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The *ompU* Paralogue *vca1008* Is Required for Virulence of *Vibrio cholerae*

Carlos G. Osorio,[1] Hector Martinez-Wilson,[2] and Andrew Camilli[2]*

Tufts University Medical School, Tufts University, Boston, Massachusetts 02111,[2] and Programa de Microbiología y Micología, Facultad de Medicina, Universidad de Chile, Santiago, Chile[1]

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We made single and combined mutations in *ompU*, *ompT*, and the two putative porin genes *vca1008* and *vc0972*. The fitness of the strains was tested in vitro and in the infant mouse model of intestinal infection. We also studied the transcriptional induction of *vca1008* in vitro and during mouse infection. We show that *vca1008* is induced during infection and is necessary and sufficient (in the absence of *ompU*, *ompT*, and *vc0972*) for infection.

Vibrio cholerae is a gram-negative bacterium and facultative pathogen that can cause an acute secretory diarrhea known as cholera. When *V. cholerae* enters a host it has to sense the new environment and induce an adaptive response that facilitates its survival and multiplication in the small intestine. ToxR is a transmembrane transcriptional activator that is part of a complex virulence gene regulon (the ToxR regulon) of more than 20 genes (9, 12, 17). The ToxR regulon is organized in two separate branches: the *toxT*-dependent and the *toxT*-independent branches. In the *toxT*-dependent branch, the transcriptional activator ToxT, controlled directly by ToxR, regulates the transcription of the cholera toxin and toxin-coregulated pilus and other factors essential for virulence (3). The *toxT*-independent branch includes two outer membrane porins called OmpU and OmpT (9). These two porins are directly and differentially regulated by ToxR in that *ompU* transcription is induced, whereas *ompT* transcription is repressed (2, 6, 9). There are some studies that suggest important functions for *ompU* during intestinal colonization, namely, increased resistance to bile and anionic detergents (13, 14), an organic acid tolerance response (7), and adhesion to epithelial cells (18). However, one study reported that OmpU does not mediate adherence to rabbit intestinal epithelia (11), and another study reported that Δ*ompU* and Δ*ompT* strains exhibited no growth defect in vitro nor any detectable attenuation of virulence in infant mice (14).

We recently used the recombination-based in vivo expression technology (RIVET) (1) to identify *V. cholerae* gene *vca1008* as being transcriptionally induced during infection of the infant mouse small intestine (C. Osorio, J. Crawford, J. Michalsky, H. Martinez-Wilson, J. Kaper, and A. Camilli, unpublished data). This gene is one of three putative porin genes located on chromosome (Chr) II, and it encodes a protein that is closely related to the Chr I-encoded OmpU porin, having 33% identity and 55% similarity. The other Chr II-encoded putative porins, OmpS and OmpW, are orthologues of the *Escherichia coli* maltose-specific LamB and uncharacterized OmpW, respectively. The OmpU and VCA1008 paralogues are more closely related to the *E. coli* nonspecific porins OmpF, OmpC, and PhoE than are the other putative or known porins of *V. cholerae* (Fig. 1). In contrast, *V. cholerae* OmpT and VC0972 porins are only distantly related to these proteins (Fig. 1).

The relatedness of OmpU and VCA1008 leads to the possibility of an overlap in their function, which might explain why mutations in *ompU* alone fail to attenuate virulence in the infant mouse host. To test this hypothesis and also to characterize the roles of porins OmpT and VC0972, we constructed single and combined in-frame deletions in each of these genes (see Table 1 for strains and plasmids used in the present study). In-frame deletions of the entire coding sequence of *V. cholerae* genes were constructed in pCVD442 by using splicing by overlap extension (SOE) PCR (16) with the oligonucleotide primers listed in Table 2. Each recombinant pCVD442 was electroporated into *E. coli* SM10α*λpir* and transferred to *V. cholerae* GOA1264 by conjugation. Allelic exchange was done as described previously (4), and the chromosomal deletion mutations were confirmed by PCR with F0 and R2 primers (Table 2), followed by DNA sequencing (data not shown). For complementation experiments, *vca1008* was amplified twice independently from GOA1264 genomic DNA by using the primer pairs GOA3-GOA4 and GOA5-GOA6. The products were cloned into pMMB67EH-neo digested with EcoRI and XbaI, producing pVCA1008-F and pVCA1008-R, respectively.

