A ToxR-dependent role for the putative phosphoporin VCA1008 in bile salt resistance in *Vibrio cholerae* El Tor N16961

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The putative phosphoporin encoded by *vca1008* of *Vibrio cholerae* O1 is expressed *in vivo* during infection and is essential for the intestinal colonization of infant mice. *In vitro*, its expression is induced under inorganic phosphate (P_i) limitation in a PhoB/R-dependent manner. In this work we demonstrated that VCA1008 has a strain-specific role in the physiology and pathogenicity of *V. cholerae* O1. Disruption of *vca1008* led to a growth defect, an inability to colonize and a high susceptibility to sodium deoxycholate (DOC; the major bile compound) in the El Tor biotype strain N16961, but did not affect the classical strain O395 in the same way. Furthermore, *vca1008* promoter activity was higher in N16961 cells grown under a low P_i supply in the presence of DOC than in the absence of the detergent. In the P_i -limited cells, *vca1008* was positively regulated by PhoB, but when DOC was added to the medium, it negatively affected the PhoB-mediated activation of the gene, and enhanced *vca1008* expression in a ToxR-dependent manner. These findings reveal for the first time a complex strain-specific interplay between ToxR and PhoB/R systems to control porin genes, as well as the influence of DOC on the expression of PhoB- and ToxR-regulated genes and pathogenesis in pandemic strains of *V. cholerae*.

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INTRODUCTION

Vibrio cholerae is a Gram-negative bacterium found free-living or in association with phytoplankton, zooplankton, crustaceans and molluscs in estuarine and other aquatic environments (Wai *et al.*, 1999). Several strains of the serogroups O1 and O139 are human pathogens and cause cholera, an often-fatal diarrhoeal disease. In the various niches of the human gastrointestinal tract, the bacterium faces distinct conditions with respect to acidity, osmolarity, oxygen levels and nutrient sources (Chowdhury *et al.*, 1996). *V. cholerae* reaches the small intestine, where it expresses virulence factors, despite the presence of inhibitory substances such as bile (Reidl & Klose, 2002), a digestive secretion constituted of bile salts, phospholipids, cholesterol and the pigment biliverdin (Begley *et al.*, 2005; Gunn, 2000). Bile acts as a biological detergent that emulsifies and solubilizes lipids and, as such, possesses antimicrobial activity that is attributed mainly to its ability to disrupt cell-membrane integrity (reviewed by Begley *et al.*, 2005). Enteric bacteria respond to bile by altering the expression of genes whose products are involved in signal sensing and transduction, in general stress adaptation

(Bernstein *et al.*, 1999; Leverrier *et al.*, 2003; Savijoki *et al.*, 2005; van Velkinburgh & Gunn, 1999) and in bile resistance mechanisms (Gänzle *et al.*, 1999; Gupta & Chowdhury, 1997; Krukons & Dirit, 2003; Pace *et al.*, 1997; Provenzano & Klose, 2000; van Velkinburgh & Gunn, 1999).

Bile salts can enter the cytoplasm of Gram-negative bacterial cells through the hydrophilic channels of outer membrane porins (Gunn, 2000; Thanassi *et al.*, 1997) or across the membrane bilayer in uncharged forms (Plésiat & Nikaido, 1992). TolC-dependent and -independent active efflux mechanisms pump bile acids out of cells (Bina & Mekalanos, 2001; Chatterjee *et al.*, 2004; Gunn, 2000; Poole, 2002; Thanassi *et al.*, 1997), but lipopolysaccharide (LPS) and porins also play roles *vis-à-vis* intrinsic bile salt resistance in Gram-negative bacteria (Gunn, 2000; Snyder & McIntosh, 2000). The involvement of pore-forming channels in resistance to bile salts is mainly due to the wide flexibility of porin expression (Achouak *et al.*, 2001). In many cases, acquired resistance in Gram-negative bacteria can be attributed to loss of porins or alterations in their expression (Poole, 2002).

In *V. cholerae*, the acquired resistance to bile *in vitro* can result from a synergy between intrinsic resistance mechanisms and ToxR modulation of the expression of OmpU

Abbreviations: DOC, sodium deoxycholate; OMP, outer membrane protein.

and OmpT porins (Provenzano *et al.*, 2000). Bile stimulates transcription of *ompU* and cells expressing OmpU alone are more resistant to bile *in vitro* than those expressing OmpT alone (Provenzano *et al.*, 2001; Provenzano & Klose, 2000). Apparently, the protection imparted to *V. cholerae* cells by OmpU is due to its higher cation selectivity and impermeability to sodium deoxycholate (DOC; the major component of bile salts) in comparison with OmpT (Duret & Delcour, 2006; Simonet *et al.*, 2003). However, neither OmpU nor OmpT is essential for expression of virulence factors *in vitro* or intestinal colonization (Osorio *et al.*, 2004).

