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Bile acids stimulate biofilm formation in *Vibrio cholerae*

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Summary

***Vibrio cholerae* is a Gram-negative bacterium that causes the acute diarrhoeal disease cholera. After the bacterium is ingested, it passes through the digestive tract, encountering various environmental stresses including the acidic milieu of the stomach and the toxic effects of bile in the duodenum. While these stresses serve as part of a host defence system, *V. cholerae* has evolved resistance mechanisms that allow it to evade these defences and establish infection. We examined the expression profiles of *V. cholerae* in response to bile or bile acids and found an induction of biofilm genes. We found that *V. cholerae* shows significantly enhanced biofilm formation in response to bile acids, and that bacteria within the biofilm are more resistant to the toxicity of bile acids compared with planktonic cells. Bile acid induction of biofilms was found to be dependent on the *vps* genes (*Vibrio polysaccharide* synthesis) and their transcriptional activator VpsR, but VpsT is not required. These results contribute to the developing picture of a complex relationship between *V. cholerae* and its environment within the host during infection.**

Introduction

Vibrio cholerae is a Gram-negative bacterium that causes the acute, severe diarrhoeal disease cholera. Its natural ecosystem includes aquatic environments in endemic locations. Ingestion of the bacterium by the human host is followed by colonization of the intestine, expression of virulence factors and a profuse, watery diarrhoea that is the hallmark of this infection. The ability of most strains of *V. cholerae* to colonize the host and cause disease requires both the potent enterotoxin cholera toxin (CT) and the toxin co-regulated pilus (TCP) (Waldor and Mekalanos, 1996).

Passage through the human digestive tract necessarily forces *V. cholerae* to encounter various noxious compounds including gastric acid in the stomach and bile in the proximal duodenal lumen. While the detergent-like characteristic of bile acids is necessary for host solubilization of lipids in digestion, they also have an immunoprotective function because they are bacteriocidal (Hofmann, 1998).

Bile is a complex, heterogenous mixture of electrolytes, bile acids, phospholipids, cholesterol and bilirubin. Bile acids themselves are a mixture of the sodium salts of taurocholic, glycocholic, deoxycholic, chenodeoxycholic and cholic acid that are secreted by the gallbladder into the proximal portion of the duodenum to an estimated concentration of about 0.2–2% for individual bile salts (Hofmann, 1998).

Various effects of bile on *V. cholerae* have been reported, including modulation of cholera toxin (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999) and outer membrane protein expression (Provenzano and Klose, 2000), motility and induction of efflux systems (Chatterjee *et al*., 2004). Some of these mechanisms appear to mediate protection against bile itself. For example, bile-modulated expression of OmpU and OmpT, two outer membrane porins, appears to confer increased resistance to bile (Provenzano *et al*., 2000). The effects of crude bile are complex, in part because of the heterogenous nature of bile itself.

One mechanism by which bacteria adapt to environmental stresses is through biofilm formation (Costerton *et al*., 1999). Biofilms are surface-associated communities of bacteria embedded in an organized, self-produced extracellular polymeric matrix that decreases their susceptibility to host immunity and antimicrobial agents (Jefferson, 2004).

Vibrio cholerae biofilm formation has been extensively studied genetically, physiologically and microscopically (Watnick and Kolter, 1999). After swimming towards the surface using a polar flagellum, the bacteria interact with abiotic surfaces, in a process that is enhanced by the mannose-sensitive haemagglutinin (MSHA) type IV pilus (Watnick *et al*., 1999). Expression of the *vps* (*Vibrio polysaccharide* synthesis) genes, encoded in two gene clusters on the large chromosome (*vpsA-K* and *vpsL-Q*), is required for synthesis of an exopolysaccharide (EPS) matrix that stabilizes the mature biofilm (Yildiz and

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Schoolnik, 1999). The regulators *vpsR* and *vpsT* activate expression of the *vps* genes (Yildiz *et al*., 2001; Casper-Lindley and Yildiz, 2004; Rashid *et al*., 2004). Overproduction of EPS results in resistance to osmotic and oxidative stress and bacteriocidal agents (Yildiz and Schoolnik, 1999).

Various cellular processes have been linked to biofilm formation. These include quorum sensing via *hapR* and *luxO* (Hammer and Bassler, 2003; Vance *et al*., 2003; Zhu and Mekalanos, 2003), nucleoside processing via the *cytR* repressor of biofilms (Haugo and Watnick, 2002) and nucleotide (cyclic diguanylate) signalling via *vieS/A/B* (Tischler and Camilli, 2004), motility via *flaA* (Watnick *et al*., 2001) and *mot* (Lauriano *et al*., 2004), and sugar metabolism in LPS core oligosaccharide synthesis via *galU* and *galE* (Nesper *et al*., 2002). In addition, the smooth to rugose colony morphotype transition has been associated with increased resistance to stresses and increased biofilm formation. A recent genome-wide approach using microarray expression profiling of rugose and smooth variants of *V. cholerae* has demonstrated a complex network of regulators of biofilm formation (Yildiz *et al*., 2004). These microarray experiments corroborated previous observations linking biofilm gene regulation and quorum sensing, nucleotide regulation, motility and chemotaxis, sugar metabolism, and other functions including protein secretion (*eps* genes) (Yildiz *et al*., 2004).

