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Bile Affects Production of Virulence Factors and Motility of *Vibrio cholerae*

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The effect of bile on the expression of cholera toxin (CT) and the major subunit of the toxin-coregulated pilus (TcpA) and on motility was examined in the *Vibrio cholerae* O1 classical-biotype strains O395 and 569B. Although the motility of the cells increased significantly in the presence of bile, transcription of the *ctxAB* genes, encoding CT, and of the *tcpA* gene was drastically reduced. In *toxR* mutant strains, motility is higher than in the wild-type strain and was further increased, by about 150%, in the presence of bile. Bile represses CT production in strain 569B-55, a *toxR* mutant of strain 569B, which normally produces more than 80% of the amount of CT synthesized in the wild-type cells. These results suggest that bile may target some factor other than ToxR that is involved in the regulation of CT production and motility. Bile has no effect on the relative amounts of the two outer membrane porins, OmpU and OmpT, which are under ToxR control.

Vibrio cholerae, a noninvasive gram-negative bacterium, is the causative agent of the diarrheal disease cholera (6). For successful infection of their human host, *V. cholerae* cells must colonize the intestine and produce copious amounts of cholera toxin (CT), a potent enterotoxin that causes the severe watery diarrhea characteristic of the disease (2). A toxin-coregulated pilus (TCP) has been shown to be necessary for colonization in both the classical and El Tor biotypes of *V. cholerae* O1 (23, 24). Besides CT and the pilus, other factors, including those necessary for survival of the bacteria in vivo, penetration of the mucous layer and adherence to the underlying epithelial cells of the intestine, binding and internalization of CT, evasion of the host defense system, etc., may also contribute to the virulence of this important human pathogen (1, 3, 7, 12, 13, 21).

In the course of infection, *V. cholerae* cells encounter a variety of biochemical, nutritional, and other parameters that constitute the microenvironment of the various niches of the human gut, and these may impose a temporal control on the expression of virulence genes of the bacterium at different stages of the pathogenesis cycle. A regulatory pathway controlling the expression of a subset of virulence factors of *V. cholerae* that has been most extensively characterized is the ToxR-ToxT system (19). The hierarchical expression of at least two activators of this regulon, ToxR and ToxT, coordinately controls the expression of a set of genes, including those coding for CT and TcpA, the major subunit of TCP (5, 17). The ToxR regulon is maximally expressed in cells grown at 30°C in medium with a starting pH of 6.6 and osmolarity equivalent to 66 mM NaCl (8). In the intestinal lumen the temperature is 37°C, the pH is alkaline, and osmolarity is thought to be equivalent to 300 mM NaCl or higher, conditions that repress the expression of ToxR-activated virulence factors in the laboratory (16, 20). It has been suggested that the ToxR regulon is repressed in the early stages of infection and is switched on at a later stage, probably in response to an as-yet-unidentified intraintestinal signal(s) (20). It has recently been reported that maltose, the most common sugar in the intestine, has significant effects on the production and secretion of virulence factors and that an intact maltose regulon is necessary for full virulence of *V. cholerae* (15). Thus, the concerted functioning of a number of regulatory systems responding to different environmental conditions encountered at different stages of infection may fine-tune the expression of virulence genes for successful infection. Bile is a major constituent of the small intestine which is secreted into the lumen of the duodenum from the gallbladder through the bile duct and is thus likely to be encountered by *V. cholerae* cells in the early stages of infection. It is in this context that the present report describes experiments to investigate the effect of bile on expression of virulence factors in *V. cholerae*. Intestinal bile is a mixture of mainly glycine and taurine conjugates of bile acids in association with cholesterol, phospholipids, etc. (11). *V. cholerae* cells are unable to deconjugate bile salts (18) and grow normally in the presence of bile, which is often incorporated in the selective media used for their isolation (22). Bile used in the present study was a crude ox bile extract which contains sodium salts of taurocholic, glycocholic, desoxycholic, and cholic acids (Sigma).

