

Characterization of the *Vibrio cholerae* *vexAB* and *vexCD* efflux systems

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Received: 23 March 2006 / Revised: 5 May 2006 / Accepted: 7 June 2006 / Published online: 28 June 2006
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Abstract *Vibrio cholerae* is an important human pathogen that causes the diarrheal disease cholera. Colonization of the human host is dependent upon coordinated expression of several virulence factors in response to as yet unknown environmental cues. Bile acids have been implicated in the in vitro regulation of several *V. cholerae* genes, including those involved in motility, chemotaxis, outer membrane protein production, and virulence factor production. Bile is toxic to bacteria and colonization of the intestinal tract is dependent upon bacterial resistance to bile acids. We have identified and characterized two bile-regulated RND-family efflux systems, named here *vexAB* and *vexCD*, that are involved in *V. cholerae* bile resistance. Mutational analysis revealed that the *vexAB* system is responsible for in vitro intrinsic resistance of *V. cholerae* to multiple antimicrobial compounds, including bile acids. In contrast, the *vexCD* efflux system was specific for certain bile acids and detergents and functioned in conjunction with the *vexAB* system

to provide *V. cholerae* with high-level bile resistance. Mutants containing deletion of *vexB*, *vexD*, and *vexB-vexD* were able to efficiently colonize the infant mouse suggesting that these efflux systems were dispensable for *V. cholerae* growth in the small intestines of infant mice.

Keywords Cholera · Efflux · Bile · RND

Introduction

Vibrio cholerae is a highly motile Gram-negative, facultative human pathogen that causes the potentially lethal diarrheal disease cholera (Reidl and Klose 2002). Cholera is acquired by ingestion of food or water contaminated with *V. cholerae*. Upon entering the human host, *V. cholerae* survives passage through the gastric acid barrier of the stomach and colonizes the mucosa of the small intestine. In response to undefined stimuli during colonization, two membrane-associated transcriptional activators, ToxR and TcpP, initiate transcription of the *toxT* gene (Krukonis et al. 2000). The ToxT protein is a transcriptional activator of the AraC family that directly activates the transcription of the genes responsible for the production of cholera toxin (CT) and the toxin-coregulated pilus (TCP). The CT is an A-B type enterotoxin that is responsible for the profuse diarrhea that is characteristic of cholera. The TCP is a type IV bundle-forming pilus that is essential for colonization of the intestinal tract of both humans and animals (Taylor et al. 1987; Herrington et al. 1988; Thelin and Taylor 1996; Tacket et al. 1998). Collectively, this regulatory system has been called the ToxR regulon.

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The specific signals encountered *in vivo* by *V. cholerae* that lead to the activation of the ToxR regulon are unknown. Several stimuli, including bile acids, have been shown to affect induction of the ToxR regulon *in vitro* (Skorupski and Taylor 1997; Bina et al. 2003). There is evidence that bile acids serve as an *in vivo* cue to modulate the expression of many factors that could be important for *V. cholerae* virulence (Fernandes and Smith 1977; Gupta and Chowdhury 1997; Schuhmacher and Klose 1999; Provenzano et al. 2000; Wibbenmeyer et al. 2002; Alvarez et al. 2003). Bile acids or crude bile extracts have been reported to function as a chemotactic signal, induce hypermotility, induce changes in outer membrane porin proteins, induce biofilm formation, and modulate the production of virulence factors in both ToxT-dependent and ToxT-independent manners (Allweiss et al. 1977; Fernandes and Smith 1977; Gupta and Chowdhury 1997; Provenzano et al. 2000; Wibbenmeyer et al. 2002; Butler and Camilli 2004; Prouty et al. 2005; Hung et al. 2006). Most of these bile-associated phenotypes are associated with *V. cholerae* virulence. Collectively, these results suggest that bile represents an important *in vivo* cue sensed by *V. cholerae*.

Bile acids are steroid carboxylic acids derived from cholesterol, produced in the liver, and stored in the gallbladder. Upon ingestion of food, bile is released from the gallbladder into the duodenum where the detergent properties of bile acids contribute to the digestion of lipids (Holt 1972). In addition to functioning in digestion, bile acids represent an important antimicrobial barrier to colonization of the small intestine by pathogens. In the upper duodenum, bile acids can reach molar concentrations that are toxic for many non-enteric bacteria (de Kok et al. 1999).

The high level of bile acid resistance found among Gram-negative enteric bacteria is generally attributed to their outer membrane (Hancock 1997; Snyder and McIntosh 2000; Nikaido 2001; Poole 2002). The outer membrane is thought to function as a permeability barrier that restricts the diffusion of bile acids across the outer membrane. However, as evidenced by the generation of bile-hypersensitive efflux mutants, the outer membrane alone is not sufficient to protect bacteria from the toxic effects of bile acids. In *Escherichia coli* and *V. cholerae*, resistance to bile acids is dependent upon the permeability properties of the outer membrane and on the production of certain cell envelope proteins, including porin proteins and energy-dependent efflux systems (Thanassi et al. 1997; Provenzano et al.

2000; Bina and Mekalanos 2001; Wibbenmeyer et al. 2002).