Recently, several of these regulatory processes were recognized to co-ordinately regulate biofilm formation and virulence expression. Quorum sensing regulation controls expression of virulence factors, including CT and TCP, and formation of biofilms (Miller *et al*., 2002; Zhu *et al*., 2002; Hammer and Bassler, 2003; Zhu and Mekalanos, 2003). In a proposed model for the *V. cholerae* infectious cycle, bacteria enter the host in an acid-resistant biofilm and are then dispersed from the biofilm at low cell density. Under these conditions, virulence gene expression is induced and intestinal colonization is enhanced. As the cells multiply to high density, HapR is expressed, repressing virulence gene expression and biofilm formation as the bacteria prepare to leave the host as planktonic cells to begin another infectious cycle (Zhu and Mekalanos, 2003). While an initial report suggested that HapR regulated the *vps* genes independently of *vpsR* (Hammer and Bassler, 2003), more recent gene expression profiling experiments suggest that *vpsR* may be involved in certain strains under certain conditions (Yildiz *et al*., 2004).

A second mechanism that couples virulence expression and biofilm formation involves the two-component regulator VieS/A/B (Tischler and Camilli, 2004). VieS/A/B activates expression of CT while repressing transcription of the *vps* genes by controlling intracellular concentrations of cyclic diguanylate (c-di-GMP) with its phosphodiesterase EAL domain (Tischler *et al*., 2002). Other members of a family of regulatory proteins containing GGDEF and EAL domains, including *mbaA* (Bomchil *et al*., 2003), *rocS* (Rashid *et al*., 2003), *scrC* in *V. parahemolyticus* (Boles and McCarter, 2002), and *wspR* (D'Argenio *et al*., 2002) and *pvrR* (Drenkard and Ausubel, 2002) in *Pseudomonas aeruginosa*, have also been implicated in regulating biofilm synthesis, suggesting a possible second messenger role for c-di-GMP in biofilm formation.

By gene expression profiling of *V. cholerae* strains in the presence of crude bile and bile salts, we have found that biofilm gene expression is affected by bile. The induction of biofilm formation by bile acids confers resistance to bile's bacteriocidal effects. This induction depends upon the *vps* operon and post-transcriptional regulation of VpsR, but does not require VpsT.

Results and discussion

Genomic analysis of the effects of crude bile and sodium cholate on the classical biotype strain O395 and El Tor biotype strain C6706

Because of the numerous reported effects of bile on *V. cholerae*, we examined the whole-genome expression profiles of *V. cholerae* in the presence of bile to assess its pleiotropic effects. RNA was isolated from cells during exponential growth under various conditions. We examined the expression profiles of both a classical biotype strain (O395) and an El Tor biotype strain (C6706) in the presence of crude bile (0.4%) in order to determine the effects of bile that are not strain-specific. Because crude bile consists of a heterogenous mixture of components, we also examined the effects of bile acids alone (purified sodium cholate, 0.1%) on the two strains. Concentrations of crude bile and sodium cholate were selected because of their relatively minimal toxicity to the *V. cholerae* strains (see *Supplementary materials*). Finally, because prior investigations have identified regulatory pathways that co-ordinate virulence and biofilm formation, and because bile is known to affect virulence (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999; Hung and Mekalanos, 2005) we examined the two strains, in the presence of crude bile or sodium cholate, under shaking and standing conditions, with standing conditions favouring biofilm formation.

Effect of crude bile and sodium cholate on biofilm gene expression

Both crude and purified sodium cholate significantly altered expression of the previously identified *vps* genes (Yildiz and Schoolnik, 1999). While some changes were observed in the *vpsI* and *vpsII* operons, the most consistent changes were observed in the genes between these