Bile decreases production of CT. The effect of bile on CT production has been examined in strains 569B and O395 of the *V. cholerae* O1 classical biotype. Environmental modulation of CT production in strain O395 is considered to be typical of the classical biotype, whereas 569B is an atypical strain where synthesis of CT is somewhat constitutive (5, 16). Both strains were grown in Luria-Bertani (LB) medium (pH 6.6) at 30°C, conditions that are optimum for CT production (16), and the amounts of the toxin in culture supernatants were determined by GM1 ganglioside-dependent enzyme-linked immunosorbent assay. About 1 mg of CT could be detected per ml of culture supernatant of cells grown in the absence of bile (Fig. 1). In contrast, when cells were grown under identical conditions except for the presence of 0.2 to 0.4% bile in the growth medium, culture supernatants contained less than 30 ng of CT per ml (Fig. 1). Thus, addition of bile to the growth medium reduces CT production by more than 97% in *V. cholerae* cells, even under conditions that normally promote high levels of CT production. The addition of bile to the growth medium at the concentration used in this study (0.2 to 0.4%) does not affect the osmolarity or pH of the medium. It has been observed that cells grown in the presence of bile are less sensitive to anionic detergents and that the uptake of crystal violet, a hydrophobic dye, is greatly reduced in these cells (unpublished observation).

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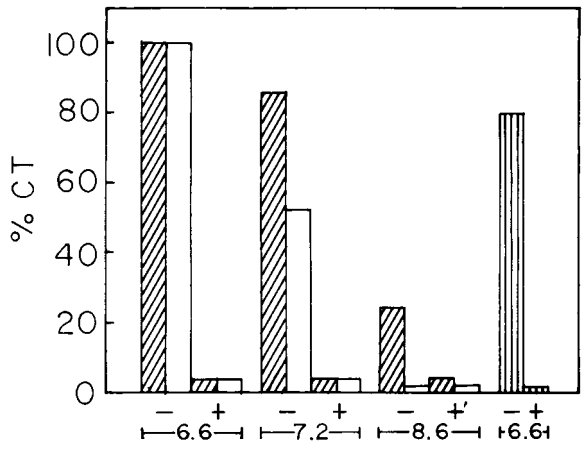

FIG. 1. Effect of bile on CT production. *V. cholerae* 569B (▨), O395 (□), and 569B-55 (▥) were grown in LB medium of pH 6.6, 7.2, or 8.6 to the early stationary phase (about 10^9 CFU/ml) in the presence (+) or absence (−) of 0.2% bile. CT was measured in culture supernatants by GM1 enzyme-linked immunosorbent assay and expressed as a percentage of the amount obtained in culture supernatants of strain 569B grown at pH 6.6 without bile.

Thus, bile may reduce cell permeability, probably by altering the outer membrane structure. In view of these observations, it was necessary to examine whether bile affects secretion of CT. The amount of CT in sonicated pellets of cells grown in the presence and absence of bile was determined, and in both cases, almost no CT could be detected in the cell pellets. Thus, bile affects the production and not the secretion of CT. The culture supernatants of cells grown in the presence and absence of bile were also used to estimate active CT in rabbit ileal loops. About 2 ml of fluid accumulated in loops inoculated with 0.1 ml of supernatants of cultures grown in medium without bile; however, no fluid accumulation was observed in loops inoculated with culture supernatants of cells grown in the presence of bile. The repression of CT production in the presence of bile could be reversed when the bile-grown cells were incubated for several hours in medium without bile.

The pH of the growth medium for optimum production of CT is 6.6 (8). At pH 7.2 only 53% of the amount of CT produced at pH 6.6 could be detected in strain O395, and no CT could be detected in cells grown at pH 8.6. In strain 569B, CT production was less sensitive to increases in pH, and at pH 7.2 and 8.6, 85 and 25%, respectively, of the amount produced in medium of pH 6.6 could be detected. Under these conditions, addition of bile caused a further reduction in CT production (Fig. 1). It has been suggested that at pH 8.6, ToxR remains in an inactive conformation in strain O395, resulting in little or no CT production (19). In strain 569B, on the other hand, although an increase in pH decreases CT production, significant amounts are still synthesized at pH 8.6, suggesting either that control of the ToxR regulon is altered in this strain or that this strain may express another regulator distinct from ToxR. In general, conditions that repress the ToxR regulon produce less pronounced effects on CT production in strain 569B than in strain O395. However, bile produces similar effects on CT production in the two strains. This may be because, unlike pH or osmolarity, bile modulates ToxR activity to the same extent in both the strains or because bile targets some other factor involved in regulation of CT production.

