Enteric pathogens have developed several resistance mechanisms to survive the antimicrobial action of bile. We investigated the transcriptional profile of *Vibrio cholerae* O1 El Tor strain C6706 under virulence gene-inducing conditions in the presence and absence of bile. Microarray analysis revealed that the expression of 119 genes was affected by bile. The mRNA levels of genes encoding proteins involved in transport were increased in the presence of bile, whereas the mRNA levels of genes encoding proteins involved in pathogenesis and chemotaxis were decreased. This study identified genes encoding transcriptional regulators from the TetR family (*vexR* and *breR*) and multidrug efflux pumps from the resistance-nodulation-division superfamily (*vexAB* and *vexD*) that were induced in response to bile. Further analysis regarding *vexAB* and *breAB* expression in the presence of various antimicrobial compounds established that *vexAB* was induced in the presence of bile, sodium dodecyl sulfate, or novobiocin and that the induction of *breAB* was specific to bile. *BreR* is a direct repressor of the *breAB* promoter and is able to regulate its own expression, as demonstrated by transcriptional and electrophoretic mobility shift assays (EMSA). The expression of *breR* and *breAB* is induced in the presence of the bile salts cholate, deoxycholate, and chenodeoxycholate, and EMSA showed that deoxycholate is able to abolish the formation of *BreR*-*BreR* complexes. We propose that deoxycholate is able to interact with *BreR* and induce a conformational change that interferes with the DNA binding ability of *BreR*, resulting in *breAB* and *breR* expression. These results provide new insight into a transcriptional regulator and a transport system that likely play essential roles in the ability of *V. cholerae* to resist the action of bile in the host.

Pathogenic bacteria continuously monitor environmental parameters (pH, osmolarity, and temperature) to adapt to their host and regulate virulence gene expression. The human gastrointestinal tract can be considered to be an extreme environment for enteric bacteria where they need to adjust to stressful conditions, including exposure to bile (2). Bile is a hepatic secretion that is stored in the gallbladder and is released into the duodenum. It is composed of bile salts, cholesterol, phospholipids, antibodies, pigments, and metals, among other components (20). Bile has the essential role of emulsifying and absorbing lipids from the diet. Moreover, bile aids in the elimination of toxins and the excretion of metabolic products and has bactericidal properties (20).

Enteric pathogens have developed several resistance mechanisms to survive the antimicrobial action of bile (2, 14). The main mechanism of bile resistance in gram-negative bacteria is mediated by the expression of multidrug resistance (MDR) efflux pumps that actively extrude bile out of the cell (14). MDR transporters belonging to the resistance-nucleation-cell division (RND) superfamily are distinct from other transporters because they transport a broad variety of compounds, such as antibiotics, dyes, and detergents, out of the cell (30). They are localized in the inner membrane and associate with a periplasmic membrane fusion protein (MFP) and an outer membrane protein to generate a three-component multidrug efflux system spanning the cytoplasmic and outer membranes that can pump toxic compounds out of the cell (30). Pioneering studies with *Escherichia coli* have shown that AcrA (an MFP), AcrB (an RND pump), and TolC (an outer membrane protein) make up one such three-component multidrug efflux system that can pump different substrates such as novobiocin, erythromycin, sodium dodecyl sulfate (SDS), cholate, taurodeoxycholate, and decaconato out of the cell (10, 36, 52). The AcrAB-like efflux system of *Salmonella enterica* serovar Typhimurium (29) and the CmeABC efflux system of *Campylobacter jejuni* are other known three-component systems for which bile salts, among other compounds, are substrates (33, 34). Since MDR transporters have broad specificities and use proton motive force, their overproduction can cause the excretion of intrinsic metabolites and the loss of membrane potential, processes that would be detrimental to the survival of the bacterial cell (13, 30). Therefore, the expression of the majority of the MDR transporters is tightly controlled (13). The TetR family members AcrR and CmeR, for example, are the transcriptional repressors of *acrAB* and *cmeABC*, respectively (31, 35).

*Vibrio cholerae* is a gram-negative, curved, rod-shaped enteric bacterium that is the causative agent of the severe diarrheal disease cholera. This pathogen has developed several mechanisms to protect against the action of bile: (i) it increases motility in the presence of bile, which is hypothesized to be important for the bacterium to swim away from...
affected in the presence of bile. In particular, we report that encoded by operon. This operon is negatively regulated by the protein vexCD vexAB response to different antibiotics and detergents was analyzed, (8), VexB, and VexD (4).

expression of genes that encode proteins involved in efflux, (porin), reducing bile uptake (4, 43, 44); and (iv) it induces the film cells to the bactericidal effect of bile (22); (iii) it enhances colonization (15, 48) (see Fig. 1A); (ii) it induces the formation layer, and gain access to the underlying epithelial cells for

TABLE 1. Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant genotype or description | Source or reference |
|-------------------|----------------------------------|---------------------|
| V. cholerae       |                                  |                     |
| C6706 str2        | El Tor; Sm'                      | Laboratory collection |
| KSK262            | C6706 str2 ΔluxZ3                 | 28                  |
| FCM164            | KSK262 P_{luxZ3}                  | This work           |
| FCM225            | FCM164 ΔvreI                     | This work           |
| FCM168            | FCM164 ΔvreI                     | This work           |
| FCM262            | FCM164 ΔvreI ΔvreI                | This work           |
| FCM211            | FCM168 pFC3                      | This work           |
| FCM213            | FCM168 pKAS178                   | This work           |
| FCM158            | KSK262 P_{luxZ3}                 | This work           |
| FCM191            | FCM158 ΔvreI                     | This work           |
| FCM214            | FCM191 pFC3                      | This work           |
| FCM216            | FCM191 pKAS178                   | This work           |