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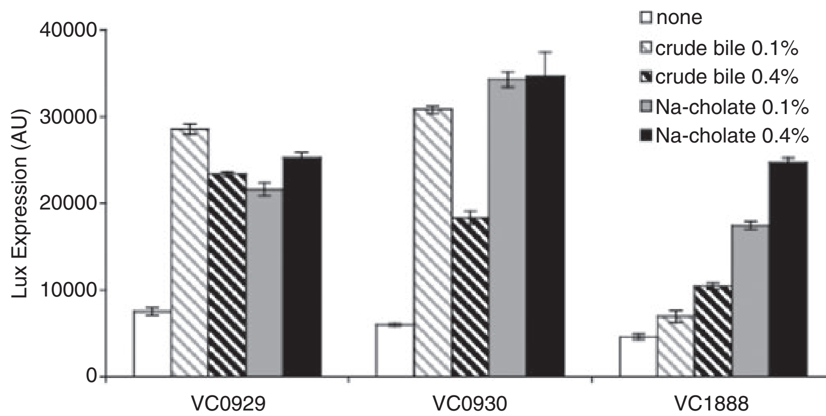

Fig. 1. Effect of bile acids on expression of biofilm-related genes. C6706 (pJZ317) (P_{VC0929}-*luxCDABE*), C6706 (pJZ318) (P_{VC0930}-*luxCDABE*) and C6706 (pJZ319) (P_{VC1888}-*luxCDABE*) were grown in 96-well plates in the absence or presence of crude bile or sodium cholate (0.1% and 0.4%) at 25°C for 36 h. Total luminescence was measured and presented in arbitrary units. Data presented are averages of three replicates and error bars represent calculated standard deviations.

two operons, VC0928–VC0930 (Table S1). Though these genes are not predicted to encode polysaccharide biosynthetic enzymes and their role in biofilm biosynthesis is not clear, their placement between the two *vps* operons suggests a role in biofilm synthesis and they are known to play a role in the rugose morphotype (Yildiz *et al*., 2004). Purified sodium cholate alone was able to induce these genes under standing conditions, suggesting that the bile acid component of crude bile is sufficient for this induction.

To confirm the microarray data, we constructed transcriptional reporters for VC0929 and VC0930 using a *lux* reporter system maintained on a plasmid. The resulting plasmids were transformed into C6706 and luminescence was measured for those strains grown in the absence or presence of crude biles or sodium cholate. As expected, both genes were induced by both crude bile and sodium cholate (Fig. 1).

Effect of crude bile and sodium cholate on biofilm formation

Based on these observations on gene expression, we examined the ability of bile to induce biofilm formation by measuring the amount of biofilm produced by O395 and C6706, as quantified by crystal violet staining. Biofilm mass formed under static conditions was examined in the presence of crude bile, sodium cholate and sodium deoxycholate (Fig. 2). Quantitative crystal violet staining of biofilms demonstrated an increase in biofilm mass with both crude bile and purified bile acids. This increase in biofilm formation was not attributed to a non-specific detergent-like effect, demonstrated by the inability of SDS to increase biofilm mass, even when cell growth of the SDS samples was comparable to that observed with 0.4% sodium cholate and 0.05% sodium deoxycholate (Fig. S1).

Bile-induced biofilm formation is dependent on the known *vps* genes. The addition of bile acids did not result in biofilm formation in a C6706 mutant with a *vps* deletion (VC0920) (Fig. 3A). Of note, this result demonstrates that sodium cholate induces biofilm in a *vps*-dependent manner, in contrast to reports of *vps*-independent biofilms formed in the presence of calcium (Kierek and Watnick, 2003).

In addition to VC0928–0930, VC1888, which is annotated as a haemolysin-related protein, was consistently induced by bile. In the genome-wide microarray study of rugosity (Yildiz *et al*., 2004), VC1888 (herein called *vcp*,

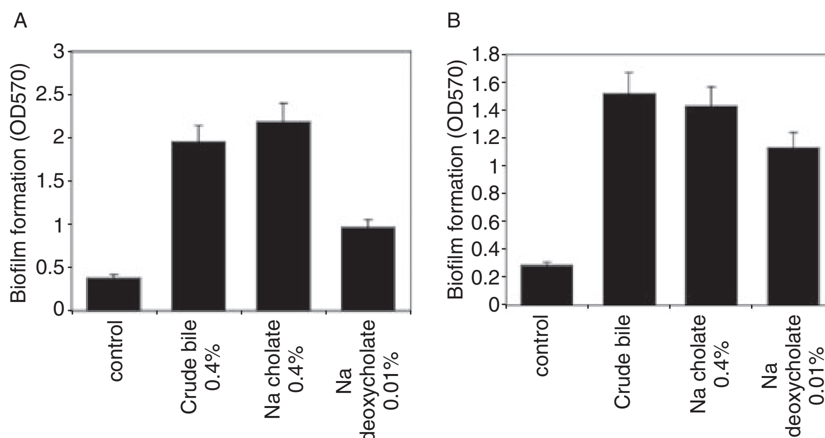

Fig. 2. Effect of bile acids on biofilm formation in *Vibrio cholerae*. (A) C6706. (B) O395. The respective strains were inoculated into culture tubes and allowed to grow without shaking at room temperature for 20 and 36 h for C6706 and O395 respectively. Samples were grown in the presence of no bile acid (control), 0.4% crude bile, 0.4% sodium cholate, or 0.01% sodium deoxycholate. Data presented are averages of three replicates and error bars represent calculated standard deviations. ■, OD_{570} of the crystal violet-stained biofilm.