Porins function as water-filled diffusion channels in the outer membrane that allow passage of soluble molecules across the otherwise impermeable outer membrane (Nikaido 2003). Bacteria produce porins with varying-sized pores in order to modulate outer membrane permeability. In *V. cholerae*, modulated expression of the ToxR-regulated OmpT and OmpU porins is associated with resistance to bile (Provenzano and Klose 2000; Miller and Mekalanos 1988; Simonet et al. 2003).

Bacterial efflux systems have been shown to contribute to antimicrobial resistance in Gram-negative bacteria and are classified into several families based on sequence similarity (Saier and Paulsen 2001). The resistance-nodulation-division (RND) family of efflux proteins is of particular interest because of their unusually broad substrate specificity (Van Bambeke et al. 2003). Several RND efflux systems have been shown to efflux chemically and structurally unrelated antimicrobial compounds. The RND efflux systems are ubiquitous transport systems found in most Gram-negative bacteria (Tseng et al. 1999). In many pathogenic bacterial, RND efflux systems are responsible for the intrinsic (basal-level) resistance to multiple antibiotics and antimicrobial compounds. In some pathogenic bacteria, RND systems are associated with high-level resistance to multiple antibiotics (Poole 2005). The RND efflux systems are three-component transporters that include an integral membrane pump protein, a periplasmic membrane fusion protein, and an outer membrane pore protein homologous to *E. coli* TolC (Fernandez-Recio et al. 2004). The crystal structures of the *E. coli* TolC outer membrane pore (Koronakis et al. 2000), the MexA periplasmic membrane fusion protein (Fernandez-Recio et al. 2004), and the AcrB RND pump (Murakami et al. 2002) have been solved and support the model that these three proteins function together to form a continuous channel for the proton motive force-dependent extrusion of substrates from within the cell envelope back into the external environment (Eswaran et al. 2004).

Accumulating evidence suggests that RND efflux systems play a role in *V. cholerae* pathogenesis. We have described a putative efflux-negative *V. cholerae* strain, created by deletion of *tolC*, the putative outer membrane component of the *V. cholerae* RND systems (Bina and Mekalanos 2001). This $\Delta tolC$ strain displays a severe colonization defect in the infant mouse model of cholera. In addition, the *vexAB* RND efflux system, encoded by open-reading frames

(ORF) VC0164 and VC0165, is upregulated in stool of cholera patients and in rabbit ileal loops as shown using microarray technology (Merrell et al. 2002; Bina et al. 2003; Xu et al. 2003).

In this study we have determined the role of the *vexAB* and *vexCD* (encoded by ORFs VC1756 and VC1757) RND efflux systems in antimicrobial resistance and colonization of the infant mouse small intestines. We show that the expression of these two RND efflux systems is regulated by bile acids and that the two RND systems have an overlapping role in resistance to bile acids. Mutants containing deletion of *vexB*, *vexD*, and *vexB-vexD* were able to efficiently colonize the infant mouse suggesting that these efflux systems were dispensable for *V. cholerae* growth in the infant mouse small intestines.

Materials and methods

Chemicals, enzymes, and bacterial strains

Chemicals were obtained from Sigma (St. Louis, MO, USA) and enzymes were purchased from New England Biolabs (Beverly, MA, USA) unless otherwise stated. Carbenicillin was purchased from Agri-Bio (North Miami, FL, USA). Bacterial strains, plasmids, and oligonucleotide PCR primers used in this study are listed in Table 1.

Growth conditions

E. coli and *V. cholerae* strains were grown in Luria-Bertani (LB) broth or on LB agar at 37°C. *E. coli*

Table 1 Bacterial strains, plasmids, and oligonucleotides used in this study

Strain, plasmid or oligonucleotide	Genotype or sequence	Source or reference
<i>Vibrio cholerae</i>		
M3	N16961 Sm ^r	Lab collection
M58	M3 Δ <i>lacZ</i>	Lab collection
M150	M3 Δ <i>tolC</i>	Bina and Mekalanos (2001)
M495	M58 Δ <i>vexB</i> (VC0164)	This study
M692	M58 Δ <i>vexD</i> (VC1757)	This study
M694	M58 Δ <i>vexB</i> (VC0164), Δ <i>vexD</i> (VC1757)	This study
<i>Escherichia coli</i>		
DH5 α λ pir	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i> (λ <i>pirR6K</i>)	Miller and Mekalanos (1988)
SM10 λ pir	<i>thi-1</i> , <i>thr</i> , <i>leu</i> , <i>tonA</i> , <i>lacY</i> , <i>supE</i> , <i>recA::RP4-2-Tc::Mu</i> , Km ^r , (λ <i>pirR6K</i>)	Miller and Mekalanos (1988)
Plasmids		
pWM91	<i>oriR6K</i> plasmid vector used for <i>sacB</i> -mediated allelic exchange	Metcalf et al. (1996)
pM471	pWM91:: Δ VC0164	This study
pM143	pWM91:: Δ VC1757	This study
pBAD18	Arabinose inducible expression plasmid	Guzman et al. (1995)
pJB <i>vexAB</i>	pBAD18 expressing <i>vexAB</i>	This study
Oligonucleotides		
164F1	TGAAAGATCTGATGCGCCC	
164F2	CGATGTGCCGAATAGCCGGTGCTCACCGTAA	
164R1	TGTTGCTTATCGCGATCCTG	
164R2	GCACCGGCTATTCGGCACATCGTCAGCTGTAA	
164F- <i>Xba</i> I	ATGCAGATGATGGGCTTCTCTTG	
164R- <i>Sal</i> I	TGTTAAGTCGACAATATTCTATGCAATTCAGCTAG	
164R- <i>Xba</i> I	GGATGGCAATTTACCGTGTC	
165F- <i>Sac</i> I	AATTGAGCTCTTTGGAGGACGCACCAGAATGA	
1757F1	AGCCGAGCTGGATAAAGTCA	
1757F2	CCAATAATGGGTCGCCAAAGCATAATCAGGC	
1757R1	GAATTTGCCCTTGCGGTTAGA	
1757R2	CTTTGGCGACCCATTATTGGGCTCCGTTGTT	
VC0328-RT1	CGACCGCTTTACTACTATCC	
VC0328-RT2	TCTGCGCCCAGCTTAGTATC	
<i>vexB</i> -RT1	CAGCGTCATTAGGTTCCAAG	
<i>vexB</i> -RT2	CCGTACTCATCCCAATTGTC	
<i>vexD</i> -RT1	GCTGCTCGGCACCTTAGTAG	
<i>vexD</i> -RT2	TTGTGGTTCGTTGCGACTCTG	
<i>ompT</i> -RT1	CTGCGTCAGAACCTTGTTG	
<i>ompT</i> -RT2	AGCTGGGCTCGTGTATATGC	