The product of *vca1008* has been identified as an outer membrane protein (OMP) whose expression is induced upon inorganic phosphate (P_i) starvation and requires the binding of PhoB to the *vca1008* regulatory region (Goulart *et al.*, 2009; von Krüger *et al.*, 2006). A prediction of VCA1008 structure has shown that the protein shares many structural similarities with PhoE of *Escherichia coli*, a porin also induced under P_i limitation. Properties such as resistance to denaturation by SDS at lower temperatures and to heat suggest that VCA1008 is a classical trimeric porin, or more precisely the phosphoporin of *V. cholerae* (Goulart *et al.*, 2009). Cells of *V. cholerae* O1 El Tor biotype deficient in VCA1008 expression are less infective in infant mice than those of their parental strain, suggesting an essential role for this protein in the colonization process (Osorio *et al.*, 2004). The observation that *vca1008* is expressed during infection in animal models and human volunteers (Lombardo *et al.*, 2007; Osorio *et al.*, 2005) corroborates this idea.

VCA1008 and OmpU are paralogues that share 33% identity and 62% similarity, and might play redundant functions in *V. cholerae* cells. Based on these findings we decided to test the hypothesis that the putative phosphoporin VCA1008 could play a role in bile resistance in the pandemic strains N16961SR and O395SR of the El Tor and classical biotypes of *V. cholerae* O1, respectively.

We demonstrate herein that VCA1008 is required for bile resistance and pathogenicity of N16961SR alone, indicating that the protein is likely to perform distinct functions in classical and El Tor strains of *V. cholerae* O1. Furthermore, interplay between ToxR- and PhoB/R-dependent regulatory pathways apparently takes place in the El Tor and classical biotypes in a bile salt-dependent and -independent manner, respectively, to control the expression of certain genes and ultimately the physiology and pathogenicity of the bacterium.

METHODS

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Strains were routinely grown at 37 °C in Luria–Bertani broth (LB) or on solid medium [Luria agar (LA), 1.5% agar in LB]. For growth under defined P_i concentrations, TG (Tris-glucose with a mix of mineral salts) was used with 6.5 mM KH_2PO_4 (high phosphate medium, TGHP) or

65 µM (low phosphate medium, TGLP; von Krüger *et al.*, 1999). Antibiotics were added to the media at the following concentrations: streptomycin (Sm), 100 µg ml⁻¹; kanamycin (Km), 50 µg ml⁻¹; ampicillin (Ap), 100 µg ml⁻¹; rifampicin (Rif), 25 µg ml⁻¹; chloramphenicol (Cm), 20 µg ml⁻¹. Because of the high content of P_i in crude bile, DOC (Sigma) at 0.1–0.2% was used to mimic bile effects.

Cloning procedures and mutant construction. Genetic manipulations were carried out in *E. coli* strains DH5α and SY327λpir. Standard methods were used to prepare plasmids, restriction enzyme digestions and DNA ligations. DNA fragments were purified from agarose gels with a Wizard SV Gel kit (Promega) in accordance with the supplier's recommendations. PCR amplifications were carried out in an automatic thermal cycler (PTC-200, MJ Research) using *V. cholerae* chromosomal DNA and Taq DNA polymerase (Promega). Amplifications were performed using 35 cycles of denaturation at 94 °C for 2 min, annealing at 56 °C (*vca1008*) or 52 °C (*toxR*) for 1 min, and extension at 72 °C for 2 min.

For the construction of mutants, fragments containing *vca1008* (1.2 kb) and *toxR* (0.9 kb) from *V. cholerae* N16961SR were PCR-amplified using the following primers (restriction sites underlined): PhoE1-*NcoI* (5'-CATAGCCATGGATTCCTCATCACTACC-3') and PhoERev-*SmaI* (5'-AAACCCGGGTCGTAACGTAGACCTAG-3'); ToxR-*BamHI* (5'-CCGGATCCATGTTCCGATTAGGACAC-3') and ToxR-*HindIII* (5'-CAAGCTTGACTCACACACCTTGATGGC-3').

DNA fragments were cloned into pGEM-T Easy (Promega) to produce pGEM-*vca1008* and pGEM-*toxR*. The plasmids were cut within the *vca1008* sequence with *EcoRV* and within *toxR* with *NruI*, where a Km-resistance cassette (1.3 kb *HincII* fragment of pUC-4K) was inserted to interrupt the target genes.

Plasmids pGEM-*vca1008*::Km and pGEM-*toxR*::Km were digested with *EcoRI*, and the fragments containing the disrupted genes were cloned into the *EcoRI* site of pGP704. To facilitate mutant selection, the *sacB* gene from *Bacillus subtilis* (1.9 kb *EcoRV/BamHI* fragment from pJG9) was cloned into both pGP-*vca1008*::Km and pGP-*toxR*::Km, digested with *EcoRV* and *BglII*, yielding pGS1 and pGS2, respectively.

Plasmids pGS1 and pGS2 were mobilized into *V. cholerae* O1 strains by conjugation with *E. coli* strain SM10λpir harbouring either pGS1 or pGS2. Mutants were obtained by allelic exchange and confirmed by Southern blot analysis, as previously described (von Krüger *et al.*, 1999). Mutants in the *vca1008* and *toxR* genes derived from strain N16961SR were named CG4 and CG5, respectively. The corresponding mutants in O395SR were, respectively, GS1 and GS2 (Table 1).