We observed no detectable growth defect in Luria-Bertani (LB) broth for the single deletion strain Δ*vca1008*, double-deletion strains Δ*ompU* Δ*vca1008* and Δ*ompT* Δ*vc0972*, or the triple-deletion strain Δ*ompU* Δ*ompT* Δ*vc0972* (data not shown). We were unable to construct the quadruple deletion strain, suggesting that the loss of all four porins is lethal to *V. cholerae*; however, this was not rigorously examined.

The single and combined deletion strains were each examined for growth in LB broth and infection of infant mice in competition assays with the virulent LacZ$^-$ strain GOA6W. Each test strain was grown to mid-exponential phase in LB broth plus 10 μg of rifampin (Rif) ml^{-1} and then mixed 1:1 with the similarly grown GOA6W. Approximately 10^5 CFU were inoculated intragastrically into 10 5-day-old mice as pre-

* Corresponding author. Mailing address: Tufts University Medical School, Tufts University, Boston, MA 02111. Phone: (617) 636-2144. Fax: (617) 636-0337. E-mail: andrew.camilli@tufts.edu.

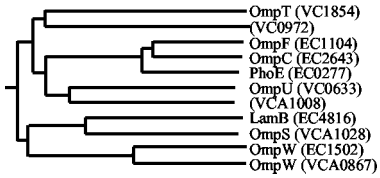

FIG. 1. Phylogenetic tree for select known and putative porins of *V. cholerae* and *E. coli* constructed by the neighbor-joining algorithm (CLUSTAL W, v1.81). Protein names (when available) and locus names obtained from The Institute for Genomic Research (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl) are listed on the right. The *V. cholerae* OmpU and paralogue VCA1008 cluster with the *E. coli* classical nonspecific porins OmpF, OmpC, and PhoE. *V. cholerae* OmpS and OmpW cluster with *E. coli* LamB and OmpW, respectively. The *V. cholerae* OmpT and VC0972 are distantly related to the other proteins.

viously described (1). In vitro competitions were done in parallel by using each of the prepared inoculae to inoculate 2 ml of LB broth with 10^4 CFU, after which the cultures were grown for 16 h at 37°C with aeration. The ratio of test strain to GOA6W in each inoculum, as well as in the resulting bacterial populations recovered from the in vitro and from the in vivo competitions after 16 and 24 h, respectively, was determined by plating serial dilutions of the outputs onto LB agar plus 10 μg of Rif and 40 μg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) ml^{-1}. The competitive indices (CI) were calculated by dividing the output ratios of test strain to GOA6W by their respective input ratios. For complementation tests, strain GOA1713 was electroporated with plasmids pMMB67EH-neo, pVCA1008-F, and pVCA1008-R.

As shown in Table 3, strain Δ*vca1008* was attenuated 40-fold for the infection of infant mice, indicating that this putative porin is necessary for infection. The Δ*vca1008* Δ*ompU* double-deletion strain was not significantly different from strain Δ*vca1008*, a finding consistent with *ompU* being dispensable for infection. The Δ*vca0972* Δ*ompT* strain was not significantly different from the wild-type, indicating that neither gene is required for infection and also that these two related proteins do not constitute a functionally redundant pair with an important role in infection. Finally, the Δ*ompU* Δ*ompT* Δ*vc0972* triple deletion strain outcompeted the parental strain by 13-fold. Together, these data suggest that *vca1008* is necessary and sufficient (in the absence of *ompU*, *ompT*, and *vc0972*) for virulence. To test for the occurrence of a spontaneous mutation in the Δ*vca1008* strain that could be causing the observed avirulent phenotype, we complemented this strain with a wild-type copy of *vca1008* and its native promoter cloned in the low-copy plasmid pMMB67EH-neo. The presence of empty vector alone did not restore virulence (data not shown). However, as shown in Table 3, both orientations of *vca1008* in the plasmid fully restored virulence, although to slightly higher levels than that of the parent strain when *vca1008* was in the same orientation as the P_{tac} promoter. It is possible that *vca1008* is being overexpressed when cloned in this orientation.

Transcriptional induction of *vca1008* in vivo would be consistent with the important role we have ascribed to this gene for infection of infant mice. To test this, we measured induction of