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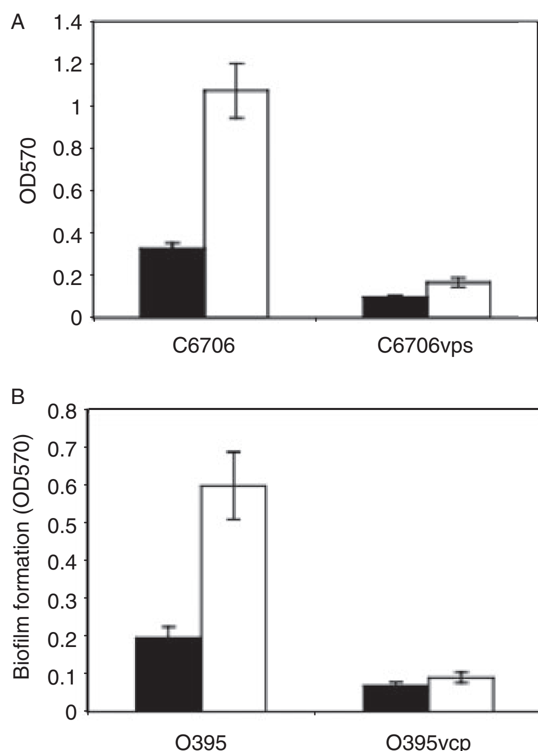

Fig. 3. Bile induction of biofilm formation in *V. cholerae* mutant strains. (A) Effect on C6706Δ*vps.* (B) Effect on O395Δ*vcp*. Borosilicate tubes were inoculated with strains of wild-type or the deletion mutant in 250 μl of LB or LB with 0.4% sodium cholate. The cultures were placed at room temperature and allowed to grow for 18 h for C6706 and 36 h for O395. The biofilms produced were stained with crystal violet and the resulting absorbance at OD_{570} was measured. Data presented are averages of three replicates and error bars represent calculated standard deviations. ■, without sodium cholate; □, with 0.4% sodium cholate.

vps co-regulated protein), was noted to increase with the *vps* gene cluster. In the presence of bile in the expression profiling experiments, expression of *vcp* also paralleled *vps* expression. We confirmed that *vcp* was induced by crude bile under standing conditions, as measured by the *lux* transcriptional reporter system (Fig. 1). To assess the role of *vcp* in biofilm formation, biofilm mass was compared between wild-type O395 and a *vcp* deletion mutant of O395 in the presence and absence of sodium cholate (Fig. 3B). O395Δ*vcp* was defective in biofilm production relative to wild-type O395 thus demonstrating the role of this gene in biofilm synthesis. The deletion mutant was also unable to respond to sodium cholate induction, thus confirming the role of *vcp* in bile-induced biofilm formation.

Bile acid induction of biofilm formation is protective against self-toxicity

Because biofilms are known to confer resistance to xenobiotics and environmental toxins, we examined the ability of the bile acid-induced biofilm to protect *V. cholerae* from its cytotoxic effects. Cell viability of planktonic cells and cells within the biofilm were compared for C6706 in the absence and presence of 0.4% sodium cholate (Table 1). Cultures that were not treated with sodium cholate had almost eightfold more viable planktonic cells compared with cultures with sodium cholate. In contrast, cholate-treated cultures had 2.5-fold more cells within the biofilm than in the corresponding biofilm without sodium cholate. This result suggests that the increased biofilm mass generated in the presence of the bile acid is able to protect cells within the biofilm from the toxic effects of the bile acid, while the planktonic cells remain highly vulnerable. This shift in population represents a change in the ratio of planktonic to biofilm-associated cells from 9.9 to 0.5 in the presence of sodium cholate.

Table 1. Planktonic cells versus biofilm cells for C6706 in the presence of sodium cholate.

	LB (cells × 10^7)	LB + Na cholate 0.4% (cells × 10^7)
Planktonic cells	18.8 ± 0.4	2.4 ± 0.3
Biofilm cells	1.9 ± 0.9	4.6 ± 0.6
Ratio of cells planktonic : biofilm	9.9	0.5

We examined the minimum inhibitory concentration (MIC) of sodium cholate for C6706 and C6706Δ*vps* under standing and shaking conditions (Table 2). Standing or stationary conditions are conducive to biofilm formation while vigorous shaking discourages biofilm formation. If biofilm formation confers resistance to bile acids, then under shaking conditions where no biofilm is made, we postulated that C6706 wild-type cells should be just as sensitive to sodium cholate as a C6706 mutant, C6706Δ*vps*, which is unable to make biofilm.