E. coli

| Origami(DE3)     | Δara-lac/7697 araD139 ΔluxX74 galE | Novagen |
|------------------|-----------------------------------|---------|
|                  | galT::aphA P_{lacI phr} F          |         |
| [lacZP{P_{lac}}]| gmr::Tn10 (Yc)                     |         |
| trcKan(DE5)      |                                    |         |
| FCM184           | Origami(DE3) pFC25 Tet' Amp' Kan'  | This work |

Plasmids

| pKAS154          | pKAS32 derivative; Kan'           | 27      |
|------------------|----------------------------------|---------|
| pKAS178          | pBAD33 derivative; Kan'           | 24      |
| pKKG344          | lacZ in pKKG344                  | This work |
| pFKG346          | lucZ in pFKG344                  | This work |
| pFC36            | P_{lux-lacZ} in pFKG346           | This work |
| pFC28            | P_{lux-lacZ} (P_{lux-lacZ}) in pFKG346 | This work |
| pFC27            | P_{lux-lacZ} in pFKG346           | This work |
| pFC32            | pKAS154 ΔvreI                    | This work |
| pFC3             | pKAS154 ΔvreI                    | This work |
| pFC33            | BreR-His construct in pKAS178     | This work |
| pFC25            | BreR-His construct in pBAD22      | This work |

The regulation of V. cholerae genes encoding efflux system components has been described previously only for the vceCAB operon. This operon is negatively regulated by the protein encoded by vceR, which is located upstream from vceCAB in a divergent orientation (8). The regulators for the vceAB or the vexCD operon, if any, are still unknown.

To further investigate the response of V. cholerae to bile and begin to define the bile reguler, we performed a microarray study to investigate the global response of V. cholerae to crude bile and determined that the expression of 119 genes was affected in the presence of bile. In particular, we report that vexB, vexCD (herein renamed breAB), and two genes encoding regulators belonging to the TetR family were upregulated in the presence of bile. When vexAB and breAB expression in response to different antibiotics and detergents was analyzed, vexAB was induced by exposure to bile, SDS, or novobiocin whereas the induction of breAB expression was specific for bile.

Given its specificity, we further characterized the regulatory mechanism of breAB. We identified BreR as the negative regulator of breAB and established that BreR is also able to regulate its own expression. Using electrophoretic mobility shift assays (EMSA), we demonstrated the direct binding of BreR to the breAB and breR promoters. Furthermore, we showed that breAB and breR expression was induced in the presence of cholate, deoxycholate, or chenodeoxycholate and that deoxycholate was able to disrupt BreR binding to the breR promoter. These findings support the hypothesis that bile plays an important role as a host signal for V. cholerae.

MATERIALS AND METHODS

Bacterial strains, plasmids, primers, antimicrobial compounds, and growth conditions. The V. cholerae and E. coli strains and the plasmids and primers used in this study are listed in Tables 1 and 2. The strains were grown in Luria-Bertani (LB) (38) or AKI (23) medium. Antibiotics (Sigma) were used at the following concentrations (except for induction experiments): ampicillin, 100 μg/ml; kanamycin, 45 μg/ml; polymyxin B, 50 U/ml; streptomycin, 100 μg/ml or 1 mg/ml (for allelic exchange experiments); and tetracycline, 15 μg/ml. For P_{lux-lacZ}, P_{lux-lacZ}, and P_{lux-lacZ} induction experiments, strains were grown in subinhibitory concentrations of erythromycin, polymyxin B, novobiocin, sodium cholate (crude bile), sodium taurodeoxycholate, sodium cholate hydrate, sodium glycocholate, sodium deoxycholate, taurocholic acid sodium salt hydrate, sodium chenodeoxycholate, sodium glycocholate hydrate, sodium glycodeoxycholate, SDS, and Triton X-100 (all from Sigma) as noted below in the description of the β-galactosidase assay methods. Antibiotic stocks were prepared according to the instructions of the antibiotic manufacturer (Sigma), while detergent stocks were prepared fresh in LB medium and filter sterilized. For allelic exchange experiments, LB agar contained 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; Gold Biotechnology Inc.)/ml.

RNA isolation, microarray analyses, and statistical evaluations. Three independent experiments were performed for the microarray analysis. For each experiment, RNA was obtained at four different time points and was prepared for hybridization; therefore, a total of three slides per time point were analyzed.
The growth conditions were as follows: *V. cholerae* C6706 str2 was grown for 15 h in LB medium at 30°C with aeration. The cultures were subsequently diluted 100-fold in AKI medium with or without 0.4% crude bile (high bile concentration) and were grown at 37°C for 3.5 h under stationary conditions and then for 2 h with aeration to induce virulence gene expression (23). Next, the cultures were diluted 100-fold in AKI medium with or without 0.02% crude bile (low bile concentration) and were grown at 37°C for 2 h under stationary conditions. Samples were obtained at four different time points: 2, 4, and 5.5 h for the high-bile-concentration cultures and 2 and 4 h for the low-bile-concentration cultures (Fig. 1B). RNA isolation, cDNA probe labeling, microarray hybridization, data detection, and statistical analyses were carried out as described previously (24).