For complementation analysis, a 1.2 kb fragment containing the whole *vca1008* gene plus an upstream region of 231 bp was PCR-amplified using the primers PhoE1-*NcoI* (described above) and PhoEStop-*SmaI* (5'-GCGCCCGGGTTATTAATAATCGTAACGTAG-3'). The fragment was first cloned into pGEM-T Easy, removed with *EcoRI* and subcloned into the *EcoRI* site of pWKS30 to generate pCG15.

To construct a promoter-*lacZ* fusion we used the promoter-probe vector pIC552. A fragment of 234 bp from the regulatory region upstream of the putative translation start codon of *phoB* (*vc0719*) was amplified by PCR using as template chromosomal DNA from *V. cholerae* strain N16961SR and the following primers (endonuclease recognition sites underlined): PhoB-*NcoI* (5'-CCCCCATGGTTTAAACCACATTGTTG-3') and PhoB-*XhoI* (5'-TCTTCTC-GAGTGTGTTTTACCAACGCCA-3'). The fragment was digested with both *NcoI* and *XhoI* and ligated into pIC552 digested with the same restriction endonucleases. It is worth pointing out that in *V. cholerae* strains N16961 and O395, *vca1008* and *toxR* gene sequences plus corresponding regulatory regions are identical.

Table 1. Bacterial strains and plasmids used in this study

Ap^R, Cm^R, Km^R, Rif^R, Sr^R: ampicillin, chloramphenicol, kanamycin, rifampicin and streptomycin resistance, respectively.

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>E. coli</i> strains		
DH5 α	F ⁻ Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i>	Raleigh <i>et al.</i> (1988)
SY327 λ <i>pir</i>	F ⁻ Δ (<i>lac pro</i>) <i>nalA argE</i> (Am) <i>rif araD recA56 λpir</i> R6K, Rif ^R	Miller & Mekalanos (1988)
SM10 λ <i>pir</i>	<i>thi thr leu tonA lacY supE recA::RP4-2Tc::Mu λpir</i> R6K	Donnenberg & Kaper (1991)
<i>V. cholerae</i> strains		
N16961SR	Spontaneous Sr ^R mutant of wild-type El Tor biotype	Laboratory stock
O395SR	Spontaneous Sr ^R mutant of wild-type classical biotype	Laboratory stock
CG4	<i>vca1008</i> mutant of N16961SR, Sr ^R and Km ^R	This work
GS1	<i>vca1008</i> mutant of O395SR, Sr ^R and Km ^R	This work
CG5	<i>toxR</i> mutant of N16961SR, Sr ^R and Km ^R	This work
GS2	<i>toxR</i> mutant of O395SR, Sr ^R and Km ^R	This work
Plasmids		
pGEM-T Easy	High-copy-number cloning vector, Ap ^R	Promega
pGEM- <i>vca1008</i>	1.2 kb fragment of <i>vca1008</i> from strain N16961SR cloned into pGEM-T Easy, Ap ^R	This work
pGEM- <i>toxR</i>	0.9 kb fragment of <i>toxR</i> from strain N16961SR cloned into pGEM-T Easy, Ap ^R	This work
pUC-4K	Km-resistance cassette of Tn903 in pUC4, Km ^R	Oka <i>et al.</i> (1981)
pGEM- <i>vca1008::Km</i>	1.3 kb <i>HincII</i> fragment of pUC-4K (Km-resistance cassette) cloned into <i>EcoRV</i> site of pGEM- <i>vca1008</i> , Ap ^R and Km ^R	This work
pGEM- <i>toxR::Km</i>	1.3 kb <i>HincII</i> fragment of pUC-4K (Km-resistance cassette) cloned into <i>NruI</i> site of pGEM- <i>toxR</i> , Ap ^R and Km ^R	This work
pGP704	Suicide vector, <i>mob</i> RP4, <i>ori</i> R6K, Ap ^R	Miller & Mekalanos (1988)
pGP- <i>vca1008::Km</i>	2.5 kb <i>EcoRI</i> fragment of pGEM- <i>vca1008::Km</i> cloned into <i>EcoRI</i> site of pGP704, Ap ^R and Km ^R	This work
pGP- <i>toxR::Km</i>	2.2 kb <i>EcoRI</i> fragment of pGEM- <i>toxR::Km</i> cloned into <i>EcoRI</i> site of pGP704, Ap ^R and Km ^R	This work
pJG9	<i>sacB</i> -containing plasmid, Cm ^R	Galen & Levine (1995)
pGS1	1.9 kb <i>EcoRV/BamHI</i> fragment of pJG9 (<i>sacB</i> gene) cloned into <i>EcoRV/BglII</i> site of pGP- <i>vca1008::Km</i> , Ap ^R and Km ^R	This work
pGS2	1.9 kb <i>EcoRV/BamHI</i> fragment of pJG9 (<i>sacB</i> gene) cloned into <i>EcoRV/BglII</i> site of pGP- <i>toxR::Km</i> , Ap ^R and Km ^R	This work
pIC552	Promoter probe vector for transcriptional fusions to <i>lacZ</i> , Ap ^R	Macián <i>et al.</i> (1994)
pICP- <i>vca1008</i>	241 bp fragment of <i>vca1008</i> promoter from N16961SR cloned into pIC552, Ap ^R	Goulart <i>et al.</i> (2009)
pIC234	234 bp fragment of <i>phoB/R</i> promoter from N16961SR cloned into pIC552, Ap ^R	This work
pGEM- <i>vca1008comp</i>	1.2 kb fragment containing the whole <i>vca1008</i> gene plus an upstream region of 231 bp cloned into pGEM-T Easy (Promega), Ap ^R	This work
pWKS30	Low-copy-number cloning vector	Wang & Kushner (1991)
pCG15	1.2 kb <i>EcoRI</i> fragment of pGEM- <i>vca1008comp</i> cloned into <i>EcoRI</i> site of pWKS30, Ap ^R	This work

