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Effect of Bile on the Cell Surface Permeability Barrier and Efflux System of *Vibrio cholerae*

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Gram-negative bacteria are inherently impermeable to hydrophobic compounds, due to the synergistic activity of the permeability barrier imposed by the outer membrane and energy dependent efflux systems. The gram-negative, enteric pathogen *Vibrio cholerae* appears to be deficient in both these activities; the outer membrane is not an effective barrier to hydrophobic permeants, presumably due to the presence of exposed phospholipids on the outer leaflet of the outer membrane, and efflux systems are at best only partially active. When *V. cholerae* was grown in the presence of bile, entry of hydrophobic compounds into the cells was significantly reduced. No difference was detected in the extent of exposed phospholipids on the outer leaflet of the outer membrane between cells grown in the presence or absence of bile. However, in the presence of energy uncouplers, uptake of hydrophobic probes was comparable between cells grown in the presence or absence of bile, indicating that energy-dependent efflux processes may be involved in restricting the entry of hydrophobic permeants into bile grown cells. Indeed, an efflux system(s) is essential for survival of *V. cholerae* in the presence of bile. Expression of *acrAB*, encoding an RND family efflux pump, was significantly increased in *V. cholerae* cells grown in vitro in the presence of bile and also in cells grown in rabbit intestine.

Vibrio cholerae, a noninvasive enteric bacterium, is the causative agent of the diarrheal disease cholera. Cholera remains a major cause of human mortality in developing countries, where conditions of poor sanitation, war, famine, and malnourishment contribute to regular episodes of cholera epidemics. For successful infection of its human host, *V. cholerae* must colonize the small intestine and produce copious amounts of cholera toxin (CT), a potent enterotoxin that causes the massive fluid loss characteristic of the disease. In addition to obvious virulence factors like CT, other toxins, toxin-coregulated pilus, hemolysins, and hemagglutinins (factors essential for survival of the bacteria in vivo and evasion of the host defense system) also contribute to the pathogenecity of *V. cholerae* (for a review, see references 12 and 24).

Enteric pathogens and normal intestinal flora must necessarily survive and colonize the intestine in the presence of bile. Bile salts are surface-active, amphipathic compounds with pronounced detergent-like activity that can cause disaggregation of the lipid bilayer structure of cellular membranes (11). However, gram-negative enteric bacteria are inherently resistant to bile, partly due to the basic, asymmetric structure of their outer membranes (OMs). Although the inner surface of the OM contains phospholipids, a characteristic lipopolysaccharide (LPS) is present on the outer leaflet that significantly retards diffusion of hydrophobic compounds across the OM. The OM thus functions as an effective permeability barrier and gives protection to enteric bacteria from potentially noxious agents present in the intestine, particularly hydrophobic and amphipathic compounds, including bile salts (18). However, the protection provided by the OM is partial; the OM must function in conjunction with energy-dependent efflux systems, which can exclude substrates with a wide range of specificity from the cell (9, 19, 20). Several families of active efflux systems in gram-negative bacteria have been described (13, 17, 22).

Bile affects expression of virulence factors, motility, and production of the OM porins OmpU and OmpT in *V. cholerae*. Although motility of the cells increase significantly in the presence of bile, expression of the major virulence factors CT and toxin coregulated pilus is drastically reduced (8). Furthermore, bile stimulates production of OmpU with concomitant repression of OmpT synthesis (26).

In this study, we report that unlike the membranes of other gram-negative enteric bacteria, the OM of smooth-type *V. cholerae* is exceptionally permeable to hydrophobic compounds. However, growth of *V. cholerae* in the presence of bile restricts entry of hydrophobic compounds, primarily due to the induction of active efflux systems.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *V. cholerae* O1 strain O395 of the classical biotype and strain N16961 of the El Tor biotype and the wild-type *Escherichia coli* strain ZK126 (4) were used in this study. The strains were grown with aeration at 37°C in Luria-Bertani (LB) medium containing 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl. The bile used was a crude ox bile extract containing sodium salts of taurocholic, glychocholic, deoxycholic, and cholic acids (catalog no. 9875; Sigma). Bile was used at a concentration of 0.4% unless otherwise stated in the text.