**Construction of in-frame deletion strains.** Deletions were achieved by the PCR amplification of ~500-bp C6706 str2 DNA fragments flanking the gene of interest while retaining several codons from the 5' and 3' ends of the gene fused in frame. The fragments were ligated into pKAS154 (27), and the genes of interest were deleted from the *V. cholerae* chromosome by allelic exchange (49). *vreR* was deleted using primers TetB with TetN2 and FC57 with TetE, and *breR* was deleted using primers TR3B with TR3N2 and TR3N1 with TR3E. The accuracy of all the constructs was confirmed by DNA sequencing.

**Construction of P*breAB-lacZ*, P*breA-lacZ*, and P*breR-lacZ* fusions. The *lacZ* plasmid pGKK344 was constructed by PCR amplification of two ~600-bp fragments flanking the *lacZ* gene from C6706 str2 by using primers CHR2 with CHR4 and GAL1 with GAL2. The fragments were joined at a NotI site and ligated into pKAS154 by using the EcoRI and BamHI sites. The pGKK344 plasmid was linearized with NotI, and a promoterless *lacZ* gene from pcvv200 (40) was ligated into pGKK344, generating pGKK346. After being screened for the correct orientation of *lacZ*, pGKK346 was linearized with XbaI between the chromosomal start and the promoterless *lacZ* gene. Approximately 500 bp of the *vreAB*, *breR*, or *breR* promoter region was amplified by PCR using FC62 with FC63, TR3E with TR3N1, or FC13F with FC13R, respectively. The resulting fragments from the *vreAB*, *breR*, and *breR* promoters were digested and ligated into the linearized pGKK346 plasmid, generating pGFC56, pFC28, and pFC27, respectively. The *lacZ* fusions were transferred into the chromosome of a *V. cholerae* Δ*breR* strain by allelic exchange (49) between the chromosomal *breR* and *lacZ* gene. The accuracy of all the constructs was confirmed by DNA sequencing.

**β-Galactosidase assays.** Different *V. cholerae* strains harboring the P*vreAB-lacZ*, P*breA-lacZ*, or P*breR-lacZ* transcriptional fusion were grown for 15 h in LB medium at 37°C with aeration. The cultures were then diluted 100-fold in LB medium with or without one of the following compounds: crude bile (0.4%), taurodeoxycholate (300 μM), cholate (300 μM), glycodeoxycholate (300 μM), deoxycholate (300 μM), taurocholate (300 μM), chenodeoxycholate (300 μM), glycocholate (300 μM), glycodeoxycholate (300 μM), SDS (300 μM), Triton X-100 (150 μg/ml), ethyromycin (0.1 μM), novobiocin (0.1 μM), or polymyxin B (5 μl/ml). The cultures were grown at 37°C with aeration until the OD600 of 0.6 at 37°C, and induced with 0.1% arabinose, with incubation for an additional 2 h. The cells were harvested by centrifugation and resuspended in buffer A (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride, pH 7.0). The suspension was sonicated and centrifuged at 14,000 rpm in a microcentrifuge at 4°C for 20 min. The supernatant was then collected. A column containing Talon metal affinity resin (Clinton) was preequilibrated with buffer B (50 mM NaH2PO4, 300 mM NaCl, pH 7.0). The column was washed with the supernatant containing BreRHis6, and washed with buffer C (50 mM NaH2PO4, 300 mM NaCl, 20 mM imidazole, pH 7.0), and BreRHis6 was eluted with buffer D (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole, pH 7.0). Fractions were collected and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). Those containing BreRHis6 were pooled and dialyzed overnight in binding buffer [20 mM HEPEs (pH 7.6), 1 mM EDTA, 10 mM (NH4)2SO4, 5 mM dithiothreitol, 0.2% Tween 20, 30 mM KCl] (31). BreRHis6 purity was estimated by SDS-PAGE to be ~90% (data not shown). Glycerol was added to purified BreRHis6 to a 10% (vol/vol) final concentration before storage at −80°C.

**EMSA.** The fragments designated ABI (the nucleotide sequence between −95 and +132 of the breA promoter region), AB2 (−382 to −76 of the breA promoter region), and AB3 (−382 to −95 of the breA promoter region) were amplified from pFC28 using FC13R, FC31F with primers CHR2 with CHR4 (26). The fragments designated ABR (−102 to +131 of the breR promoter region) and R2 (−370 to −83 of the breR promoter region) were amplified from pFC27 using TR3N1 with FC31 and FC32 with TR3E, respectively. The penicillin V amide (PVA) gene fragment (+18 to +191 of the pva promoter) was obtained by PCR as described previously (25). The fragments were gel purified and 3' end labeled with digoxigenin (DIG) as described previously (26).