Preparation of outer membrane samples and identification of OMPs. OMPs were prepared as previously described (von Krüger *et al.*, 1999). Briefly, cells were disrupted by sonication and cell membranes were recovered after ultracentrifugation (66 000 *g* for 40 min). The insoluble fraction was first washed with 1 % Sarkosyl and subsequently with 2 % SDS. Insoluble proteins were resuspended in 1 % octyl-polyoxyethylene and analysed by SDS-PAGE. The gels were stained with Coomassie brilliant blue G-250 and proteins were identified by MALDI-TOF analysis, after tryptic digestion, as previously described (von Krüger *et al.*, 2006).

DOC susceptibility analysis. Overnight cultures were diluted 1 : 100 in fresh LB and incubated in a shaking water bath at 37 °C to early exponential growth phase (OD₆₀₀ ~0.5). Cells were harvested by centrifugation (5000 *g* for 5 min at room temperature), washed, resuspended to the same cell density in TGHP or TGLP, and

incubated for 12–14 h with agitation at 37 °C. Cells were collected by centrifugation, as before, and resuspended in fresh TGHP or TGLP. Viable cell counts (c.f.u.) were determined before (control) and 1, 2 and 5 min after the addition of 0.2 % DOC to the cultures. The c.f.u. of the control was used as the 100 % value and the survival rates were expressed as percentages of the control. The survival rates of the cells pre-cultivated in TGHP and TGLP were compared using the *t* test and considered different at *P* < 0.05.

Enzymic assays. β -Galactosidase activities in strains carrying the recombinant plasmids pICP-*vca1008* and pIC234 were measured as previously described (Goulart *et al.*, 2009). Alkaline phosphatase (PhoA) activity was determined by a variation of the permeabilized whole-cell assay (von Krüger *et al.*, 1999). Activities of both enzymes were expressed in Miller units (μ g protein)⁻¹, as determined using the Protein Assay Dye Reagent concentrate (Bio-Rad).

Infant mouse colonization assay. The infant mouse colonization assay was performed as described by Gardel & Mekalanos (1996). Briefly, *V. cholerae* cells were grown overnight at 37 °C in LB supplemented with the appropriate antibiotics. Infant Swiss albino mice (3–4 days old) were orogastrically inoculated ($\sim 10^6$ bacteria in 50 μ l per mouse), separated from their mother and left at 30 °C for 18 h. A central fraction of the small intestine was removed and homogenized in 2 ml LB. The homogenate was serially diluted and plated onto LA plates containing appropriate antibiotics. For the *in vivo* competition assay, an equal number of cells was mixed and inoculated (total $\sim 10^6$ bacteria in 50 μ l per mouse). The total c.f.u. of Km-sensitive (wild-type) and Km-resistant (*vca1008* mutant) bacteria was calculated and expressed per gram of tissue. Mean colonization was determined for three replicates with 6–12 animals per experiment.

RESULTS AND DISCUSSION

Strain-dependent role for *vca1008* in growth rate and colonization ability of *V. cholerae* O1

Disruption of the gene encoding the putative phosphoporin VCA1008 generated mutants that clearly differed from their parental strains. The *vca1008* null mutant of N16961SR, strain CG4, presented a reduced growth rate in rich LB medium, but eventually reached a cell density comparable with that of the wild-type strain at the stationary phase (Fig. 1a). Growth deficiency was also observed in TG defined media under both low (TGLP) and high (TGHP) phosphate levels (Fig. 1b, c). These findings correlate well with the reduced intestinal colonization ratio exhibited by the *vca1008* mutant, when tested independently or in the presence of the wild-type strain (Table 2). In contrast to CG4, GS1, the *vca1008* null mutant of the classical strain O395SR, grew similarly to the parental strain in either LB or TG medium under high or low levels of P_i (Fig. 1). Furthermore, GS1 colonized the infant

mouse intestine nearly as efficiently as the wild-type strain when inoculated independently, and out-competed O395SR in mixed-infection assays (Table 2).

These are surprising results, considering that the sequences of genes *vca1008* (from the El Tor strain N16961) and VC0395_0230 (from the classical strain O395) and their 250 bp corresponding upstream regions are identical. The distinct phenotypes of *vca1008* mutants CG4 and GS1 suggest that this gene might play divergent P_i -independent roles in the physiology and pathogenicity of classical and El Tor biotypes of *V. cholerae* O1. In addition, they further emphasize the differences between these two strains and suggest that they contrast markedly in the mechanisms whereby they colonize the host intestine.