DH5 α pir was employed as a host for all cloning experiments and *E. coli* SM10pir was used for the transfer of plasmids into *V. cholerae* by conjugation. The analysis of CT and TCP production in *V. cholerae* was accomplished by growth of strains in AKI broth under AKI-inducing conditions as described below (Iwanaga and Yamamoto 1985; Iwanaga et al. 1986). L-arabinose was added to growth media to a concentration of 0.1% for complementation experiments. Antibiotics were added to growth media when needed at the following concentrations: carbenicillin (cb), 100 μ g/ml; streptomycin (sm), 100 μ g/ml.

In vitro growth kinetics of *V. cholerae* strains were determined in a Biotek LX808IU microplate reader as follows. Overnight cultures of each strain were diluted (10^{-4}) in fresh prewarmed LB broth or LB broth containing the indicated concentrations of bile acids. Aliquots (150 μ l) of the diluted culture were subsequently transferred to individual wells of a 96-well microtiter plate. The plate was positioned in the microplate reader prewarmed to 37°C and growth was monitored by change in optical density at 630 nm over 21 h. Growth curves were performed in triplicate for each strain and the OD₆₃₀ measurements were averaged for each time point to generate the growth curves.

In vitro growth competition assays were performed as follows. Approximately 10^5 colony forming units (cfu) of each test strain (*lac*⁻) plus the wild type reference strain (*lac*⁺) were inoculated into 5 ml of LB. Immediately following inoculation an aliquot of the mixed culture was removed, serially diluted, and plated on LB X-gal agar plates for cell enumeration. The remainder of the culture was incubated at 37°C on a rotary shaker overnight. The following day, aliquots were removed, serially diluted, and plated onto LB X-gal agar plates. Following an overnight incubation at 37°C, the resultant colonies were scored as mutant (white) or wild type (blue). The competitive index (CI) was calculated as the ratio of wild type (wt) to mutant in the input divided by the ratio of the wt to mutant in the output.

Strain and plasmid construction

Deletion of *vexB* was accomplished by crossover PCR as previously described (Imai et al. 1991; Bina and Mekalanos 2001). Briefly, oligonucleotide PCR primer pairs 164F1/164R2 and 164F2/164R1 (Table 1) were used in separate PCR reactions using N16961 chromosomal DNA as a template. The resulting PCR products were purified and pooled. The pooled PCR products were used as the template for a second PCR reaction using the flanking 164F1/164R1 PCR primers. The amplicon from this PCR reaction was

cloned into pCR2.1 with the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. The deletion construct was excised from pCR2.1 by restriction endonuclease digestion and cloned into the counter-selectable suicide vector pWM91 (Metcalf et al. 1996) to generate pM471. Construction of pM143 was accomplished analogously. Next, pM471 was conjugated into M58 and co-integrants were selected for resistance to cb and sm. Several cb/sm resistant co-integrants from the mating were then inoculated onto LB agar without antibiotics and allowed to grow for 1–3 h. The outgrowth from the LB agar plates was then streaked for individual colonies on LB (without NaCl) agar containing 5% sucrose to select for excision of the integrated plasmid. Several sucrose-resistant colonies were selected and screened for cb sensitivity. Deletion of *vexB* was confirmed by PCR using the flanking PCR primers (164F1/164R1, Table 1). Deletion of *vexD* was accomplished in an identical manner using pM143 and appropriate flanking PCR primers listed in Table 1.