Measurement of drug susceptibility. The MIC of antibiotics was determined by serial twofold dilutions of drugs in LB medium with an inoculum of 5×10^4 exponential-phase cells per ml. Turbidity at 600 nm (optical density at 600 nm) was measured after 16 h of incubation at 37°C with aeration. Optical density at 600 nm of less than 0.05 was considered negative.

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Uptake of hydrophobic compounds. *V. cholerae* was grown in LB medium or LB medium containing 0.4% bile to the logarithmic phase (2×10^8 to 3×10^8 CFU ml^{-1}), washed with phosphate-buffered saline (PBS), and suspended in an equal volume of PBS. Crystal violet (CV) (5 μg ml^{-1}) was added to the cells, aliquots were removed at regular intervals, and CV remaining in the supernatant was estimated by measuring absorbance at 590 nm. In some experiments, 50 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added prior to the addition of CV. To measure uptake of *N*-phenyl naphthylamine (NPN), 10 μM NPN was added to exponentially growing cells in PBS or in PBS containing 1 mM KCN, and fluorescence was monitored with a fluorescence spectrophotometer (F-4500; Hitachi) set as follows: excitation, 350 nm; emission, 420 nm; slit widths, 2.5 nm (28). NPN binding by isolated LPS was measured by adding 10 μM NPN to LPS aggregates (400 μg ml^{-1}) in PBS (2).

LPS preparation. Crude LPS preparations obtained by hot phenol-water extraction (16) were dispersed in 0.05 M $MgCl_2$–0.125 M sodium acetate buffer (pH 7), digested with nucleases, and freeze-dried. Samples were then suspended in 0.01 M $CaCl_2$–0.1 M Tris-HCl (pH 7.3) buffer, digested with proteinase K, dialyzed extensively, freeze-dried, suspended in water, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. To measure the binding of NPN to LPS aggregates, the LPS was thoroughly dispersed by sonication, supplemented with an equal volume of 0.2% sodium deoxycholate in 0.1 M Tris-HCl (pH 8.5), incubated at room temperature for 15 min, and precipitated with 6 volumes of ethanol at −20°C for 18 h. The precipitate was sedimented by centrifugation, washed with ethanol, suspended in water, and dialyzed, and the concentration was adjusted to 400 μg ml^{-1} (2). NPN binding by LPS aggregates was determined as described for whole cells.

Dansylation of whole cells. *V. cholerae* cells grown to the logarithmic phase in the presence or absence of bile were dansylated following the method of Paul et al. (21). Briefly, dansyl chloride-cyclodextrin complex was added to cells suspended in 1 mM $MgCl_2$–50 mM borate buffer (pH 8.5) and stirred in darkness for 60 min at room temperature. Cells were washed repeatedly, and OMs and inner membranes (IMs) were isolated from the treated cells as previously described (8). Phospholipids were extracted from both membranes, separated by thin-layer chromatography (TLC), and visualized under UV light (21, 25).

RNA isolation and RT-PCR. For isolation of RNA, cells were grown to the logarithmic phase and total RNA was extracted and purified with guanidium isothiocyanate (1). The RNA was treated with RNase free DNase 1 (amplification grade; Gibco-BRL) and reverse transcription-PCR (RT-PCR) was performed with a single-tube RT-PCR kit (Gibco-BRL) in the presence of an RNase inhibitor (RNasin; Gibco-BRL). A total of 200 ng of DNase-treated RNA was used in all reaction mixtures. Amplification was for 25 to 35 cycles (each cycle consisted of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by a 7-min extension at 72°C). Genomic DNA served as a positive control, and DNase-treated RNA that had not been reverse transcribed was used as a negative control. Primers for RT-PCR, corresponding to internal regions of the open reading frames, were designed based on the *V. cholerae* genome sequence (10). Aliquots removed at 25, 30, and 35 cycles of each PCR were electrophoresed, and the gels were analyzed with a Gel Doc 1000 system (Bio-Rad Laboratories). PCR products were normalized according to the amount of 16S rRNA detected in the same cDNA sample. Each set of experiments was repeated at least thrice. Comparisons between the relative intensities of PCR products were made with the two-sample *t* test.