**RESULTS**

Global gene expression in *V. cholerae* in response to crude bile. Studies with *V. cholerae* have shown that in the presence of bile the expression of virulence genes is repressed and motility and/or chemotaxis is increased (15, 48). From these studies, Schuhmacher and Klose formulated a model hypothesizing the effect of bile on *V. cholerae* motility and/or chemotaxis to mobilize the bacterium into the intestinal lumen (48). When the bacterium is within the lumen, bile induces motility and/or chemotaxis (15, 48). From these studies, Schuhmacher and Klose formulated a model hypothesizing the effect of bile on *V. cholerae* motility and/or chemotaxis to mobilize the bacterium into the intestinal lumen (48). When the bacterium is within the lumen, bile induces motility and/or chemotaxis (15, 48). Study with *V. cholerae* have shown that in the presence of bile the expression of virulence genes is repressed and motility and/or chemotaxis is increased (15, 48). From these studies, Schuhmacher and Klose formulated a model hypothesizing the effect of bile on *V. cholerae* motility and/or chemotaxis to mobilize the bacterium into the intestinal lumen (48). When the bacterium is within the lumen, bile induces motility and/or chemotaxis (15, 48).
of the epithelial cells takes place (Fig. 1A). We investigated the coordinated response of *V. cholerae* gene expression to the presence of crude bile by using a microarray analysis. The proposed in vivo conditions were simulated by growing *V. cholerae* O1 El Tor strain C6706 str2 under virulence gene-inducing conditions (in AKI medium) in vitro in the presence of a high bile concentration (0.4% crude bile) for 5.5 h and then diluting the cultures (100-fold) in AKI medium in the presence of a low bile concentration (0.02% crude bile) for 2 h (Fig. 1). Since the effect of bile on *V. cholerae* has been determined previously using 0.4% crude bile (15, 21, 22, 48), a concentration within the estimated range of concentrations of individual bile salts in the intestine (0.2 to 2%) (20), we utilized this concentration as the high bile concentration, whereas 0.02% crude bile was employed as the low bile concentration because it was 10-fold lower than the lowest individual bile salt concentration in the intestine (0.2%) (20). Samples were obtained at 2, 4, and 5.5 h from the high-bile cultures and at 2 h from the low-bile cultures (Fig. 1B). The same scheme was followed for the reference cultures grown without bile. RNA was extracted, reverse transcription was performed, and cDNA from control and bile-treated samples was used for microarray analysis. A ≥2-fold change in the mRNA level in high-bile cultures at two or more of the three time points and the reverse trend in the low-bile cultures were used as the criteria to indicate genes for which expression was affected by bile. By these criteria, a total of 119 genes were found to be differentially expressed in the presence and absence of bile. Forty-eight genes showed an increase in the RNA level, while 71 genes showed a decrease. Figure 2 shows the distribution of the corresponding gene products within cluster of orthologous group classifications (from the TIGR genome database). The majority of the genes that showed an increase in the RNA level and encode products with assigned functions belong to the group encoding transport and binding proteins (14 genes), whereas the majority of those that displayed a decrease in the RNA level encode products that belong to the cellular processes category (19 genes in total, 8 being part of the pathogenesis subset and 11 belonging to the chemotaxis and motility subset) (Fig. 2; see also Tables S1 and S2 in the supplemental material). The pathogenesis genes tcpA, ctxA, and ctxB showed a decrease in the mRNA level in the presence of the high bile concentration, consistent with previous findings (15, 48), and reversed this decrease in the presence of the low bile concentration, consistent with the hypothesis of Schuhmacher and Klose (48). The same pattern was observed for the following genes: tcpQ, tcpD, tcpS, pspA, and hlyA (see Table S2 in the supplemental material). Studies with the OmpU and OmpT porins have demonstrated previously that *ompU* transcription is stimulated in the presence of crude bile or deoxycholate (44) and that the expression of *ompT* is repressed (4). Neither *ompU* nor *ompT* mRNA was identified as being affected by bile by our criteria. However, the *ompT* mRNA level in the presence of the high bile concentration was decreased 24-fold compared to the baseline level at the 5.5-h time point, and this trend was reverted in the presence of the low bile concentration, although we did not detect changes in *ompU* mRNA levels at any of the different time points. None of the genes involved in biofilm formation in the presence of bile (*vps* genes and *vpsR*) (22) were identified using our criteria. This outcome was anticipated, though, since our experimental conditions did not promote biofilm formation. The *vexB* and *vexD* genes, encoding efflux components that have been shown previously to play a role in resistance to deoxycholate and other compounds (4), and the *vexC* gene, encoding a putative MFP, showed an increase in the mRNA level in the presence of a high bile concentration and reversed this increase under the low-bile conditions (see Table S1 in the supplemental material). Using our criteria, we did not identify the *vceA*, *vceB*, or *vceA* gene, encoding an MFP, a multidrug efflux pump, and a putative MFP, respectively, as being affected by bile, even though these genes encode proteins involved in bile resistance (4, 8). However, *vceA* and *vceA* showed three- and twofold increases in the mRNA level, respectively, only in the presence of the high bile concentration at the 5.5-h time point, and this trend was reverted in the presence of the low bile concentration. In addition, three genes, *vexR*, VC1746, and VCA0933, classified as having regulatory functions, showed an increase in the mRNA level in the presence of the high bile concentration and a reversion of this trend in the presence of the low bile concentration (see Table S1 in the supplemental material). Since the aim of this study was to identify genes for which expression is affected by bile and genes encoding proteins involved in the regulation of these genes in response to bile, we

**FIG. 1.** (A) Model illustrating the effect of bile on *V. cholerae* colonization based on a hypothesis proposed by Schuhmacher and Klose (48). When *V. cholerae* is within the lumen of the intestine, the high bile concentration inhibits the transcription of the virulence genes and induces motility and/or chemotaxis to mobilize the bacterium into the mucus layer. Upon migration through the mucus layer, where the bile concentration is low, motility and/or chemotaxis is inhibited and virulence gene expression is induced, facilitating the colonization of the epithelial cells by *V. cholerae*. (B) Schematic representation of the microarray experimental design.
further explored the expression of vexAB and vexCD to determine their response specificities.