The *vexAB* genes were cloned into pBAD18 (Guzman et al. 1995) in a two-step process as follows. The amplicon from a PCR reaction using primers 164R-*SalI* and 164F-*XbaI* was digested with *SalI* and *XbaI* restriction enzymes and ligated into similarly digested pBAD18 to generate pBAD18-VC0164. Likewise, the amplicon resulting from a PCR amplification using 164R-*XbaI* and 165F-*SacI* primers was digested with *SacI* and *XbaI* restriction enzymes and ligated into similarity-digested pBAD18-VC0164 to generate pBAD18-*vexAB*.

Protein detection

The production of CT and TCP from *V. cholerae* strains grown under AKI conditions (Provenzano and Klose 2000) was determined as follows. Overnight cultures of *V. cholerae* grown in LB broth were inoculated (1:10,000) into 10 ml of AKI broth in a 150 \times 15 mm test tube and the cultures were incubated statically at 37°C for 4 h. The cultures were then aseptically transferred to 125 ml Erlenmeyer flasks and incubated with shaking at 37°C for 18 h before the cultures were processed for determination of CT and TCP production. The CT production was determined by GM1 enzyme-linked immunosorbent CT assays (CT-ELISA). Briefly, the OD₆₀₀ of the culture was recorded and supernatant and controls were applied to the first row of GM1 ganglioside-coated 96-well plates and serially diluted. After a 30-min incubation at 37°C, the wells were washed three times in PBS containing 2 mg/ml bovine serum albumin (BSA) and CT mouse antiserum was

added to each well. Incubation and washes were repeated twice and followed with the addition of anti-mouse/alkaline phosphatase antiserum and 2 mg/ml *p*-nitrophenylphosphate in 1 M Tris-HCl, pH 8.0 for development. Plates were read at 405 nm in a microtiter plate reader (Bio-Rad, Hercules, CA, USA) and CT secretion expressed in ng/ml of supernatant/OD₆₀₀. Production of TCP was determined by Western blot. Overnight AKI-grown cultures were collected by centrifugation. The cell pellets were resuspended in 0.2 ml of Laemmli solubilization buffer, adjusted by OD₆₀₀, and were heated at 100°C for 10 min. The proteins from the resulting whole cell lysates were individually resolved by sodium dodecyl sulfate 12.5%-polyacrylamide gel electrophoresis (SDS-PAGE) before proteins were transferred to nitrocellulose membrane (Bio-Rad). TcpA production was visualized using polyclonal rabbit anti-TcpA antibody and the ECL Enhanced Chemiluminescence Western blotting detection kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's directions.

Antimicrobial susceptibility tests

Antimicrobial susceptibility was determined by the disk diffusion method on LB agar using antibiotic disks purchased from Becton Dickinson (Franklin Lakes, NJ, USA). The disk diffusion experiments were performed according to the manufacturer's directions. Antibiotic disks containing test compounds that were not commercially available were prepared by spotting 20 µl aliquots of concentrated stock solutions of the test compounds onto the surface of blank disks (Becton Dickinson). Next, overnight cultures of the strains were evenly spread on the surface of 100 × 150 mm LB agar plates by streaking with a Dacron tipped swab to generate a lawn of cells. Finally, the antimicrobial disks were aseptically placed on the agar surface and the plates were incubated at 37°C overnight. Zones of growth inhibition were measured. All antibiotic disks measured 6 mm in diameter. Minimum inhibitory concentrations (MIC) for deoxycholate (DOC) were determined by spotting 10⁴ cfu of each strain onto LB agar plates containing various concentrations of DOC. Following inoculation, the plates were incubated at 37°C for 18 h and the MIC was recorded as the concentration of DOC that inhibited visible growth of the strain.

Infant mouse competition assay

Overnight cultures of mutant (*lac*⁻) and wild type reference (*lac*⁺) *V. cholerae* strains were individually

diluted 1:100 into LB broth. Subsequently, the inoculum was generated by adding 10 µl of each diluted mutant strain and the control strain into 980 µl 0.15 M NaCl containing 8 µl of blue food coloring for visualization of gastric inoculation. This inoculum (50 µl) was delivered perorally to lightly anesthetized 5-day-old infant mice that had been separated from their mothers approximately 2 h prior to inoculation. An aliquot of the inoculum was also serially diluted and plated onto LB agar plates containing 50 µg/ml X-gal for colony enumeration of the input. The mice were incubated at 30°C for 18 h, killed, and small intestines above the cecum removed immediately. The intestines were homogenized in 10 ml 0.15 M NaCl, serially diluted, and plated on LB agar plates containing sm and X-gal. Colonies were enumerated following an overnight incubation at 37°C and scored as mutant (white colonies) or wild type (blue colonies). The CI was calculated as the ratio of wt to mutant in the input divided by the ratio of wt to mutant in the output.

RNA isolation, transcriptional profiling, and data analysis

V. cholerae N16961 was used for all transcriptional profiling experiments. In these experiments, fresh overnight broth cultures of N16961 were used to inoculate 10 ml of LB broth in a 125 ml Erlenmeyer flask. The inoculated cultures were then incubated with shaking at 37°C until they reached an OD₆₀₀ of approximately 0.9. A stock solution of bile acids (B8756, Sigma) prepared in LB broth was then added to a final concentration of 0.2% and the cultures were incubated with shaking at 37°C for additional 20 min before RNA was isolated as described below. Control cultures received an equal volume of LB broth.