Comparing the MICs of sodium cholate for wild-type and Δ*vps* strains of C6706, under stationary conditions where wild-type but not the mutant is able to make biofilm, wild-type had an MIC of 2% compared with 0.3% for the mutant. However, under shaking conditions where not only the Δ*vps* strain but also the wild-type strain could not make biofilm, the MICs were the same, 0.2–0.3%, and were no different than the Δ*vps* strain under standing conditions. (We confirmed that C6706 did not make biofilm relative to C6706Δ*vps* under shaking conditions; data

Table 2. MIC of sodium cholate for wild-type and Δ*vps* strain of C6706 in standing versus shaking conditions.

	Wild type (%)	Δ*vps* mutant (%)
Standing conditions[a]	2	0.3
Shaking conditions[b]	0.3	0.2

a. Stationary at 30°C, 22 h, 200 μl in 96-well plate.
b. Shaking 260 r.p.m. at 22°C, 22 h, 1 ml in 12 mm test-tube.

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not shown.) These results suggest that the induction of biofilm by sodium cholate confers to *V. cholerae*, increased resistance to cholate's own toxic effects.

Bile acid induction of biofilm is independent of previously described regulators of virulence and biofilm formation

The regulation of virulence and, more recently, biofilm formation in *V. cholerae* has been the subject of extensive study with the identification of several regulation pathways. These pathways include the ToxRS regulon, which has been previously described to be affected by bile or bile acids (Provenzano *et al.*, 2000; Hung and Mekalanos, 2005), quorum sensing regulation (Zhu and Mekalanos, 2003) and the VieS/A/B three-component regulatory system (Tischler and Camilli, 2004). We examined whether these pathways are involved in sodium cholate induction of biofilm in a variety of different mutants.

All three regulatory systems did not appear to be involved in biofilm indution by bile acids (Figs S2–S4). Deletion in C6706 of the known virulence regulators *toxRS*, *tcpPH*, or the downstream regulator *toxT*, had no effect on the ability of sodium cholate to induce biofilm. Similarly, bile acids had no effect on biofilm formation in C6706 mutants that contained deletions of quorum sensing regulators *luxO* and *hapR* (Hammer and Bassler, 2003; Zhu and Mekalanos, 2003), or deletion or overexpression of dinucleotide regulator *vieA* (Tischler and Camilli, 2004).

The vps *regulator* vpsR *but not* vpsT *is required for biofilm induction by sodium cholate*

Given that cholate induction of biofilms is *vps*-dependent, we examined the role of the positive transcriptional activators of the *vps* genes, *vpsR* (VC0665) and *vpsT* (VCA0952). We examined the effects of sodium cholate on *vpsR* and *vpsT* transcription in C6706 using transcriptional reporter fusions with *lacZ* and found no cholate-dependent difference in transcription levels of either regulator (Fig. 4A). However, comparison of biofilms formed by C6706 wild-type, *vpsR* deletion mutant and *vpsT* deletion mutant, in the absence and presence of sodium cholate, demonstrated that cholate induction of biofilm requires *vpsR* (Fig. 4B). Deletion of *vpsT* does not prevent cholate induction of biofilm thus demonstrating that it is not essential for cholate's effect. However, the amount of biofilm produced in the presence of cholate is somewhat reduced relative to wild-type, suggesting that *vpsT* can contribute to full induction. Together, these results suggest that cholate induction of the *vps* genes occurs not by activation of *vpsR* transcription, but by a post-translational mechanism, such as activation of VpsR. Post-translational activation of VpsR would be entirely consistent with its