VCA1008 is required for bile salt resistance in *V. cholerae* O1 El Tor strain N16961SR

Earlier studies on *V. cholerae* O1 classical strain O395 have revealed that the ToxR-regulated porin OmpU is critical for bile resistance *in vitro* (Provenzano *et al.*, 2000; Provenzano & Klose, 2000). However, this porin is not required for efficient intestinal colonization of animal models by either El Tor or classical strains of *V. cholerae* O1 (Osorio *et al.*, 2004; Provenzano *et al.*, 2001). The VCA1008 and OmpU genes are paralogues but, in contrast to *ompU*, *vca1008* is essential for intestinal colonization by *V. cholerae* El Tor strains and is expressed in the gastrointestinal tract of animal models and human volunteers (Lombardo *et al.*, 2007; Osorio *et al.*, 2005), where the bacteria encounter bile salts.

These findings lead us to suspect that the putative phosphoporin VCA1008 plays a role in the *V. cholerae* O1 bile resistance mechanism. To investigate this hypothesis, N16961SR and CG4 cells cultivated in both TGLP and

Fig. 1. Growth kinetics of wild-type strains N16961SR and O395SR and their respective isogenic *vca1008* mutants CG4 and GS1. *V. cholerae* cells were grown overnight in LB, inoculated into fresh LB (a), TGHP (b) or TGLP (c) medium and incubated at 37 °C with aeration for several hours. Growth was monitored by OD₆₀₀ measurement. Data are representative of three experiments; error bars, SD. Note that time and OD₆₀₀ scales are different among the three panels. ●, N16961SR; ○, CG4; ■, O395SR; □, GS1.

Table 2. Colonization efficiency of *V. cholerae* strains

The ratio of wild-type to mutant strains is shown. A ratio greater than 1.0 means that the wild-type colonizes more efficiently than the mutant strain.

Strains	Independent	Mixed infection
N16961SR/CG4	5.13	17.91
O395SR/GS1	1.55	0.47

TGHP were subjected to short time exposures (up to 5 min) to 0.2 % DOC. Wild-type cells pre-grown in TGLP better tolerated exposure to DOC in the first 2 min than those from TGHP (Fig. 2). The N16961SR *vca1008* mutant CG4, on the other hand, presented higher DOC sensitivity than its parental strain, independently of the pre-growth condition (Fig. 2).

Complementation of CG4 with a wild-type *vca1008* containing its own regulatory region in plasmid pCG15 restored the ability to survive in the presence of 0.2 % DOC, independently of the P_i level in the pre-culture medium. Interestingly, CG4/pCG15 cells from TGLP tolerated the bile salt much better in the first 2 min than the wild-type grown under similar conditions. This is probably due to the high copy number of *vca1008* within these cells. However, 5 min later, the CG4/pCG15 DOC survival rate reached that of the parental strain (about 30 %). Taken together, these findings highlight that *vca1008* is essential for the survival of N16961SR cells upon exposure to DOC.

To compare the effect of *vca1008* mutation on the bile resistance phenotypes of *V. cholerae* O1 from distinct biotypes, similar experiments were carried out with cells of the classical strain O395SR and its isogenic *vca1008* mutant

GS1. In contrast to N16961SR and CG4 (Fig. 2), O395SR and GS1 cells grown in TGHP were equally sensitive to DOC (Fig. 3). However, a comparison of the survival rates of O395SR and GS1 cells grown in TGLP (Fig. 3) showed that the mutation in *vca1008* affected the bile salt resistance of the classical strain, but not so severely as in the El Tor strain N16961SR (Fig. 2). The higher survival rate of GS1 cells in comparison with CG4 is most likely due to another DOC resistance strategy induced in O395SR cells upon P_i starvation. Altogether, the above results showed that the *vca1008* mutants of the *V. cholerae* O1 classical and El Tor biotypes differ not only in the ability to colonize the intestinal mucosa of infant mice but also with respect to bile salt resistance and the mechanisms whereby they survive the antimicrobial action of bile salts.

It is well known that outer membrane porins contribute to bile resistance in *V. cholerae* (Provenzano *et al.*, 2001; Provenzano & Klose, 2000). Hence, to better understand the molecular basis of DOC resistance/sensitivity described above, and to check the involvement of VCA1008 in the process, the porin composition of the wild-type and mutant cells exposed to DOC was analysed. The relatively high DOC resistance of N16961SR cells pre-grown in TGHP could, at least in part, be attributed to OmpU, the major porin in their outer membrane (Fig. 4a). However, OmpU, also the major porin of CG4 cells cultivated in either TGHP or TGLP (Fig. 4a), apparently did not contribute to the cells' resistance to bile, in contrast to earlier results for the classical strain O395 (Provenzano *et al.*, 2000; Provenzano & Klose, 2000). In fact, as mentioned above, CG4 cells pre-grown in both TGHP and TGLP showed significantly lower bile resistance than the parental strain (Fig. 2). The phosphoporin VCA1008, on the other hand, seems to play an essential role in the bile resistance of the El Tor strain N16961SR (Fig. 2), in perfect

Fig. 2. DOC susceptibility of the wild-type strain N16961SR and *vca1008* (CG4) and *toxR* (CG5) mutants. *V. cholerae* cells of strains N16961SR, CG4 (*vca1008*), CG4/pCG15 (complemented *in trans* with *vca1008*) and CG5 (*toxR*) were cultivated in TG medium supplemented with high (HP) or low (LP) P_i concentrations for 14 h and then treated with 0.2 % DOC for up to 5 min. Strain CG4/pCG15 presented a wild-type resistance phenotype. Data are representative of three experiments; error bars, SD. Asterisks denote significantly ($P < 0.05$) different survival rates.