RNA was purified from N16961 cell cultures by the direct addition of three volumes of Trizol (Gibco BRL) to the 10 ml cell culture followed by mixing on a vortex mixer for 1 min. Trizol-isolated RNA was treated with DNase before purification using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions. The resulting RNA was used as the template for quantitative real-time PCR (qPCR) as follows. RNA was reverse transcribed using the First Strand cDNA Synthesis Kit (Fermentas, Hanover, MD, USA) according to the manufacturer's directions. qPCR was performed on a Perkin Elmer 7700 sequence detector using the SYBR Premix Ex Taq (Takara, Madison, WI, USA) using PCR primers designed with the Clone Manager software (Scientific & Educational Software, Cary, NC, USA). The qPCR primers are listed in Table 1. Expression

ratios were determined using the comparative C_T method.

Results

Identification of the *vexRAB* and *vexCD* operons

The TIGR annotation of the N16961 genome lists six independent RND efflux systems that are organized into respective operons. In this report we have characterized two of these RND efflux systems (Fig. 1). The *vexAB* genes, encoded by ORFs VC0165 and VC0164, were previously shown to be induced in the population of *V. cholerae* transcripts recovered from human cholera patient stool samples and from inoculated rabbit ileal loops (Merrell et al. 2002; Bina et al. 2003; Xu et al. 2003). The second RND system, named *vexCD*, is encoded by ORFs VC1756 and VC1757. Each of these RND systems contains genes encoding for two of the three components required for the assembly of a functional RND transporter: genes for the production of the membrane fusion protein (*vexA* and *vexC*) and the RND pump protein (*vexB* and *vexD*). Absent from both operons is a *tolC* homologue that could function as the outer-membrane pore protein of the RND efflux system. We presume that in *V. cholerae* this function is provided by the previously characterized and unlinked *tolC* (Bina and Mekalanos 2001). A linked transcriptional regulatory gene is often found associated with RND efflux systems, and a putative TetR-family transcriptional regulator (*vexR*) is associated with the *vexRAB* operon (Fig. 1). No transcriptional regulators are present or associated with the *vexCD* locus.

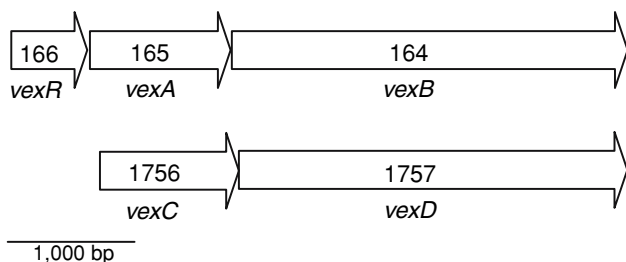


Fig. 1 The *vexRAB* and *vexCD* loci. The *vexRAB* operon is arranged as a three-gene operon. A TetR-family transcriptional regulator (*vexR*, VC0166) is located upstream of the gene encoding a membrane fusion protein (*vexA*, VC0165) followed by an RND pump protein (*vexB*, VC0164). The *vexCD* locus does not have a linked transcriptional regulator but, like the *vexAB* operon, has a membrane fusion gene (*vexC*, VC1756) upstream of the gene encoding the RND pump protein (*vexD*, VC1757)

Production of *vexB* and *vexD* in response to bile acids

We hypothesized that bile acids may function as an environmental cue involved in the regulated expression of *vexAB* and *vexCD*. This hypothesis was tested by determining the effect of bile acids on the production of *vexB* and *vexD* RNA transcripts. Transcription from *vexB* and *vexD* genes and the gene encoding *ompT*, known to be repressed by bile acids, were analyzed following growth of *V. cholerae* in LB and in LB plus 0.2% bile salts. The qRT-PCR results revealed that the expression of both *vexB* and *vexD* was up-regulated in response to bile acids and transcription of *ompT* was repressed (Table 2). These results suggest that expression of the *vexAB* and *vexCD* RND efflux systems is regulated by bile acids. The finding that the RND efflux systems are responsive to bile is consistent with a recent report by Chatterjee et al. (2004) on the upregulation of a *V. cholerae* RND efflux system in response to bile acids; due to the lack of gene identification or DNA sequence information the ORF analyzed in the Chatterjee et al. report could not be identified.

Role of *vexB* and *vexD* in antimicrobial resistance

Many RND efflux systems are characterized by broad substrate specificity. We determined the substrate specificity of the *vexAB* and *vexCD* systems by screening for changes in antimicrobial susceptibility using the disk diffusion assay or the agar dilution method. Deletion of *vexB* resulted in increased susceptibility to SDS, Triton X-100, erythromycin, novobiocin, and polymyxin B suggesting that these compounds are substrates for efflux by VexAB (Table 3). The susceptibility of the *vexB*, *vexD*, and *vexB-vexD* mutants to other antibiotics that are known substrates for RND efflux systems in other pathogenic bacteria was unchanged (data not shown). The list of antibiotics tested included amikacin, ciprofloxacin, norfloxacin, gentamycin, bacitracin, chloramphenicol, tetracycline, doxycycline, vancomycin, kanamycin,