To investigate the role of ToxR in bile-mediated repression of CT production, the effect of bile on ToxR regulatory mutants O395-55 (16) and JJM43 (10) of strain O395 and 569B-55 of strain 569B (16) was examined. Compared to strain O395, strain 569B is less dependent on ToxR for expression of some genes of the ToxR regulon, and the *toxR* insertion mutant 569B-55 showed little decrease in toxin production (5). Whereas the wild-type strain produced about 1.0 μg of CT per ml of culture supernatant under permissive conditions, the mutant strain 569B-55 produced about 0.8 μg of CT per ml (Fig. 1). A similar insertion mutant in strain O395 almost completely abolished CT production (5). Addition of bile to the culture medium reduced CT production by about 90% in strain 569B-55, similar to the reduction in cultures of the wild-type strain (Fig. 1). It would be expected that, if the repression of CT production by bile is mediated by ToxR, very little repression would be observed in strain 569B, since this strain is partially independent of ToxR for CT production. The drastic repression of CT in this strain, as well as in the mutant strain 569B-55, suggests that bile may target some factor, other than ToxR, that is involved in the regulation of virulence in this strain.

Transcriptional repression of *ctx* and *tcpA* genes in the presence of bile. To examine whether the inhibition of CT production in cells grown in medium containing bile is at the level of transcription, RNA was isolated from cells grown in the absence or presence of bile and probed for *ctx*-specific mRNA by using a labeled 1.9-kb *Hin*dIII-*Xba*I fragment of plasmid pCVD15 carrying the *ctxAB* genes (14) in Northern blot experiments. A transcript of approximately 1,400 nucleotides was detected in cells grown in the absence of bile (Fig. 2A, lane a). With the same probe, however, very little transcript could be detected in cells grown in medium containing bile (Fig. 2A, lanes b and c).

Environmental modulation of the expression of the *tcpA* gene follows the same pattern as that of *ctx*, both genes being under the coordinate regulation of ToxR (23). To examine the effect of bile on the expression of the *tcpA* gene, RNA from cells grown in the presence or absence of bile was hybridized with a PCR-amplified fragment of the *tcpA* gene (Fig. 2B). The *tcpA* mRNA was barely detectable in cells grown in the presence of bile (Fig. 2B, lanes b and c). Thus, bile represses transcription of the major virulence genes, *ctxAB* and *tcpA*, of the ToxR regulon. However, transcription of *toxR* itself was not affected in the presence of bile (Fig. 2C, lanes a and b).

Outer membrane proteins. The relative levels of the two major outer membrane porins of *V. cholerae*, OmpT (40 kDa) and OmpU (38 kDa), depend on the osmolarity of the growth

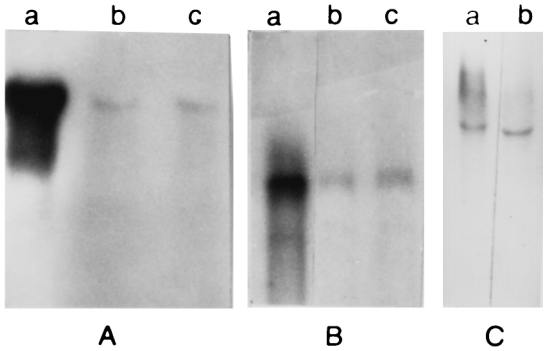

FIG. 2. Northern blot analysis of *ctx*-, *tcpA*-, and *toxR*-specific mRNA in *V. cholerae* cells grown in the presence or absence of bile. Cells were grown in LB medium (pH 6.6) to late logarithmic phase at 30°C in the absence (lane a) or presence of 0.2% (lane b) or 0.4% (lane c) bile. RNA was isolated from the cells, and Northern blots were prepared and probed with a ^{32}P-labeled *ctxAB* gene fragment of plasmid pCVD15 (A), a PCR-amplified *tcpA* gene fragment (B), or a *Bam*HI fragment of plasmid pVM7 (C) (16).

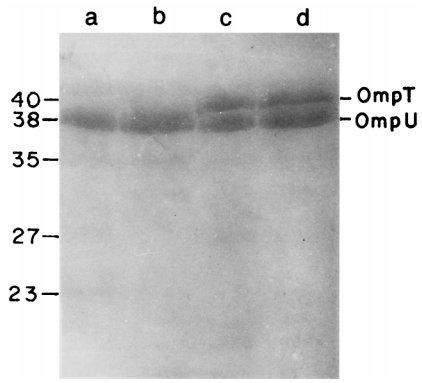

FIG. 3. Outer membrane proteins of *V. cholerae* cells grown in the presence or absence of bile. *V. cholerae* O395 (lanes a and b) and 569B (lanes c and d) were grown in the absence (lanes a and c) or presence (lanes b and d) of 0.2% bile in LB medium, pH 6.6, at 30°C to early stationary phase. Outer membranes were isolated, and the outer membrane proteins were analyzed by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis.