Ligated rabbit ileal loop model. In vivo expression of *acrA* in *V. cholerae* was assayed by using the ligated rabbit ileal loop model (5, 7). Strain O395 or N16961 (~5×10^6 CFU) was inoculated into individual ligated rabbit ileal loops; after 16 h, the animals were sacrificed. Bacteria were recovered from each loop, and RNA was isolated and used for RT-PCR. Each bacterial strain was inoculated into at least two loops in each animal, and each strain was tested in at least three individual animals.

RESULTS

Sensitivity of *V. cholerae* to hydrophobic compounds is reduced when grown in the presence of bile. *V. cholerae* is extremely sensitive to a wide variety of compounds, particularly hydrophobic and amphipathic agents (21). It is about 100 times more sensitive than *E. coli* to novobiocin, a hydrophobic antibiotic that has been used as an indicator of bacterial OM permeability to hydrophobic compounds (Table 1). However, when *V. cholerae* was grown in the presence of 0.4% bile, the MIC of novobiocin increased by more than 10-fold compared to the novobiocin MIC for cells grown under identical conditions without bile. The presence of up to 5% bile in LB had no effect on the growth rates of strains O395 and N16961. The wild-type *E. coli* strain ZK126 also grew normally in the presence of bile; but unlike *V. cholerae*, growth of ZK126 in the presence of 0.4% bile had very little effect on the MIC of novobiocin for *E. coli* (Table 1).

TABLE 1. Novobiocin sensitivity of *V. cholerae* and *E. coli*[a]

Strain	Novobiocin MIC (μg ml^{-1})	
	− Bile	+ Bile
V. cholerae O395	2	35
V. cholerae N16961	3	40
E. coli ZK126	300	400

[a] Experiments were performed with triplicate samples from two independent cultures grown in the absence (−) or presence (+) of 0.4% bile. Standard errors were less than 10% in all cases.

Uptake of hydrophobic compounds into bile-grown *V. cholerae* cells. To examine whether growth of *V. cholerae* in the presence of bile had any effect on permeability to hydrophobic compounds, uptake of the hydrophobic dye CV and the fluorescent hydrophobic probe NPN by cells grown in the presence or absence of bile was estimated. To measure CV uptake, *V. cholerae* strains O395 and N16961 were grown to the logarithmic phase in LB with or without 0.4% bile, washed, and suspended in buffer containing 5 μg of CV ml^{-1}. At regular time intervals, samples were withdrawn and centrifuged, and the amount of dye in the supernatant was estimated by measuring turbidity at 590 nm. More than 70% of the added CV was taken up in about 10 min by strain O395 grown without bile, although less than 20% CV uptake was observed in cells grown in the presence of the bile (Fig. 1A). In strain N16961 of the El Tor biotype, about 40% CV uptake was observed when the cells were grown without bile, and the uptake was reduced to about 15% in bile-grown cells (Fig. 1B). Fractionation of the cells into membrane and cytoplasmic constituents indicated that the incorporated dye was distributed almost equally be-

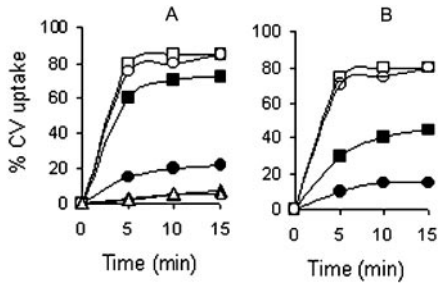

FIG. 1. CV uptake by *V. cholerae* O395 and *E. coli* ZK126 (A) and *V. cholerae* N16961 (B). Cells were grown to the logarithmic phase in the absence or presence of 0.4% bile and suspended in buffer containing CV (5 μg ml^{-1}) with or without 50 μM CCCP. CV uptake was measured at regular time intervals and expressed as a percentage of added CV taken up by 10^8 CFU of each strain. Results represent the average of five independent experiments. Symbols: *V. cholerae* strains (solid squares), *V. cholerae* plus CCCP (open squares), *V. cholerae* plus bile (solid circles), *V. cholerae* plus bile plus CCCP (open circles), *E. coli* ZK126 (solid triangles), *E. coli* ZK126 plus bile (open triangles).