Table 2 Expression ratios of efflux genes

	N16961	
	LB ^a	0.2% Bile salts ^a
<i>vexB</i>	1	3.17
<i>vexD</i>	1	1.92
<i>ompT</i>	1	0.12

^aExpression ratios for each gene are relative to the expression level observed of genes from N16961 grown in LB

Table 3 Effect of VexB and VexD on *V. cholerae* antimicrobial susceptibility

Genotype ^a	Plasmid ^b	Zone of growth inhibition ^c (STD) ^d					
		DOC	SDS	TX-100	ERT	NOV	PXB
Wild type	pBAD18	6.0 (0)	9.0 (0)	6.0 (0)	15.3 (0.6)	19.3 (0.6)	11.7 (0.6)
$\Delta vexB$	pBAD18	6.0 (0)	12 (0)	15.3 (0.6)	20 (0)	23.3 (0.6)	14 (0)
$\Delta vexB$	pJBvexAB	6.0 (0)	9.0 (0)	6.0 (0)	15.0 (0)	19 (0)	11 (0)
Wild type		6.0 (0)	9.7 (0.6)	6.0 (0)	15.3 (0.6)	19.3 (0.6)	11.7 (0.6)
$\Delta vexB$		6.0 (0)	12.0 (0)	14.3 (0.6)	19.0 (1.0)	22.0 (0)	14.0 (0)
$\Delta vexD$		6.0 (0)	8.7 (0.6)	6.0 (0)	17.3 (0.6)	18.3 (0.6)	11.0 (1.0)
$\Delta vexB-\Delta vexD$		24.7 (0.6)	12.3 (0.6)	17.7 (0.6)	19.3 (0.6)	19.0 (0)	14.0 (0)
$\Delta tolC$		27.7 (0.6)	13.3 (0.6)	23.3 (0.6)	23.3 (0.6)	24.0 (0)	15.0 (0)

^aActual strains used (see **Materials and methods**): El Tor biotype N16961 (wild type), M495 ($\Delta vexB$), M433 ($\Delta vexN$), M150 ($\Delta tolC$)

^bStrains carry either the control expression vector pBAD18 or plasmid pBAD18 expressing *vexAB* (pJBvexAB) from the P_{BAD} promoter. Expression from the P_{BAD} promoter was induced by the addition of 0.3% L-arabinose to the culture medium

^cZone of growth inhibition (measured in mm) to deoxycholate (DOC), sodium dodecyl sulfate (SDS), Triton-X 100, erythromycin (ERT), novobiocin (NOV), and polymixin B (PXB)

^dSTD is standard deviation

nalidixic acid, rifampicin, trimethoprim, and a number of β -lactam antibiotics. In addition, none of the efflux mutants displayed altered sensitivity to Cd, Al, Ni, Cu, Co, or Hg metal cations (data not shown). The *vexB* mutant was complemented by expression of the *vexAB* genes from a plasmid. Deletion of only *vexD* did not affect resistance to any of the tested antimicrobial compounds. The role of *vexD* was illuminated during analysis of the *vexB-vexD* double deletion mutant. The *vexB-vexD* double mutant showed elevated susceptibility to deoxycholate and Triton X-100, but not to the other tested antimicrobial compounds, relative to the *vexB* mutant. The dramatic increase in deoxycholate susceptibility of the *vexB-vexD* mutant suggested that both *vexB* and *vexD* contributed to N16961 deoxycholate resistance. These results also suggested that *vexD* has a narrow substrate specificity, limited to deoxycholate and Triton X-100.

There was no apparent change in susceptibility of the *vexB* or *vexD* mutants to deoxycholate using the disk diffusion method due to limitations in the amount of deoxycholate that can be applied to the paper disks. Therefore, we determined the minimal inhibitory concentration of deoxycholate for the *vexB*, *vexD*, *vexB-vexD*, and *tolC* deletion mutants by agar dilution. The MICs for each strain were as follows: N16961, 6%; *vexB* and *vexD*, 5%; *vexB-vexD* and *tolC* < 0.25%. Thus, there was a modest increase in sensitivity in the *vexB* and *vexD* mutants supporting the role of these RND systems in deoxycholate resistance.