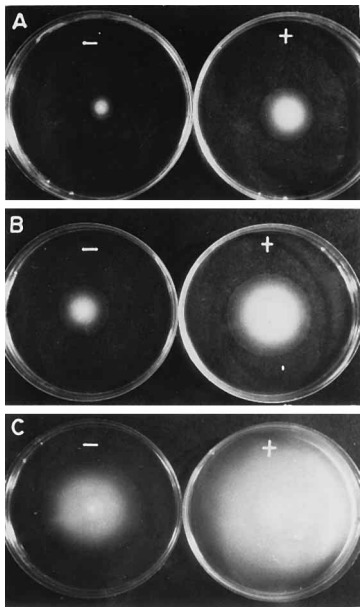

FIG. 4. Swarming behavior of *V. cholerae* 569B (A), O395 (B), and JJM43 (Δ*toxR*) (C) on motility agar with (+) or without (−) 0.2% bile.

medium, one of the parameters sensed by ToxR, but appear to be less affected by changes in pH and temperature, factors that influence the expression of other genes of the ToxR regulon (4, 16). ToxR acts as a positive regulator of OmpU but appears to negatively regulate OmpT (16). Since bile reduces expression of Ctx and TcpA, the effect of bile on the relative amounts of OmpU and OmpT was examined. Outer membranes were isolated from cells grown in LB medium containing about 90 mM NaCl, and the outer membrane proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). The outer membrane of strain 569B contained both OmpU and OmpT in almost equal amounts (Fig. 3, lane c). In contrast, OmpU is the major porin in strain O395 and OmpT was not detectable at all under conditions used in this study (Fig. 3, lane a). In both the strains, no change in the relative amounts of OmpU and OmpT could be detected in the outer membrane when the cells were grown in the presence of bile (Fig. 3, lanes b and d).

Swarm plate assay. *V. cholerae* cells are in general highly motile, and motility is thought to be important for virulence, at least in the early stages of infection. It has recently been reported that motility and the major virulence factors are oppositely regulated in *V. cholerae* (9). In view of the fact that the expression of the *ctxAB* and *tcpA* genes is drastically reduced in the presence of bile, the swarming ability of these cells in semisolid motility medium containing 0.3% agar in LB medium (pH 6.6) at 30°C was examined. Both strains 569B and O395 displayed little motility in the swarm plates (Fig. 4A and B). However, when motility was assayed in the same medium containing 0.2% bile, a 150% increase in the swarm diameters was observed (Fig. 4A and B). In addition to motility, the ability of bacteria to swarm also depends on growth and chemotaxis. The presence of bile did not have any effect on the growth rates of strains 569B and O395. It is not yet clear if the increased motility observed in semisolid medium is due to hypermotility per se or to an increase in chemotaxis-directed motility.

It has been postulated that ToxR may have a negative regulatory effect on motility (9). Under conditions that activate the ToxR regulon, while the wild-type strain O395 displayed little motility, the mutant strain JJM43 (Δ*toxR*) (10) was more than 100% more motile (Fig. 4C). The motility of strain JJM43 was further increased, by 150%, in the presence of bile (Fig. 4C), suggesting that the bile-mediated increase in motility may not be due to depression of ToxR activity alone and that some other factor responsible for the increase in motility may also be affected. It may be mentioned in this context that Class I hyperswarming mutants isolated by Gardel and Mekalanos (9) exhibit increased swarming activity and reduced CT and TCP production. The level and size of the ToxR protein in the mutant were similar to those in wild-type cells, and the mutant phenotypes could not be complemented by the introduction of *toxR* and *toxS* genes, suggesting that the Class I hyperswarming phenotype may result from new regulatory mutations. It would be interesting to investigate if the effect of bile is mediated by such a regulatory gene product(s).

Conclusions. It has been proposed from studies on the ToxR regulon that at early stages of infection by *V. cholerae*, production of CT, TCP, and possibly other ToxR-activated factors is repressed. Motility of the cells, which is oppositely regulated, increases at this stage, probably assisting the bacteria to penetrate the mucous layer and gain access to the underlying epithelial cells. The significant increase in motility, with concomitant reduction in synthesis of CT and TcpA, in the presence of bile, an important constituent of the intestinal lumen, observed in the present study supports the predictions made from the ToxR model regarding the expression of virulence factors in the pathophysiology of *V. cholerae* infection.

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