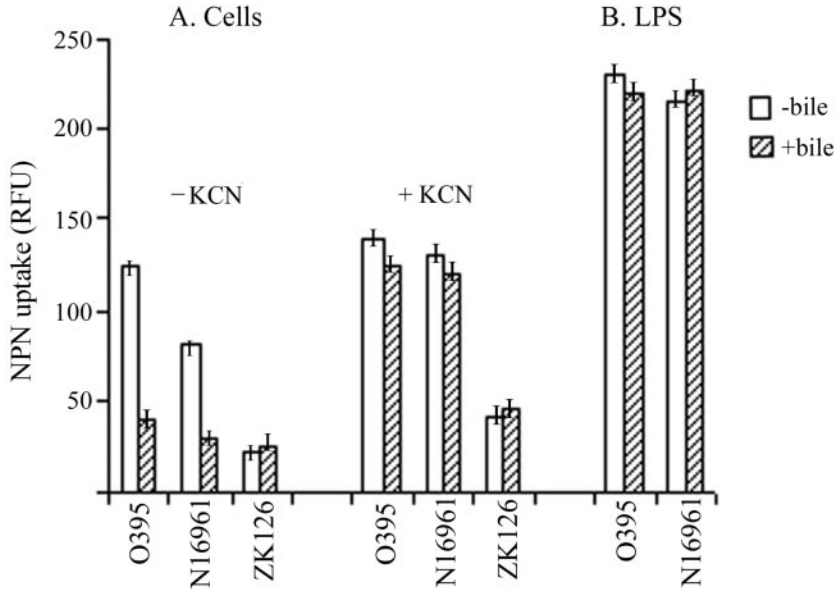

FIG. 2. NPN uptake. (A) *V. cholerae* strains O395 and N16961 and *E. coli* ZK126 were grown without or with 0.4% bile, and NPN uptake was measured in the presence or absence of KCN. (B) NPN binding by LPS aggregates isolated from *V. cholerae* strains O395 and N16961 grown in the presence or absence of 0.4% bile. The results represent the average of three independent experiments, and error bars indicate standard errors of the means.

tween the cell envelope and the cytoplasmic fractions in cells grown in the absence of bile. In bile-grown cells, however, most of the incorporated dye could be detected in the membrane fraction. *E. coli* cells took up very little CV, and the uptake was similar irrespective of whether the cells were grown in the absence or in the presence of bile (Fig. 1A). These results demonstrate that *V. cholerae* is unusually permeable to the hydrophobic dye CV, but growth of the cells in the presence of bile restricts entry of CV into the cells. Similar results were obtained with NPN. Quantum yield of the fluorescent probe NPN increases upon transfer from a hydrophilic to a hydrophobic environment (28). When NPN was added to cells grown to the logarithmic phase, only a small increase in fluorescence was observed with the wild-type *E. coli* strain ZK126. A significantly higher increase was observed with both the *V. cholerae* strains O395 and N16961, although NPN uptake by strain O395 was higher than that of strain N16961. However, when the *V. cholerae* strains were grown in the presence of 0.4% bile, NPN uptake was significantly reduced to similar levels in both strains ($P = 0.02$) (Fig. 2A).

Taken together, these results clearly indicated that although the OM of *V. cholerae* did not constitute an effective permeability barrier against hydrophobic compounds, entry of these compounds into the cells was significantly reduced when the cells were grown in the presence of bile. No statistically significant difference ($P = 0.25$) was detected in the partitioning of NPN into LPS aggregates isolated from *V. cholerae* strains O395 and N16961 grown with or without bile (Fig. 2B). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis also did not reveal any difference between LPS isolated from cells grown with or without bile (data not shown).