Fig. 3. DOC susceptibility of the wild-type strain O395SR and *vca1008* mutant (GS1). O395SR and GS1 cells were cultivated in TG medium with high (HP) or low (LP) P_i concentrations for 12 h and then treated with 0.2% DOC for up to 5 min. Data are representative of three experiments; error bars, SD. Asterisks denote significantly ($P < 0.05$) different survival rates.

agreement with the reduced intestinal colonization ability of CG4 cells (Table 2).

Strain O395SR and its *vca1008* mutant, GS1, grown in TGHP presented OmpT and OmpU as their main porins (Fig. 4b). However, under P_i limitation, the parental strain produced VCA1008 and a reduced amount of OmpT, whereas GS1 expressed OmpT as its major porin (Fig. 4b).

Fig. 4. OMP profiles of El Tor and classical strains and their respective *vca1008* mutants grown in TGLP and TGHP. *V. cholerae* El Tor strains N16961SR and CG4 (*vca1008*) (a), and classical strains O395SR and GS1 (*vca1008*) (b) were cultivated in TG medium with high (HP) or low (LP) concentrations of P_i for 14 h. Their OMP profiles were analysed by 11% SDS-PAGE and proteins were identified by MS.

Therefore, it is likely that the higher apparent DOC resistance of O395SR cells pre-grown in TGLP in comparison with those from TGHP (Fig. 3) is in part due to the presence of VCA1008 in the outer membrane of the cells.

The fact that cells of both strains, O395SR and GS1, barely expressed OmpU under P_i -limiting conditions was a great surprise, and might be an indication that PhoB directly or indirectly represses *ompU* expression in these strains. Moreover, it is possible that PhoB activates bile resistance mechanisms in the classical strain other than the modulation of porin expression, which could explain the relatively lower susceptibility to DOC observed in GS1 when compared with the El Tor *vca1008* mutant CG4 (Fig. 3).

ToxR-dependent expression of *vca1008* and *phoB/R* and susceptibility to DOC

ToxR is a transmembrane transcriptional factor that regulates expression of many known virulence genes of *V. cholerae* O1 in response to certain environmental cues (reviewed by Childers & Klose, 2007). It also controls positively and negatively the expression of two major porins, OmpU and OmpT, respectively (Provenzano *et al.*, 2000). However, the mechanisms underlying the ToxR control of outer membrane porin gene expression remain unknown.

Herein, we propose that regulatory pathways controlled by ToxR and PhoB could overlap to modulate the outer membrane porin composition of *V. cholerae* O1 under P_i -limiting conditions and, consequently, susceptibility to bile salts. To test this hypothesis, cells of the wild-type N16961SR and CG5, an isogenic *toxR* mutant, were grown in either TGHP (control condition) or TGLP and their porin composition was analysed. Cells of both strains under P_i limitation presented VCA1008 as one of their main porins (Figs 4a and 5a), showing that *vca1008* expression is apparently not affected by the mutation in *toxR* under this condition.

The sensitivity to DOC and porin composition of the *toxR* mutant CG5 were compared with those of N16961SR, as done with CG4. CG5 cells pre-cultivated in TGHP presented OmpT as the major porin (Fig. 5a) and proved to be highly sensitive to DOC (Fig. 2), confirming published results (Provenzano *et al.*, 2000). CG5 cells grown under low P_i supply, on the other hand, demonstrated DOC resistance comparable with that of N16961SR cells grown in TGHP, after 5 min exposure to the detergent (Fig. 2). In TGLP, the CG5 outer membrane presented VCA1008 and OmpT as major porins (Fig. 5a). As the presence of OmpT does not appear to have a role in DOC resistance, the evidence convincingly suggests that VCA1008 is part of the mechanism of strain CG5 DOC resistance under P_i -limiting conditions.