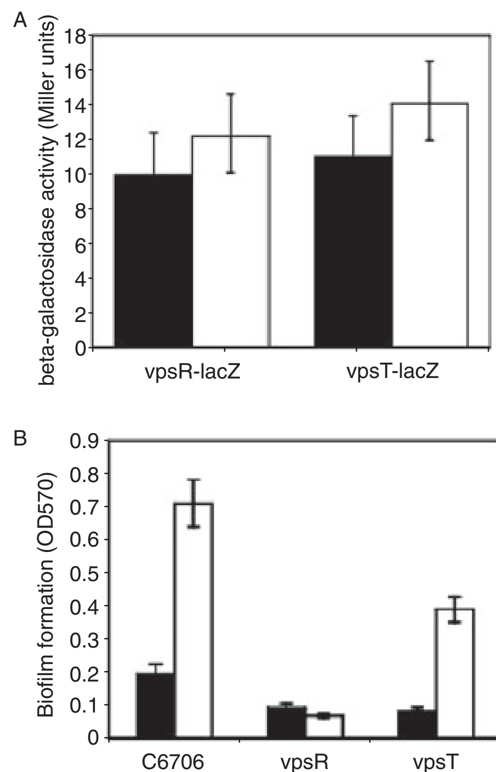

Fig. 4. A. Effect of sodium cholate on *vpsR* and *vpsT* transcription. Borosilicate tubes were inoculated with C6706 bearing *vpsR–lacZ* or *vpsT–lacZ* transcriptional fusion reporters in 1 ml of LB or LB with 0.4% sodium cholate. The cultures were placed at room temperature and allowed to grow for 22 h. The subsequent cultures were vortexed with glass beads to homogenize the biofilms and the resulting culture of planktonic and biofilm cells were assayed for β-galactosidase activity. The activity was normalized for OD_{600} of the homogenate, which was approximately the same as normalizing for viable cell number as determined by plating out the resulting cultures. Data presented are averages of three replicates and error bars represent calculated standard deviations. ■, without sodium cholate; □, with 0.4% sodium cholate.
B. Bile induction of biofilm formation is *vpsR*-dependent but *vpsT*-independent. Borosilicate tubes were inoculated with strains of wild-type C6706, a Δ*vpsR* mutant or a Δ*vpsT* mutant strain of C6706 in 250 μl of LB or LB with 0.4% sodium cholate. The cultures were placed at room temperature and allowed to grow for 22 h. The biofilms produced were stained with crystal violet and the resulting absorbance at OD_{570} was measured. Data presented are averages of three replicates and error bars represent calculated standard deviations. ■, without sodium cholate; □, with 0.4% sodium cholate.

predicted behaviour, given its homology to the response element of two-component regulatory systems (Yildiz *et al.*, 2001). Additionally, given the diversity of the VpsR regulon (Yildiz *et al.*, 2004), modulation of VpsR activity would be consistent with the pleiotropic effects described for bile and bile acids (see the model presented in Fig. 5).

Conclusion

Bile is an important physiologic component of the human intestine. It probably plays a role in host immunity to

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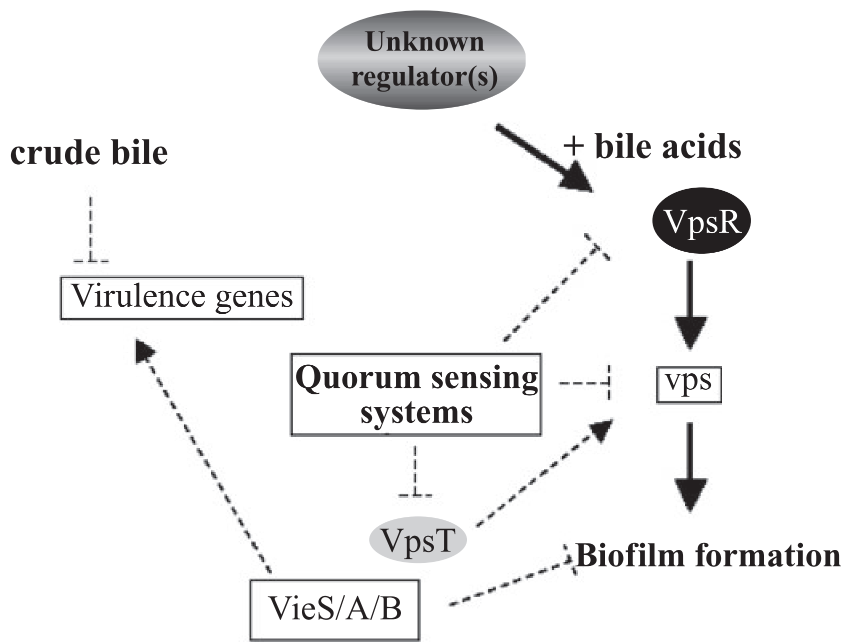

Fig. 5. Model for bile effect on biofilm formation and virulence. Bile acids activate VpsR post-transcriptionally, thus resulting in increased biofilm formation. This activation occurs through some unknown regulator of VpsR. Meanwhile, crude bile contains some components other than bile acids that repress virulence directly.

V. cholerae infection because of its bacteriocidal effects. It also induces a range of pleiotropic responses by the bacteria, due in part to the heterogeneity of bile composition (Hung and Mekalanos, 2005). Expression profile analysis of the effects of bile on *V. cholerae* demonstrated that some of the *vps* genes (and *vcp* gene) are upregulated as part of a bacterial response that results in increased biofilm formation.