The expression of vexAB can be induced by structurally and chemically unrelated compounds, whereas vexCD expression is induced exclusively by bile. The vexB and vexD genes encode efflux pumps belonging to the RND superfamily, while the vexA and vexC genes each encode an MFP (4). In E. coli (36), C. jejuni (32), Neisseria gonorrhoeae (46), Pseudomonas aeruginosa (37), and Pseudomonas putida (50), the expression of genes that encode RND efflux pumps is induced by the different substrates extruded by the products of these genes. Bina et al. demonstrated that bile increases the mRNA levels of the vexB and vexD transcripts (4). Additionally, after a thorough study testing several antibiotics, detergents, and metals, it was shown previously that a vexB mutant is susceptible to structurally dissimilar compounds: SDS, Triton X-100, erythromycin, novobiocin, polymyxin B, and deoxycholate. This study also showed that a vexD mutant is susceptible only to deoxycholate (4). Therefore, we used these compounds to further determine the specific transcriptional induction of the efflux pump-encoding genes. The expression of vexAB and vexCD was examined using strains containing lacZ fusions integrated elsewhere in the chromosome so as not to interfere with the resistance to the compound being tested. A promotorless lacZ gene was fused to either ~520 bp of DNA upstream of vexR codon 3 or ~520 bp of DNA upstream of vexC codon 4, generating the P_{vexRAB-lacZ} or P_{vexCD-lacZ} fusion, respectively (Fig. 3A and B). The specific β-galactosidase activities from strains grown in the presence or absence of subinhibitory concentrations of the compounds mentioned above were measured. Exposure to crude bile, SDS, and novobiocin induced the expression of vexAB between 2.2- and 4.3-fold (Fig. 4A), whereas vexCD expression was induced exclusively by bile, with a 10-fold increase (Fig. 4B). Similar induction results were obtained with strains harboring lacZ fusions to the wild-type promoter of the vexRAB or vexCD operon constructed by the insertion/deletion of lacZ at the vexR or vexC locus, respectively (data not shown). Overall, these results suggest that the vexCD operon responds specifically to bile. Therefore, we renamed this operon breAB for bile response genes and continued to characterize its expression and identify regulatory proteins associated with it.

BreR represses the expression of the breAB operon. Genes encoding efflux pumps from the RND family are usually regulated by TetR family members (30). From the microarray data, we identified two genes, vexR and VC1746 (see Table S1 in the supplemental material), that encode regulators belonging to the TetR family as determined by BLASTP. We hypothesized that vexR and/or VC1746 may regulate breAB expression in response to bile. In order to test this hypothesis, we deleted vexR, VC1746, or both genes in the strain harboring the P_{breAB-lacZ} fusion at the lacZ locus. The results showed that the vexR deletion did not affect lacZ expression, either in the absence or the presence of 0.4% crude bile (Fig. 5). In contrast, the deletion of VC1746 increased lacZ expression 24.5- and 2.8-fold in the absence and in the presence of crude bile, respectively, compared to that in the wild type (Fig. 5). These results indicated that VC1746 deletion caused the derepression of P_{breAB-lacZ} expression and the loss of the majority of bile responsive-
ness, suggesting that VC1746 encodes a bile-responsive repressor. Finally, the vexR VC1746 double mutant showed an induction pattern that was the same as that associated with the VC1746 single deletion (Fig. 5). Since the results (including those discussed below) indicated that VC1746 encodes a transcriptional repressor of the breAB operon, VC1746 was named breR. Further analysis using the TIGR database showed that the protein encoded by breR is composed of 209 amino acids and has a predicted helix-turn-helix DNA binding motif near its N terminus, characteristic of transcriptional regulators in the TetR family (45).

BreR binds to the breAB promoter region. To determine if BreR directly interacts with the breAB promoter region, we initially determined the breAB transcriptional start site. RACE (11) determined that the +1 nucleotide of the breAB promoter is an A located 121 bp upstream of the predicted transcriptional start site.

FIG. 3. Diagrams showing the promoter regions and fragments employed to generate lacZ transcriptional fusions and DIG-dUTP-labeled fragments. (A) Fragment of ~520 bp from the upstream region of the putative vexAB ATG start codon. (B) breAB (vexCD) promoter region. Fragments AB1 and AB2 were used for EMSA. (C) breR promoter region. The R1 and R2 fragments were used for EMSA. The breAB and breR transcriptional start sites are indicated by gray arrows.

FIG. 4. Induction of P_vexRAB-lacZ (A) and P_vexCD-lacZ (P_vexR-lacZ) (B) expression by different compounds. β-Galactosidase expression was measured by growing the strains in the absence or presence of subinhibitory concentrations of crude bile (0.4%), SDS (300 μM), Triton X-100 (150 μg/ml), erythromycin (0.1 μM), novobiocin (0.1 μM), or polymyxin B (5 U/ml) in LB at 37°C until the OD_600 of the cultures reached 0.8 to 1.0. The amount of change (n-fold) in β-galactosidase activity was calculated by dividing the level of β-galactosidase activity obtained in the presence of each compound by the activity obtained in the absence of the compound. The results shown are from three independent experiments. Error bars indicate standard deviations.