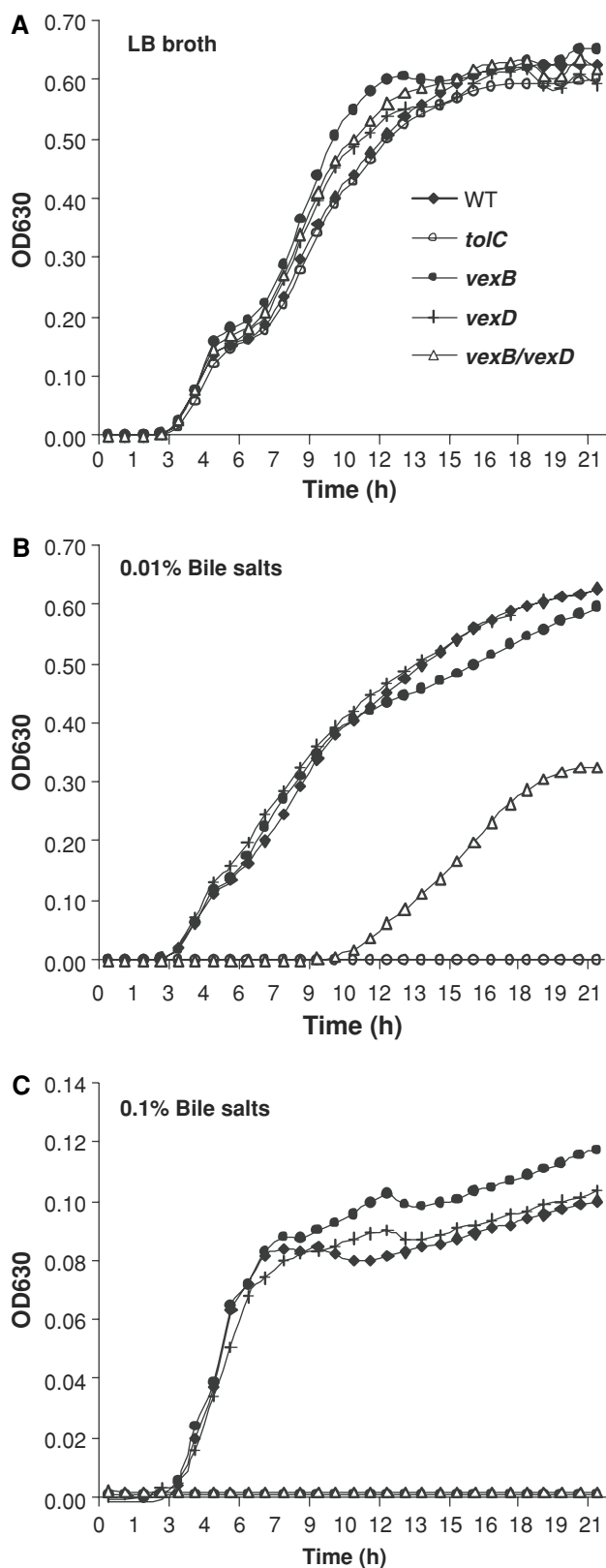
Growth kinetics in the presence of bile

Two concentrations of bile salts were used for analysis of the in vitro growth kinetics of all mutant

strains. The *vexB*, *vexD*, and *vexB-vexD* mutants produced growth curves that were nearly identical to the wild type control strain when grown in LB broth (Fig. 2a), suggesting that *vexB* and *vexD* were dispensable for growth under standard laboratory conditions. The presence of a sub-inhibitory concentration of bile salts (0.01%) resulted in attenuated growth of the *vexB-vexD* double mutant (~10 h lag phase), whereas the growth of the *vexB* and *vexD* mutants was similar to growth of the wild type control strain (Fig. 2b). The addition of bile salts to 0.1% inhibited the growth of the *vexB-vexD* double mutant, whereas the *vexB* and *vexD* mutant strains grew similarly to the wild type control strain (Fig. 2c). The results of these experiments reveal the importance of *vexB*, *vexD*, and *tolC* in resistance of *V. cholerae* to bile acids.

In vitro virulence factor expression and colonization of the infant mouse

We then tested whether the *vexB*, *vexD*, or *vexB-vexD* mutants would display a colonization defect in the infant mouse colonization model. In this model, infant mice were inoculated perorally with wild type or mutant *V. cholerae* and the rate of infection in the intestine was determined. Surprisingly, the *vexB* and *vexD* mutants and the *vexB-vexD* double mutant colonized the infant mouse to levels comparable to that of the wild type strain (data not shown). In contrast, the *tolC* mutant was unable to colonize the infant mouse. In in vitro growth competition assays, the *vexB*, *vexD*, and *vexD-vexB* mutants were similar to the wild type strain. Lastly, CT-ELISA and TcpA immunoblots confirmed that the efflux mutants were not defective



for synthesis of CT or TCP when the strains were grown in vitro under AKI-inducing conditions (data not shown).

Fig. 2 Growth kinetics of *V. cholerae* efflux mutants in the presence of bile acids. Growth curves were obtained by diluting overnight cultures of the listed *V. cholerae* strains 1:10,000 into LB broth (a), LB broth plus 0.01% bile salts (b), or LB broth plus 0.1% bile salts (c). The diluted cultures were distributed as 150 μ L aliquots into individual wells of a flat-bottomed 96-well microtiter plate and growth was monitored by OD₆₃₀ on a BioTek EL808IU microplate reader. Strains in all three panels are as listed in a legend

Discussion

There is a substantial amount of data to suggest that *V. cholerae* responds to the presence of bile acids by modulating the production of genes that are important for resistance to bile acids and pathogenesis (Gupta and Chowdhury 1997; Provenzano and Klose 2000; Hung and Mekalanos 2005; Prouty et al. 2005). However, only a few genes involved in *V. cholerae* bile acid resistance have been defined, including the ToxR-regulated reciprocal production of the OmpT and OmpU porin proteins (Miller and Mekalanos 1988; Provenzano and Klose 2000; Provenzano et al. 2000; Wibbenmeyer et al. 2002; Simonet et al. 2003), the *vceAB* efflux system (Colmer et al. 1998), *tolC* (Bina and Mekalanos 2001) and the *vexAB* and *vexCD* RND efflux systems reported here. Evidence for the importance of the RND efflux systems in *V. cholerae* pathogenesis consisted of the observations that an efflux-deficient *tolC* strain was colonization deficient and that the *vexAB* RND efflux system was upregulated in vivo in human-shed vibrios and the ileal loop of infected rabbit (Merrell et al. 2002; Bina et al. 2003; Xu et al. 2003). We have now characterized the roles of two *V. cholerae* RND efflux systems, *vexAB* and *vexCD*.