Induction of biofilm formation by bile acids, one component of bile, appears to confer resistance to bacteria within the biofilm, relative to planktonic cells. While this biofilm induction is independent of known biofilm regulatory pathways, it is dependent on the *vps* genes and *vpsR*, possibly involving post-translational activation of VpsR.

Vibrio polysaccharide synthesis production and biofilm formation have been suggested to play an important role in the survival of *V. cholerae* in the environment (Watnick and Kolter, 1999; Yildiz and Schoolnik, 1999). Biofilm formation may be significant however, not only in the environment, but also during infection. Animal studies suggest that this phenomenon is important in some models of infection (Rashid *et al.*, 2004); other studies suggest that it is not essential for infant mouse colonization (Zhu and Mekalanos, 2003). Interestingly, bile is not yet made in the infant intestine at that age and could account for this lack of effect in that particular system (Li-Hawkins *et al.*, 2000).

These *in vitro* studies of the effects of bile on *V. cholerae* biofilm formation and pathogenesis are provocative because the bacterium inevitably encounters bile in the human intestine during infection. While much remains to be elucidated regarding the *in vivo* regulation of *V. cholerae* genes during infection, the continued study of these mechanisms is central to understanding how *V. cholerae* survives and persists in this intestinal environment.

Experimental procedures

Bacterial strains and plasmids

The *V. cholerae* El Tor biotype strain C6706 and the classical biotype strain O395 (Mekalanos *et al.*, 1979) were used in all experiments. The method of Skorupski and Taylor (1996) was used to construct an in frame deletion of *vcp* (VC1888) in O395. The construction of the mutant carrying a deletion in *vps* in C6706 has been previously described (Zhu *et al.*, 2002; Zhu and Mekalanos, 2003).

The chromosomal *vpsR–lacZ* and *vpsT–lacZ* transcriptional reporters were constructed by polymerase chain reaction (PCR) amplification of the 5′ DNA of *vpsR* and *vpsT*, respectively, and cloning these fragments into pVIK112 (Kalogeraki and Winans, 1997). The resulting plasmids were then integrated into the chromosome at the *vpsR* and *vpsT* loci by homologous recombination. Laboratory stocks of *Escherichia coli* DH5$\alpha\lambda$pir and SM10λpir were used for cloning and mating into *V. cholerae* respectively.

Expression profiling of V. cholerae

Vibrio cholerae strains O395 and C6706 were grown to mid-log phase in the absence of bile, in the presence of crude bile (0.4%; Sigma) or purified sodium cholate (0.1%; Sigma). For O395 shaking experiments, an overnight culture of O395 was inoculated at a 1:1000 dilution into 5 ml LB broth pH 6.5 and shaken at 260 r.p.m. for 6 h at 30°C prior to RNA isolation. For O395 standing cultures, an overnight culture of O395 was inoculated at a 1:1000 dilution into 5 ml LB broth

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pH 6.5 and divided into 1 ml aliquots into five test-tubes (12 × 75 mm). The cultures were allowed to grow standing at 30°C for 18 h prior to RNA isolation.

For C6706 shaking experiments, an overnight culture of C6706 was inoculated at a 1:10 000 dilution into 5 ml of AKI media and grown for 4 h stationary at 37°C, followed by shaking at 260 r.p.m. for 6 h prior to RNA isolation. For C6706 standing experiments, an overnight culture of C6076 was inoculated at a 1:1000 dilution into 5 ml of AKI media and divided into 1 ml aliquots into five test-tubes (12 × 75 mm). The cultures were allowed to grow standing at 37°C for 14 h prior to RNA isolation.

Cells from the shaking cultures were harvested by centrifugation at 22°C of the 5 ml culture and the cells were immediately placed in 5 ml TRIzol reagent (Gibco-BRL). Cells from the standing cultures were harvested by vortexing the cultures with glass beads (1 mm) for 1 min to disrupt the biofilm. The subsequent cellular suspection was then centrifuged at 22°C and the resulting cell pellet was taken up in 5 ml TRIzol reagent.

The production of spotted microarrays containing full-length open reading frames (ORFs) derived from *V. cholerae* strain N16961 has been described elsewhere (Dziejman *et al.*, 2002). RNA was isolated from cells grown under the appropriate conditions using TRIzol reagent extracted with hot phenol (pH 5.2, 65°C), and purified using RNeasy kit (Qiagen). Fluorescently labelled cDNA was prepared by direct incorporation of fluorescent nucleotide analogues (Cy3-dCTP and Cy5-dCTP) during a first-strand randomly primed reverse transcription reaction. The differentially labelled cDNAs were combined and subsequently applied to the array surface under conditions that favour hybridization (Dziejman *et al.*, 2002). Microarray slides were scanned using a ScanArray 5000 apparatus (GSI Lumonics). For every ORF-specific spot, the resulting fluorescence intensity of each of the labels was measured and compared using the GenePix Pro 3.0 software system (Axon).