FIG. 5. Induction of P_vexR-lacZ expression in various strain backgrounds by crude bile. β-Galactosidase expression was measured by growing the strains in the absence or presence of 0.4% crude bile in LB at 37°C until the OD_600 of the cultures reached 0.8 to 1.0. The results shown are from three independent experiments. Error bars indicate standard deviations.
breA ATG start codon (Fig. 6A). Further examination of the upstream region revealed the presence of putative −35 (TTTACT) and −10 (TATGAT) regions (Fig. 6A) separated by 18 bp. The −35 sequence has two mismatches from the consensus sequence TTGACA, and the −10 sequence has only one mismatch from the consensus sequence TATAAT.

Subsequently, EMSA was performed using a purified BreR-His6 fusion protein. BreR-His6 was overexpressed from pFC25 in E. coli Origami(DE3) (Novagen) and purified by nickel-nitrilotriacetic acid affinity column chromatography to approximately 90% purity as judged by SDS-PAGE on a gel stained with Coomassie blue. The apparent molecular mass of purified BreR-His6 is 23 kDa, consistent with the predicted size (data not shown). The breAB promoter region utilized for the lacZ transcriptional fusions was divided into two slightly overlapping fragments, an 230-bp fragment (the nucleotide sequence between −95 and +132) named AB1 and an 300-bp fragment (−382 to −76) named AB2 (Fig. 3B). In addition, an 175-bp fragment from the unrelated PVA gene (pva) promoter (the PVA fragment) (25) was utilized as a negative control. BreR-His6 was incubated with the AB1, AB2, or PVA fragment in the presence of poly(dI-dC) and, surprisingly, caused a mobility shift of both the AB1 and AB2 fragments at low (50-ng) and high (250-ng) protein levels (Fig. 6B, lanes 5, 6, and 7), whereas no shift of the PVA control fragment was observed (Fig. 6B, lanes 2 and 3). These data provide evidence for the direct binding of BreR to the breAB promoter region. Furthermore, they suggest that BreR binds the breAB promoter at two independent sites. However, the AB1 and AB2 fragments overlap by 20 bp, and this may account for BreR's binding of both fragments. Therefore, an 280-bp fragment (−382 to −95) named AB2s was designed (see Fig. S1A in the supplemental material). The AB1, AB2s, and PVA fragments were incubated with BreR-His6, and a mobility shift was observed with both the AB1 and AB2s fragments (see Fig. S1B in the supplemental material), thus demonstrating the ability of BreR to bind to the breAB promoter at two independent sites, one distal from the −1 site (within AB2 or AB2s) and one proximal (within AB1).

Expression of breR is induced in response to bile in a BreR-dependent manner. To analyze the regulation of breR expression, we used a PbreR-lacZ transcriptional fusion. We designed a reporter strain containing the wild-type breR operon and 500 bp of DNA upstream of breR codon 30 fused to a promoterless lacZ gene and integrated into the V. cholerae chromosome at the lacZ locus (Fig. 3C). The transcription of the PbreR-lacZ fusion was increased 6.6-fold when the strain was grown in the presence of crude bile (Fig. 7). Similar induction was obtained with strains harboring lacZ fusions to the wild-type promoter of the breR operon constructed by the insertion/deletion of lacZ at the breR locus (data not shown).

Several transcriptional regulators from the TetR family, such as TetR, AcrR, UidR, and MexR, negatively regulate their own expression (5, 18, 35, 41). To investigate if breR is subject to autoregulation, we generated a breR deletion derivative of the reporter strain harboring the PbreR-lacZ fusion at the lacZ locus. The deletion of breR led to 13- and 2.4-fold increases in lacZ expression in the absence and presence of...
crude bile, respectively, compared to the expression in the wild-type background (Fig. 7).

**BreR binds to the breR promoter region.** To study the potential interaction of BreR with the breR promoter, the transcriptional start site at the breR promoter was first identified. By using 5’ RACE, it was determined that the +1 nucleotide of the breR promoter is a G located 40 bp upstream of the predicted ATG start codon (Fig. 8A). The inspection of the upstream region revealed a putative −35 (TGTACT) region with three mismatches from the consensus sequence TTGACA and a putative −10 (TATAGT) region with only one mismatch from the consensus sequence TATAAT. The putative −35 and −10 sequences (Fig. 8A) are separated by 17 bp.

Consequently, EMSA was performed utilizing BreR-His$_6$ and two fragments: a ~230-bp fragment (−102 to +131) named R1 and a ~290-bp fragment (−370 to −83) named R2 obtained from the division of the breR promoter region used for the lacZ fusions (Fig. 3C). The results showed that after the incubation of BreR-His$_6$ with the R1, R2, or PVA (negative control) fragment, BreR bound only to the R1 fragment (Fig. 8B, lanes 8 and 9) and not to the R2 or PVA fragment (Fig. 8B, lanes 2, 3, 5, and 6). Given that no supershift was observed with the R1 fragment in the presence of BreR, it is highly probable that BreR binds at only one site at this promoter. From these data, we can conclude that BreR binds differently at the breR promoter and the breAB promoter (Fig. 7), utilizing a single binding region for the former and two for the latter.