Amino acid sequence analysis of VexAB and VexCD RND efflux systems revealed that they shared strong similarity to the *E. coli* AcrAB RND efflux system (Ma et al. 1995). *AcrAB* encodes a stress-induced multiple drug efflux system that is important for *E. coli* resistance to bile. Based on this, we hypothesized that bile acids may be involved in the regulated expression of *vexAB* and *vexCD*. Our qRT-PCR analysis of transcripts from *V. cholerae* grown in vitro confirmed that the expression levels of both *vexAB* and *vexCD* increased in response to bile acids. This result suggested that these two efflux systems were likely involved in bile resistance. We subsequently characterized *vexB*, *vexD*, and *vexB-vexD* deletion strains. The broad substrate specificity of the VexAB system was typical of many RND efflux systems and included detergents and antibiotics. In contrast, the VexCD efflux system had a substrate specificity that was limited

to bile salts and the nonionic detergent Triton X-100. There was a dramatic increase in bile acid susceptibility of the *vexB*–*vexD* double mutant relative to the individual *vexB* and *vexD* mutants.

The growth of the *vexB*–*vexD* mutant was highly attenuated in vitro in the presence of low concentrations of bile salts (0.01%). Since the concentration of bile salts present in the small intestine is estimated to reach 4% (de Kok et al. 1999), it was surprising that the *vexB*–*vexD* mutant was able to efficiently colonize the small intestine of the infant mouse. One possible explanation for this result could be the in vivo induction of one or more of the four as yet uncharacterized RND efflux systems. In this scenario the in vitro bile hypersensitivity exhibited by the *vexB*–*vexD* mutant would not reflect in vivo susceptibility. The observation that the *tolC* mutant was more sensitive to bile acids than was the *vexB*–*vexD* mutant is consistent with this hypothesis.

The mechanisms involved in the transcriptional regulation of *vexAB* and *vexCD* are unknown. TetR-family transcriptional regulators are often found in association with RND efflux operons. A TetR-family transcriptional regulator, *vexR*, is encoded upstream of the *vexA* gene (Fig. 1). However, the gene arrangement of the *vexRAB* operon differs from that observed in other RND efflux systems where the TetR-family repressor is divergently transcribed. The role played by *vexR* in the regulated expression of the *vexAB* and *vexCD* remains to be determined.

Although the regulatory mechanisms involved in the induction of genes involved in *V. cholerae* bile acid resistance are largely unknown, the *V. cholerae* response to bile acid is somewhat similar to that of *E. coli*. In *E. coli*, environmental sensing of bile occurs through the MarR transcriptional regulator (Cohen et al. 1993; Prouty et al. 2004) that activates transcription of the *mar* operon. The expression of the *mar* operon results in production of MarA, a transcriptional activator that directly activates expression of the *acrAB* RND efflux system. The *mar* operon also modulates the production of the OmpF porin (Pratt et al. 1996) via expression of the *micF* antisense RNA (Delihias and Forst 2001).

A *mar*-like regulatory system has not yet been identified in *V. cholerae*. The bile-mediated reciprocal regulation of the *V. cholerae* OmpT/OmpU porins is reported to occur through ToxR (Miller and Mekalanos 1988); however, expression of the RND efflux systems does not seem to be under control of the ToxR regulon (Merrell et al. 2002; Bina et al. 2003). Based on the results presented here, it is tempting to speculate that bile acids play a role in the

observed upregulation of *vexAB* in the ileal loop model (Xu et al. 2003) and in human shed-vibriosis (Merrell et al. 2002; Bina et al. 2003). However, given the broad substrate specificity of VexAB, we cannot rule out other environmental cues as inducers of this efflux system.

Expanding drug resistance among pathogenic *V. cholerae* strains is a growing concern for the treatment of cholera. The mechanisms responsible for resistance remain largely unknown. The RND efflux systems contribute to the development of multiple antibiotic resistance in other pathogenic bacteria (Poole 2002; Van Bambeke et al. 2003; Webber and Piddock 2003). Analysis of *vexB*, *vexD*, and *vexB*–*vexD* mutants did not reveal significant contributions to the resistance of *V. cholerae* grown in vitro to antibiotics commonly used for the treatment of cholera (for example, fluoroquinolone and tetracycline antibiotics). This observation does not exclude the possibility that these antibiotics may act as substrates for these or other *V. cholerae* efflux systems. It is possible that in vivo-induced efflux activity and/or reduced outer membrane permeability could result in observed resistance.

Acknowledgements We thank Angel Olguin for assistance with the CT and TCP assays. This study was supported by a fellowship from the Cystic Fibrosis Foundation (J.E.B.), a grant from University of Tennessee Pathogenesis Center (J.E.B.), and NIH grants AI-18045 (J.J.M) and GM068855 (D.P).

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