To further analyse a putative interaction between ToxR- and PhoB/R-dependent regulatory pathways, the activity of the *vca1008* promoter was evaluated in the *toxR* mutant CG5 and in the wild-type N16961SR cells under high and

Fig. 5. Effect of DOC on OMP profiles of *toxR* mutants of El Tor and classical strains and on the activity of the *vca1008* promoter in N16961SR and in its *toxR* mutant CG5. Cells were grown for 14 h in TG medium with high (HP) or low (LP) levels of P_i in the presence or absence of 0.1 % DOC. OMP profiles were analysed by 11 % SDS-PAGE and proteins were identified by MS: OMP-enriched samples of the El Tor *toxR* mutant CG5 (a) and classical *toxR* mutant GS2 (b). (c) β -Galactosidase activity measured in wild-type N16961SR and *toxR* mutant CG5 strains transformed with pICPvca1008 (containing the *vca1008* promoter fused to *lacZ*) grown as described above.

low levels of P_i in the absence and presence of 0.1 % DOC. The detergent was added to the media because it is well known that the ToxR transcriptional activity in both biotypes of *V. cholerae* O1, El Tor and classical, is modulated by bile salts (Provenzano *et al.*, 2000). Moreover, we hypothesized that some of these *in vitro* conditions mimic aspects of the intestinal environment. These are the presence of bile salts, pH above 7.0, 37 °C and P_i limitation, since it has been observed that some *V. cholerae* PhoB/R-regulated genes are induced *in vivo* (Merrell *et al.*, 2002; Osorio *et al.*, 2004, 2005).

The putative regulatory region of *vca1008* was fused to *lacZ* in a low copy plasmid (pICPvca1008) and its promoter activity was evaluated in N16961SR and CG5 cells grown as described above. Virtually no β -galactosidase activity was detected in the cells of either strain transformed with pICPvca1008 and grown in TGHP in either the absence or

presence of DOC (Fig. 5c). Similar results were obtained with cells harbouring the promoterless pIC552 (control; data not shown).

In contrast, a high level of β -galactosidase activity was observed in N16961SR/pICPvca1008 cells grown in TGLP, and even higher *lacZ* expression (1.5-fold the expression in TGLP) was detected in cells cultivated in TGLP/DOC (Fig. 5c). This result indicates that DOC increased the transcription rate from the *vca1008* promoter in the wild-type cells under P_i limitation. However, the *vca1008* promoter activity was 1.4-fold higher in CG5 than in N16961SR in TGLP, suggesting a negative control of *vca1008* expression by ToxR, under low P_i supply (Fig. 5c). Taken together, these findings show that under P_i -limiting conditions, ToxR apparently reduces the expression of the porin gene *vca1008*, but in TGLP in the presence of DOC its expression is enhanced.

In CG5/pICPvca1008 cells grown in TGLP, β -galactosidase activity was high and comparable with that in N16961SR cultured in TGLP/DOC (Fig. 5c). Surprisingly, however, the activity of β -galactosidase in cells of CG5/pICPvca1008 cultivated in TGLP/DOC was 10-fold lower than in the same cells grown in TGLP and also in N16961SR/pICPvca1008 cultivated in TGLP/DOC (Fig. 5c). This result confirms the above observation that ToxR/DOC indeed has a positive effect on the expression of *vca1008* in N16961SR.

Curiously, the bile salt seems to inhibit the activation of the *vca1008* promoter by PhoB, otherwise its activity level in CG5 in TGLP/DOC would have been similar to that observed in the cells grown in TGLP alone (Fig. 5c). Accordingly, the low activation of the *vca1008* promoter in CG5/pICPvca1008 in TGLP/DOC (Fig. 5c) correlates well with the negligible amount/absence of VCA1008 in the outer membrane of these cells (Fig. 5a).

Altogether, these results suggest that in N16961SR under P_i -limiting conditions, *vca1008* expression is under the control of the transcription factors PhoB and ToxR, whose activities towards the gene promoter are modulated by the bile salt. Under P_i limitation (TGLP), *vca1008* promoter activation by PhoB seems to overcome the negative effect of ToxR on the promoter (Fig. 5c) and a high amount of VCA1008 is produced (Fig. 4). However, when DOC is added to the medium (TGLP/DOC), the PhoB-mediated activation of *vca1008* expression is negatively affected, although *vca1008* expression via ToxR is enhanced. How PhoB and ToxR/bile salt interact to control *vca1008* expression is not known. Nevertheless, it is apparent that PhoB is required *in vitro* for the ToxR/DOC-mediated mechanism of regulation of *vca1008* expression, since it was only observed under P_i -limiting conditions.

VCA1008 expression was also evaluated in the *toxR* mutant GS2, derived from the classical strain O395SR (Fig. 5b). Contrary to the results observed for the El Tor strain CG5, disruption in the *toxR* gene did not affect VCA1008

biosynthesis in GS2 cells under P_i limitation in the presence of DOC. These findings clearly demonstrated that regulation of *vca1008* expression is different in classical and El Tor strains of *V. cholerae* O1.

To investigate whether the increased expression of *vca1008* in the presence of bile salt is correlated with alterations in the expression of other Pho regulon genes, the activity of the promoter of the *phoB/R* operon (fused to *lacZ* in the low copy plasmid pIC234) was determined in N16961SR and CG5 cells grown in TGLP and TGLP/DOC (Fig. 6).