**Cholate, deoxycholate, and chenodeoxycholate are inducers of breAB and breR expression.** Since bile salts are abundant components of crude bile (2, 20), we tested individual bile salts to determine if any could induce breAB and/or breR expression. The reporter strains harboring the P$^{breAB}$-lacZ or the P$^{breR}$-lacZ fusion at the lacZ locus were grown in the presence of a subinhibitory concentration (300 μM) of eight different bile salts. The expression of P$^{breAB}$-lacZ and P$^{breR}$-lacZ was significantly induced (P < 0.05) in the presence of deoxycholate (17- and 5-fold, respectively) and chenodeoxycholate (10- and 4-fold, respectively) (Fig. 9A), whereas cholate produced lower-level inductions of the expression of breAB (3-fold) and breR (1.4-fold) (Fig. 9A). These results demonstrate that the induction of breAB and breR expression can be accomplished by the specific bile salts cholate, deoxycholate, and chenodeoxycholate and that the hierarchy of stronger to weaker inducers is as follows: deoxycholate > chenodeoxycholate > cholate. To determine if the response to individual bile salts was also mediated through BreR, we examined the expression of the breAB and breR promoters in a ΔbreR background with and
without subinhibitory concentrations of cholate, deoxycholate, chenodeoxycholate, and glycocholate (negative control). The deletion of \textit{breR} led to constitutive expression of \(P_{\text{breAB}}\)-\textit{lacZ} and \(P_{\text{breR}}\)-\textit{lacZ} (Fig. 9B) in the absence and presence of the different compounds tested.

Deoxylolate inhibits the binding of \textit{BreR} to the \textit{breR} promoter. It has been shown previously that the tetracycline repressor (TetR) binds to the \textit{tetA} operator in the absence of tetracycline, repressing the expression of \textit{tetA}, which encodes an efflux pump. When tetracycline enters the cytoplasm, it binds to TetR, inducing a conformational change that dissociates TetR from the \textit{tetA} operator, allowing the production of TetA and the active efflux of tetracycline (18, 19). This general regulatory mechanism has also been described previously for several members of the TetR family (6, 9, 12, 31, 39, 47, 51).

Given that cholate, deoxycholate, and chenodeoxycholate induce \textit{breAB} and \textit{breR} expression, we hypothesized that these bile salts may interact with \textit{BreR}. To test this possibility, we performed EMSA with the R1 fragment and \textit{BreR} in the presence of deoxycholate because this bile salt gives the most robust induction of \(P_{\text{breR}}\)-\textit{lacZ} expression (Fig. 9A). In addition, we tested \textit{BreR} binding in the presence of glycocholate, a noninducing bile salt, and glycodeoxycholate, a noninducing bile salt with structural similarity to deoxycholate. Initially, we determined the concentration at which deoxycholate abolished the formation of the \textit{BreR}-R1 complex (Fig. 10A). The result showed that in the presence of 10 mM deoxycholate, \textit{BreR} was unable to bind to R1. We selected 10 mM deoxycholate as the concentration to be used in our binding assay given that it was the lowest concentration at which we observed the inhibition of binding. In addition, we used 10 mM glycodeoxycholate and glycocholate as control bile salts. Figure 10B shows that 10 mM deoxycholate abolished the formation of the \textit{BreR}-DNA complex but that the same concentration of glycodeoxycholate or glycocholate did not disrupt this interaction. These results suggest that deoxycholate is able to specifically prevent the formation of the \textit{BreR}-R1 complex.

**DISCUSSION**

In the present study, we investigated the mechanism of transcriptional activation of genes that encode components involved in facilitating the resistance of \textit{V. cholerae} to bile. We initially used microarray technology to investigate the changes in \textit{V. cholerae} global gene expression during growth in the presence of different bile concentrations. The results confirmed the repression of the virulence regulon and the induction of the \textit{vexB} and \textit{breAB} (\textit{vexCD}) genes, which encode efflux system components. Furthermore, they revealed the induction...
of genes associated with transcriptional regulation, vexR and breR (see Table S1 in the supplemental material).

Since it has been shown previously that the expression of genes that encode components of RND efflux pumps are inducible by the various substrates extruded by the pumps (32, 36, 37, 46, 50), we analyzed the expression of vexAB and breAB in the presence of various compounds. In doing so, we established that crude bile, SDS, and novobiocin induced the expression of the vexAB genes but that the induction of the expression of breAB was specific to bile, suggesting that this operon responded exclusively to bile, unlike vexAB, which responded to several molecular signals. We therefore pursued the study of the breAB regulatory mechanism.

Genes encoding components of RND efflux systems are tightly regulated by regulators of the TetR family (30). We showed that BreR, a TetR-like regulator, repressed breAB expression, while VexR, another TetR regulator, did not affect breAB expression. It was also demonstrated that BreR, as other TetR members, was able to repress the expression of its own gene. VexR does not repress or activate the breR promoter to indirectly affect breAB expression since P_{breAB-lacZ} expression was not affected in the ΔbreR strain. β-Galactosidase assays demonstrated that a P_{breAB-lacZ} fusion exhibited high-level expression in the presence of crude bile; however, in a ΔbreR strain, the expression was even greater regardless of the presence of bile. Usually, local regulators play a modulating role, while the principal transcriptional expression is controlled by global regulators (13). These data support the hypothesis that BreR functions as a repressor of the breAB operon by acting as a local modulator preventing the excessive production of the BreAB efflux pump and that VexR is neither a global nor a local regulator of the breAB promoter. In addition, they strongly suggest that there is no global activator that regulates the expression of the breAB operon, such as the global activators MarA, SoxS, and Rob that induce the expression of the genes encoding the AcrAB efflux system in E. coli (13). Moreover, since the β-galactosidase assays indicated that the level of expression of breAB was highest in the breR mutant, it is possible that a ΔbreR strain may be more resistant to bile than the wild-type strain. MBC experiments with the wild-type and ΔbreR strains determined that there was a 1.5-fold increase in the resistance of a breR mutant to bile compared to that of the wild type (data not shown), as may be expected.