Presence of exposed phospholipids. It has been reported that unlike most gram-negative bacteria, *V. cholerae* contains phospholipids on the outer surface of the OM (21). Labeling of intact cells with the membrane impermeant probe dansyl chloride-cyclodextrin complex was used to examine whether exposed phospholipids were present in the OMs of cells grown in the presence of bile. In intact cells, the probe binds to the amino group of phosphatidylethanolamine only when phosphatidylethanolamine is exposed on the outer leaflet of the OM (25). When phospholipids were isolated from the OMs and IMs of dansylated cells of strains O395 and N16961 and analyzed by TLC, similar fluorescent spots were detected in phospholipids isolated from the OMs of cells grown in the absence or presence of 0.4% bile (Fig. 3). Phospholipids from the IM were not dansylated, confirming the impermeant nature of the probe. Thus, the extent of phospholipid bilayer zones in the OM is similar in cells grown in the presence or absence of bile.

Accumulation of hydrophobic compounds in de-energized cells. To examine if the lower accumulation of hydrophobic compounds in bile-grown cells is due to the activation of energy-dependent efflux systems, *V. cholerae* strains O395 and N16961 grown in the absence or presence of 0.4% bile were treated with NPN in a buffer containing KCN, which inhibits

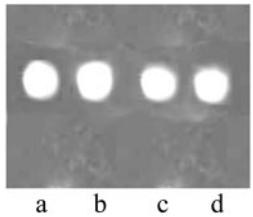

FIG. 3. TLC of phospholipids isolated from dansylated *V. cholerae* strains O395 (lanes a and b) and N16961 (lanes c and d) grown in the absence (lanes a and c) or presence (lanes b and d) of 0.4% bile.

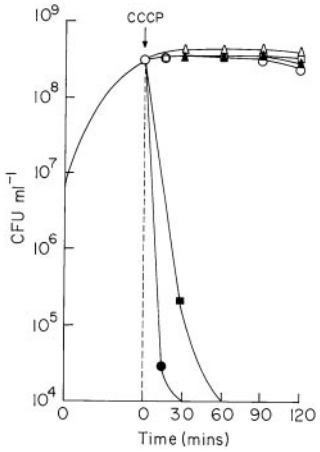

FIG. 4. Cell growth in the presence of CCCP. *V. cholerae* O395 (circles), *V. cholerae* N16961 (squares), and *E. coli* ZK126 (triangles) were grown to the logarithmic phase in the absence (open symbols) or presence (solid symbols) of 0.8% bile. CCCP was added, and viability was assayed at regular time intervals. The time of addition of CCCP is indicated.

respiration and prevents active efflux of NPN. Although in the absence of KCN, the rate of NPN binding to bile-grown cells was about threefold lower than the binding to cells grown without bile, in the presence of KCN, no difference in NPN uptake was observed between cells grown in the presence or absence of bile (Fig. 2A).

Furthermore, when the cellular electrochemical proton gradient, which energizes efflux processes, is dissipated by the addition of CCCP, rapid accumulation of CV occurred even in bile-grown *V. cholerae*. In the presence of CCCP, no difference in CV accumulation was observed between cells of either biotype grown in the presence or absence of bile (Fig. 1).

These results indicate that energy-dependent active efflux is required for restricting the entry of hydrophobic compounds into bile-grown *V. cholerae*.

***V. cholerae* is extremely sensitive to CCCP in the presence of bile.** *V. cholerae* strains O395 and N16961 were grown in LB in the presence or absence of 0.8% bile; when the cultures reached logarithmic phase (2×10^8 to 3×10^8 CFU ml^{-1}), 75 μM CCCP was added, and viability was assayed at regular intervals. When CCCP was added to cells grown in LB without bile, viability of the culture remained almost constant during the 2 h it was examined (Fig. 4). Interestingly, a drastic reduction in the number of CFU was observed when CCCP was added to *V. cholerae* cultures in LB containing bile. Within 15 min of addition of CCCP, the viability of the strain O395 decreased 10^4 fold. Strain N16961 was relatively less sensitive to CCCP in the presence of bile, and the number of CFU decreased about 10^4 fold in 1 h (Fig. 4). However, when up to 100 μM CCCP was added to cultures of *E. coli* ZK126, although cell growth was inhibited, no loss of viability was observed for up to 2 h, irrespective of whether or not the cultures contained 0.8% bile (Fig. 4).