Growth conditions

All *V. cholerae* strains were grown in LB broth or AKI media (Iwanaga *et al.*, 1986) at 22, 30 or 37°C as indicated. MICs for the varying detergents were measured under stationary conditions by inoculating LB with a 1:1000 dilution of an overnight culture, plating 200 μl of the resulting culture into 96-well plates and monitoring growth at 30°C in a Spectramax plus plate reader (Molecular Devices) for 22 h. Under shaking conditions, a 1 ml culture was grown in a 100 mm × 12 mm culture tube at 22°C, shaking at 260 r.p.m. OD_{600} of the samples was measured at 22 h. MIC was defined as the concentration of sodium cholate that resulted in less than 10% of the OD_{600} measured for the strains in the absence of sodium cholate at 22 h.

Lux transcriptional reporter system for vps *genes*

To make transcriptional *lux* fusions, DNA fragments of the promoter regions of VC0929, VC0930 and VC1888 were amplified by PCR and cloned into a vector pHKBS1 (Hoang *et al.*, 2000) resulting in pJZ317 (P_{VC0929}-*luxCDABE*), pJZ318 (P_{VC0930}-*luxCDABE*) and pJZ319 (P_{VC1888}-*luxCDABE*) respectively. Overnight cultures of C6706 (pJZ317), C6706 (pJZ318) and C6706 (pJZ319) were inoculated 1:100 into 200 μl LB broth containing 2 μg ml^{-1} of tetracycline in the absence or presence of two concentrations of crude bile or sodium cholate in 96-well white plates. The samples were incubated at room temperature for 36 h and luminescent production was then measured using a Synergy HT multidetection reader (Bio-tek Instruments, VT, USA).

Biofilm assays

The *V. cholerae* strains were grown overnight on LB agar plates. Colonies were resuspended in LB broth at OD_{600} of 0.6. A 1:100 dilution of this suspension was inoculated in LB broth into 10 × 75 mm borosilicate glass test-tubes and incubated for 20–22 h at 22°C. Subsequently, the tubes were rinsed with distilled water then filled with crystal violet stain. After 5 min, the tubes were rinsed. The biofilm-associated crystal violet was resuspended with dimethyl sulphoxide (DMSO), and the OD_{570} of the resulting suspension was measured (Zhu and Mekalanos, 2003). All experiments were performed at least three independent times and samples were performed in triplicate. Data shown are a representative experiment and error bars represent standard deviations within that single experiment.

Measuring viability of planktonic versus biofilm cells

The *V. cholerae* strains were grown overnight on LB agar plates. Colonies were resuspended in LB broth at OD_{600} of 0.6. A 1:100 dilution of this suspension was inoculated in 250 μl LB broth in the presence or absence of 0.4% sodium cholate in 10 × 75 mm borosilicate glass and incubated for 22 h at 22°C. The number of planktonic cells in the liquid broth were plated out and counted. The cells in the biofilm were vortexed with glass beads (1 mm) for 1 min to disrupt the biofilm and then were plated out and counted. All experiments were performed at least three independent times and samples were performed in triplicate. Data shown are a representative experiment and error bars represent standard deviations within that single experiment.

β-Galactosidase assay for vpsR–lacZ *and* vpsT–lacZ

The strains C6706*vpsR–lacZ* and C6706*vpsT–lacZ* were grown overnight on LB agar plates. Colonies were resuspended in LB broth at OD_{600} of 0.6. A 1:100 dilution of this suspension was inoculated into 10 × 75 mm borosilicate glass tubes containing 1 ml of LB. These cultures were incubated at 22°C for 18 h. The biofilm structures were disrupted by adding large glass beads (1 mm) to the culture and vortexing for 1 min. The β-galactosidase activity of the resulting suspensions was measured by the method of Miller (Miller, 1972) and the activity was corrected for OD_{600} and surviving cell number (determined by plating on LB agar). While the results reported are for samples in which planktonic and biofilm cells were combined, qualitatively, there was no difference when biofilm cells were measured alone. All experiments were performed at least three independent times and samples were performed in triplicate. Data shown are a rep-

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resentative experiment and error bars represent standard deviations within that single experiment.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Growth curve of C6706 and O395DtcpA in the presence of bile.

Fig. S2. Effect of C6706 virulence regulators on bile acid-induced biofilm formation.

Fig. S3. Effect of C6706 quorum sensing regulators on bile acid-induced biofilm formation.

Fig. S4. Effect of C6706 VieA regulator on bile acid-induced biofilm formation.

Table S1. VPS gene cluster expression in O395 and C6706 in the presence and absence of crude bile or sodium cholate under shaking or standing conditions.*

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