EMSA confirmed that BreR directly binds to the breAB promoter region at two independent sites, one (ABI fragment) proximal to and one (AB2 or AB2s fragment) distal from the transcriptional start site. The finding that BreR completely shifted the ABI fragment at a level (250 ng) that produced only a fractional shift of the AB2 or the AB2s fragment may indicate that the affinity for the distal site is lower. Finally, the results presented here indicate that BreR is able to repress the expression of its corresponding gene and interacts directly with the breR promoter region at a single site.

It is known that a number of regulators belonging to the TetR family act as transcriptional repressors by binding to their own operator sequences in the absence of effector/inducer molecules. Once the effector enters the cell, it will bind to a nonconserved domain on the C terminus of the repressor and cause a conformational change resulting in the dissociation of the repressor from the DNA and the transcription of the negatively regulated genes (45). Figure 9A shows that of all the bile salts tested, cholate, deoxycholate, and chenodeoxycholate, induce the expression of the P_{breAB-lacZ} and P_{breR-lacZ} transcriptional fusions, suggesting that these bile salts can serve as an environmental signal(s) necessary for the activation of breAB and breR expression. In addition, MBC tests showed that BreB mediates resistance to these bile salts (data not shown). Previous studies with C. jejuni demonstrated that CmeR, a TetR repressor, binds to the cmeABC promoter and represses its expression (31). When bile is incubated with the CmeR-cmeRAB complex, it interacts with CmeR, causing it to dissociate from the promoter region (32). In V. cholerae, VceR, a TetR family repressor of the vceAB operon, dissociates from its operator sequence in the presence of 77.2 mM deoxycholate (6). The findings of these studies demonstrate that bile or bile salts can act as effectors/inducers of TetR family regulators. We performed EMSA using deoxycholate, glycocholate, or glycodeoxycholate, which demonstrated that deoxycholate, at 10 mM, specifically disrupted the binding of BreR to the breR promoter. Similar results have been observed previously with MarR, an S. enterica serovar Typhimurium transcriptional regulator belonging to the MarR family (42). MarR represses the marRAB operon, which is involved in decreasing OmpF porin levels and increasing AcrAB-ToIC levels to reduce the influx and enhance the efflux of antibiotic compounds, respectively (30). Most importantly, it has been shown previously that deoxycholate specifically induces marR expression, and gel shift experiments have demonstrated that this bile salt specifically interacts with MarR, disrupting binding to the marRAB operon (42) in parallel to the interaction with BreR described here.

Based on the results that show (i) breAB and breR induction by cholate, deoxycholate, and chenodeoxycholate and (ii) the inhibition of BreR binding to the breR promoter by deoxycholate, as well as the data in the supporting literature, we propose a model wherein BreR is continuously associated with the breR and breAB promoters, repressing their expression. Once cholate, deoxycholate, and/or chenodeoxycholate enters the cell, it binds to BreR, causing the dissociation of the BreR-DNA complex, resulting in breR and breAB expression.

It has been demonstrated previously that the tetracycline repressor (TetR) binds to the tetA operator in the absence of tetracycline (an effector/inducer molecule), repressing the expression of tetA, which encodes an efflux pump, and that the tetR gene is expressed simultaneously with the tetA gene. This synchronized expression ensures that there is enough repressor available to inactivate the expression of tetA when tetracycline has been completely secreted out of the cell (18). Our results show that breR and breAB share this feature since both $P_{vexR-lacZ}$ and $P_{breR-lacZ}$ showed higher levels of expression in the presence of bile, specifically in the presence of cholate, deoxycholate, and chenodeoxycholate, than in the absence of bile.

Finally, the organization of breR with respect to breAB represents a novel arrangement for these systems, since the genes that encode TetR regulators that control the expression of the cognate genes encoding RND efflux systems are localized either in a divergent orientation adjacent to the genes they regulate (1, 17, 31, 35) or in the same operon (39, 53). In contrast, breR is located 8.99 kb upstream, positioned several genes away from the breAB operon. Genes encoding hypothetical proteins, paraquat-inducible protein A and B, and a putative
lipoprotein are among the genes between breR and the breAB operon. Interestingly, three of the genes encoding hypothetical proteins were also identified in our microarray study as being induced in the presence of bile (see Table S1 in the supplemental material). These genes are currently under investigation.

The findings reported here demonstrate that BreR is the transcriptional repressor of the breAB efflux system operon and that this repression is probably accomplished by binding at two independent binding sites at the breR promoter. In addition, BreR negatively regulates its own expression by binding to one site at the breR promoter. The mechanism of BreR repression at these promoters is currently under investigation. Lastly, we propose that BreR requires an effector/inducer molecule(s) to dissociate from the breAB and/or breR promoter and that the effector/inducer molecule(s) may be cholate, deoxycholate, and/or chenodeoxycholate.

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