Similarly to the results observed for the *vca1008* promoter (Fig. 5c), the activity of the *phoB/R* promoter was 1.4-fold higher in CG5 than in N16961SR in TGLP, suggesting a negative control of *phoB/R* expression by ToxR, under low P_i supply (Fig. 6a). Moreover, the activity of the *phoB/R* promoter in each strain was reduced in TGLP/DOC in comparison with TGLP (Fig. 6a), indicating that the expression of the *phoB/R* operon is negatively affected by the bile salt under P_i limitation. Interestingly, the effect of

DOC on *phoB/R* promoter activity was greater in the *toxR* mutant than in the wild-type, leading us to believe that ToxR/DOC acts synergistically to upregulate the expression of *phoB/R*, as observed for *vca1008* (Fig. 5c). Taken together, these findings prompted us to suggest that in TGLP, the expression of *phoB/R* in N16961SR cells is controlled positively by PhoB and negatively by a ToxR-dependent pathway. DOC somehow changes the balance between these regulatory systems and ultimately their activity towards the *phoB/R* promoter, with a consequent alteration in the operon expression in N16961SR under P_i -limited conditions.

In this scenario, lower levels of PhoB might be expected in N16961SR cells cultivated in TGLP/DOC than in those cultivated in TGLP. Hence, the increased activation of the *vca1008* promoter in N16961SR cells cultured in TGLP/DOC (Fig. 5c), in a background of reduced *phoB/R* expression, might indeed be due to the positive effect of ToxR/DOC, also observed with respect to the *phoB/R* promoter, but at a lower level (Fig. 6a).

PhoA, a protein with alkaline phosphatase activity, is also induced in *V. cholerae* cells under P_i limitation (von Krüger *et al.*, 1999) and is considered a natural reporter of the Pho regulon. Thus, to evaluate expression of the regulon in N16961SR and CG5, PhoA activity was measured in the cells grown under P_i limitation, in either the presence or absence of DOC. PhoA activity was detected in both N16961SR and CG5 cells, and did not change by addition of DOC to the culture medium. However, in the *toxR* mutant cells, PhoA activity was slightly lower (about 20 %) than in N16961SR cells (Fig. 6b). Putting our results together, we conclude that another regulatory molecule/system besides PhoB is involved in the regulation of *vca1008* and *phoB/R* expression in *V. cholerae* O1 strain N16961SR, and that ToxR appears to be the most suitable candidate. Further studies are under way to determine whether ToxR interacts directly with the regulatory regions of *vca1008* and *phoB/R*, or whether some other indirect mechanism is involved.

Concluding remarks

This study revealed many surprising findings on the gene regulation, physiology and pathogenicity of the pandemic strains of *V. cholerae* O1, N16961SR and O395SR, of the El Tor and classical biotypes, respectively. As far as we know this is the first report of an interplay between ToxR and PhoB regulatory systems to control porin gene expression, of the influence of DOC on the expression levels of PhoB- and ToxR-regulated genes, and of the requirement for the putative phosphoporin VCA1008 for bile resistance in *V. cholerae*.

In the El Tor strain N16961SR, ToxR and PhoB regulatory systems seem to control in a bile salt-dependent manner the expression of *vca1008*, whose product is absolutely required for the bile resistance and pathogenicity of the

Fig. 6. Effect of DOC on the expression of the Pho regulon (PhoA) and on the activity of the *phoB/R* promoter in N16961SR and its *toxR* mutant CG5 grown in low- P_i medium. Cells were grown for 14 h in TGLP in the absence (white bars) or presence of 0.1 % DOC (black bars). (a) β -Galactosidase activities were measured in N16961SR and *toxR* mutant CG5 transformed with pIC234 (containing the *phoB/R* promoter fused to *lacZ*). (b) PhoA activities were measured in the same cells. Data are representative of three independent experiments; error bars, SD.

bacterium. The same was not observed with strain O395SR, indicating that the classical and El Tor strains differ markedly with respect to the strategies that they use to deal with the toxic effects of bile *in vitro* and, most likely, *in vivo* as well, to cause cholera. The classical and El Tor strains also seem to differ in the regulation of OmpU expression under P_i limitation. Under such conditions the cells of classical strains present high levels of VCA1008 and reduced amounts of OmpU. The molecular mechanisms behind *ompU* gene downregulation are not known, but they might involve a synergy between ToxR- and PhoB-dependent regulatory pathways. As far as is known, neither OmpU nor VCA1008 is essential to the pathogenicity of classical strains of *V. cholerae* O1. Therefore, a role for this particular bile salt-independent ToxR–PhoB system interaction in *V. cholerae* O1 O395SR *in vivo* is not clear. However, it is possible that in the natural habitat, where the P_i supply is limited, members of the ToxR and PhoB regulons team up to upregulate *vca1008* and downregulate *ompU*, as a strategy to increase the P_i -uptake rate of *V. cholerae* O1 cells.

The data presented herein also permit us to speculate that upon infection of the intestinal tract by N16961SR, bile salts somehow modulate the transcription rate of the *phoB/R* operon and the transcriptional activity of the regulators PhoB and ToxR to allow the appropriate expression of genes required for bile resistance and virulence. The molecular mechanisms by which bile salts control ToxR- and PhoB-regulated genes are not clear, and how the low P_i -induced porin protects *V. cholerae* O1 cells from the cytotoxic effects of bile remains to be found. Therefore, further work will be needed to clarify these intriguing and exciting issues, which could greatly contribute to our understanding of the regulatory mechanisms involved in the gut–*V. cholerae* interaction.

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