Induction of *acrAB* expression in bile-grown *V. cholerae*. In view of the fact that the *acrAB* efflux system has been shown to be necessary for efflux of hydrophobic compounds in *E. coli* (14), expression of *acrAB* in *V. cholerae* was examined. RT-PCR analysis of RNA extracted from strain O395 grown to the exponential phase in the absence or presence of 0.4% bile indicated a three- to fourfold increase in *acrAB* expression in bile-grown cells compared to cells grown without bile (Fig. 5A). A progressive increase in *acrAB* expression occurred with increasing bile concentrations, and a maximum induction of six- to eightfold was observed in the presence of 1.6% bile (Fig. 5B). With El Tor biotype strain N16961, the basal level of *acrAB* expression was higher than in strain O395, and a further 1.5- to 2-fold induction was observed in the presence of bile (Fig. 5A). Induction of *acrAB* was also observed in *V. cholerae* grown in vivo in rabbit intestine. The expression of *acrAB* in strain O395 grown in rabbit ileal loops was comparable to that in cells grown in the presence of 0.4% bile and about three- to fourfold higher than that in cells grown in vitro in LB without bile (Fig. 5C). In strain N16961, in vivo expression of *acrAB* was about 1.5-fold higher than expression in vitro (Fig. 5C). The relatively low levels of *acrAB* induction by bile in strain

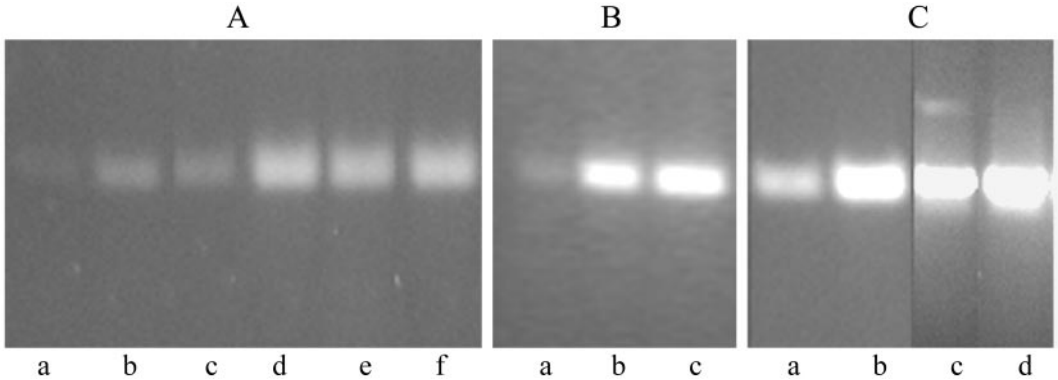

FIG. 5. Effect of bile on *acrA* expression. (A) RT-PCR was performed for 25 (lanes a and b) and 30 (lanes c to f) cycles with RNA isolated from strain O395 (lanes a to d) or strain N16961 (lanes e and f) grown without (lanes a, c, and e) or with (lanes b, d, and f) 0.4% bile for estimation of *acrAB* mRNA. (B) RNA was isolated from *V. cholerae* O395 grown in the absence of bile (lane a) or in the presence of 0.8% bile (lane b) or 1.6% bile (lane c). RT-PCR was performed for estimation of *acrAB* mRNA. (C) RT-PCR analysis of *acrAB* expression in *V. cholerae* strains O395 (lanes a and b) and N16961 (lanes c and d) grown in LB (lanes a and c) or in rabbit intestine (lanes b and d). RT-PCR for estimation of 16S rRNA and PCR with DNase-treated RNA samples that had not been reverse transcribed were used as controls.

N16961 were similar to the bile salt-dependent induction of *acrAB* expression in *E. coli* (23).

DISCUSSION

The ability to restrict the entry of hydrophobic compounds through the OM is crucial for enteric bacteria that must necessarily survive in the intestine in the presence of bile salts and other noxious hydrophobic agents. OM impermeability is generally considered to be a characteristic feature of enteric bacteria. The enteropathogen *V. cholerae* appears to be a curious exception, since its OM is relatively permeable to hydrophobic agents. Levels of novobiocin sensitivity and accumulation of the hydrophobic probes CV and NPN in *V. cholerae* were significantly higher than in *E. coli* (Table 1;, Fig. 1 and 2) and were similar to those reported for *Brucella*, a pathogen that uses a nonenteric route for infection (15). This observation raises fundamental questions about how *V. cholerae* survives in the intestine. We report here that when *V. cholerae* was grown in the presence of bile at concentrations similar to those present in the intestine, accumulation of hydrophobic probes in the cells was very significantly reduced. The lower level of accumulation of hydrophobic compounds in bile-grown *V. cholerae* was not due to alterations in the passive diffusion across the exposed phospholipid bilayer zones present in the OM (Fig. 3) but to the bile-dependent induction of an active energy-requiring efflux process.

