discussed by Nikaido (19), great care has to be taken in the interpretation of channel sizes derived from relative rates of diffusion and from hydration radii of sugars because of the very large differences in penetration rates, even among solutes whose sizes are within the exclusion limit. In addition, calculation of channel size is based on the assumption that the

FIG. 4. Concentration dependence of the bile effect. The ordinate indicates the ratio of the rate of antibiotic degradation in the presence of various external bile concentrations to the rate of antibiotic degradation in the absence of bile, determined with the same cell suspension for each strain. Symbols: $\circ$, KKV1107 expressing only *ompU*; $\bullet$, KKV1108 expressing only *ompT*. Measurements were made in triplicate in each experiment, and the experiments were repeated three to seven times. The error bars indicate standard errors of the means and sometimes lie within the thickness of the symbol.

channel is a perfect cylinder through which the substrates diffuse as they do in water, without interacting with the protein, which is known not to be the case. As stated above, a definite answer regarding the relative sizes of OmpT and OmpU will have to wait for more refined measurements.

Within a human host, *V. cholerae* colonizes the intestinal epithelia, and the virulence cascade is triggered by an as-yet-undefined environmental signal(s). Bile has been shown to induce ToxR-dependent (and *toxT*-independent) expression of *ompU* in the outer membrane in vitro (20) and may be one such signal found in the intestine. In addition, *V. cholerae* Δ *toxR* strains expressing *ompU* from a plasmid had higher growth rates in the anionic detergents deoxycholate (also found in bile) and SDS than Δ *toxR* strains synthesizing plasmid-encoded OmpT (21). This suggests that OmpU may play a role in virulence by selectively excluding the toxic bile anions from the cell and allowing *ompU*-expressing cells to survive in the intestine. These observations are consistent with our finding that bile does not seem to interact with OmpU, as the rate of diffusion of the β -lactam is essentially unaffected. Since we have shown that bile itself reduces the activity of the β -lactamase, the lack of reduction of the rate in the *ompU*-expressing strains also indicates that bile, but not cephaloridine, is excluded from this passageway.

The reduced activity of β -lactamase in OmpT-expressing cells in the presence of bile is probably due to a combination of factors, including (i) the effect of bile on the β -lactamase itself (accounting for ~35% of the reduction in the presence of 0.6% external bile) and (ii) reduced entry of cephaloridine into the periplasm. The latter phenomenon may itself have two distinct origins, which may act together: (i) as bile penetrates through OmpT, it effectively competes with cephaloridine; and (ii) bile acts as an allosteric inhibitor of OmpT, effectively decreasing the number of open porins available for antibiotic entry. The second scenario does not necessarily preclude entry into OmpT, as bile components might bind to multiple sites on the porin or even exert multiple types of effects from the same internal binding site. This is exactly what is observed with the binding of spermine inside the OmpF pore in *E. coli* (10, 11). The intermediate value for rate reduction observed in the presence of bile in wild-type cells expressing both OmpT and OmpU is further evidence that the two porins respond differently to bile.

Since the activity of released β -lactamase is reduced 35% in the presence of bile, the total lack of an effect of bile on antibiotic degradation in OmpT-deficient (i.e., OmpU-containing) cells suggests that very little, if any, bile permeates the outer membrane in such cells. Not only does bile not inhibit the remaining porin (i.e., OmpU), but it also does not permeate the outer membrane (either through OmpU or lipids) and come in contact with the periplasmic β -lactamase (otherwise, we would have seen a 35% reduction in the enzymatic rate due to the effect of bile on the enzyme itself). This is a very significant observation. The resistance of OmpU to inhibition by bile and the apparent lack of bile permeation through OmpU, even at concentrations as high as 1.2%, provide an explanation for the observation that cells expressing only *ompT* have lower growth rates in the presence of deoxycholate (a major component of bile) than cells expressing only *ompU*. As bile moves through the outer membrane of OmpT-containing cells, it

competes with nutrient entry (by inhibiting OmpT), causes damage to the inner membrane, and has deleterious effects in the cell. This is not the case in OmpU-containing cells, as our results suggest that OmpU may not provide a pathway for bile across the outer membrane.

Thus, our results confirm that OmpU plays a protective role in *V. cholerae* in the bile-containing environment of the intestine. While neither OmpU nor OmpT is essential for virulence factor expression in vitro or colonization in vivo (23), the preferential expression of *ompU* appears to play an important role in pathogenicity by providing a pathway for nutrient import into the intestine without compromising cellular integrity in the presence of bile. It is interesting that bile itself may be responsible for triggering increased *ompU* expression in *V. cholerae*. It is possible that the presence of OmpT in the early stages of infection allows some small bile influx, which acts as a triggering environmental signal. Only a detailed mechanistic investigation of the interactions between each of the two porins and bile components will be able to verify this proposition.

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