In gram-negative bacteria, efflux pumps complement the barrier function of the OM (13, 17, 22). The efflux systems, which can pump out a variety of structurally diverse hydrophobic and amphiphilic compounds, are generally energized by an electrochemical proton gradient. In *V. cholerae* grown in the presence of bile, when the proton gradient was dissipated by the addition of CCCP, hydrophobic permeants accumulated to high levels, comparable to the accumulation in cells grown without bile (Fig. 1 and 2) and suggesting that the activity of the energy-dependent efflux system is solely responsible for the restricted entry of hydrophobic compounds into bile-grown *V. cholerae* (27). Indeed, active efflux systems are essential for survival of *V. cholerae* in the presence of bile. Addition of 75 μM CCCP to cells in the presence of bile causes a drastic reduction in viability. Under identical conditions, no loss of viability was observed for a wild-type *E. coli* strain (Fig. 4). It is likely that even 100 μM CCCP cannot completely dissipate the proton motive force in *E. coli*, due to the intrinsically lower OM permeability and relatively high constitutive levels of expression of efflux pumps in *E. coli* compared to *V. cholerae*.

Although *V. cholerae* strain N16961 of the El Tor biotype takes up less CV and NPN than strain O395 of the classical biotype, uptake of the probes was similar in both strains when treated with CCCP or KCN (Fig. 1 and 2), suggesting that the relatively lower accumulation of the hydrophobic probes in the El Tor biotype strain N16961 was due to higher basal activity of efflux pumps than in strain O395 of the classical biotype. Indeed, RT-PCR analysis indicated that the basal level of expression of the *acrAB* efflux pump in strain N16961 was higher than in strain O395 (Fig. 5). In strain O395, the a*crAB* efflux system was strongly induced when cells were grown in vitro in the presence of bile and also in cells grown in vivo in rabbit intestine; under these conditions, *acrAB* expression in strains O395 and N16961 was similar (Fig. 5). The modest induction of *acrAB* in the presence of bile in strain N16961 is similar to the pattern observed with *E. coli* (23).

It would be relevant to mention in this context that TolC, which functions as an OM pore protein for several RND family efflux systems, is necessary for the survival of *V. cholerae* in the presence of bile (3), suggesting that a member(s) of the TolC-dependent RND family of efflux systems is essential for bile resistance in *V. cholerae*. Also, a TolC-independent efflux system, VceAB, an EmrAB homolog, has been shown to contribute modestly to the resistance of *V. cholerae* to deoxycholate (6).

In conclusion, it may be stated that the OMs of smooth-type *V. cholerae* of both the classical and El Tor biotypes are not effective barriers to hydrophobic permeants, probably due to the presence of exposed phospholipids on the outer surface of the OM. Also, efflux pumps are not active in the classical biotype, since their inactivation by the addition of CCCP caused only a marginal increase in the permeability of hydrophobic compounds. The basal activity of efflux pumps is higher in strain N16961 of the El Tor biotype, which accounts for the somewhat lower accumulation of hydrophobic compounds in strain N16961 than in strain O395. When *V. cholerae* was grown in the presence of bile, entry of hydrophobic compounds into the cells was very significantly reduced, not from alterations in the OM barrier function per se but from the induction of efflux systems. Although induction of efflux pumps in the presence of bile decreases the accumulation of hydrophobic compounds in *V. cholerae*, even bile-grown *V. cholerae* does not acquire the high level of resistance to hydrophobic drugs observed with *E. coli* (Table 1; Fig. 1 and 2), where optimum coordination between efflux pump activity and OM barrier function appears to have been achieved.

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