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype[a]	Source or reference
Strains		
V. cholerae		
CVD110	E7946 El Tor biotype Δ(*cep-ctxB*) *hlyA*::*mer*::*ctxB* Hg^r	8
GOA1264	Spontaneous Rif^r of CVD110; Rif^r	This work
GOA1713	GOA1264 Δ*vca1008*	This work
GOA1714	GOA1264 Δ*ompU*	This work
GOA1715	GOA1264 Δ*vca1008* Δ*ompU*	This work
GOA1716	GOA1264 Δ*ompT* Δ*vc0972*	This work
GOA1717	GOA1264 Δ*ompT* Δ*vc0972* Δ*ompU*	This work
GOA6W	GOA1264 *lacZ*::pGP704; $LacZ^-$	This work
GOA1245	GOA1264 *lacZ*::*res-neo-sacB-res* Rif^r Km^r Suc^s	C. Osorio, J. Crawford, J. Michalsky, J. Kaper, and A. Camilli, unpublished data
GOA1705	GOA1245 *vca1008*::$tnpR^{135}$ *lacZ*::*res-neo-sacB-res* Rif^r Km^r Suc^s	C. Osorio, J. Crawford, J. Michalsky, J. Kaper and A. Camilli, unpublished data
E. coli		
SM10αλ*pir*	*thi recA thr leu tonA lacY supE* RP4-2-Tc::Mu λ::*pir* Km^r	Laboratory strain
DH5αλ*pir*	F^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR1 supE44 thi-1 gyrA96 relA1*::*pir*	5
Plasmids		
pCVD442	*oriR6K mobRP4 sacB* Ap^r Suc^s	4
pGP704	*oriR6K mobRP4* Ap^r	9
pMMB67EH	*oriV mobRP4* Ap^r	10
pMMB67EH-neo	pMMB67EH Δ*bla*::*neo;* Km^r	This work
pVCA1008-F	pMMB67EH-neo::*vca1008* forward	This work
pVCA1008-R	pMMB67EH-neo::*vca1008* reverse	This work

[a] Rif^r, Rif resistant; Km^r, kanamycin resistant; Suc^r, sucrose resistant; Ap^r, ampicillin resistant.

TABLE 2. Oligonucleotide primers

Primer[a]	sequence (5′ - 3′)[b]	Use[c]
*vca1008*F1	CAGGACGCAATGGAGTAGTC	SOE PCR
*vca1008*F2	GGTAGAAAAATGTAATCACACTGCCTTAAAC	SOE PCR
*vca1008*R1	AGTGTGATTACATTTTTCTACCCTATTAG	SOE PCR
*vca1008*R2	TGGCGTGATCAAACGGCTGGAC	SOE PCR
*ompU*F1	CAGCATGGTATTCCGCATTC	SOE PCR
*ompU*F2	ATGGACAATACTTCAGGTCACACGCCAAAC	SOE PCR
*ompU*R1	GACCTGAAGTATTGTCCATAAATTTG	SOE PCR
*ompU*R2	GATCAGGTTGTCGGACTCTTG	SOE PCR
*ompT*F1	TGATCACTGATCCTGCGA	SOE PCR
*ompT*R1	GATCTTACAACTCTTTGTTTGGTCACCAC	SOE PCR
*ompT*F2	CAAAGAGTTGTAAGATCTCGAACACGTTTA	SOE PCR
*ompT*R2	CATCCCTCTTGCCAAGCCAG	SOE PCR
*vc0972*F1	GGTAGACTCTGCTCTAGC	SOE PCR
*vc0972*R1	CGTCTGATTACATGGATAACTCCTAAAAATG	SOE PCR
*vc0972*F2	GTTATCCATGTAATCAGACGAACCCTGC	SOE PCR
*vc0972*R2	AAGAGTGAGCATGGCTTTAG	SOE PCR
*vca1008*F0	CAAGCAATTAAATTGCACAC	Deletion confirmation
*ompU*F0	TAGCTTGTATTCGATATCAC	Deletion confirmation
*ompU*R0	AGTAAAAACGGTGTCCCAAGG	Deletion confirmation
*ompT*F0	ACGTTCGCTACAACAATAAC	Deletion confirmation
*vc0972*F0	ACCGTAACCAACAAGTGATC	Deletion confirmation
GOA3	TCTAATGAATTCTGCGTGGCAACATTGATGTG	*vca1008* cloning
GOA4	TCTAATTCTAGAGGTGATGTTTAATGCTCATG	*vca1008* cloning
GOA5	TCTAATTCTAGATGCGTGGCAACATTGATGTG	*vca1008* cloning
GOA6	TCTAATGAATTCGGTGATGTTTAATGCTCATG	*vca1008* cloning

[a] The beginning of each primer name corresponds to the *V. cholerae* N16961 gene being deleted, and the F and R indicate forward and reverse primers, respectively.
[b] Underlined bases indicate a noncomplementary 5′ tail and restriction site.
[c] SOE PCR refers to splicing-by-overlap-extension PCR used to construct gene deletions.

vca1008 after growth in vitro and after infection of infant mice by using RIVET essentially as described previously (1). The resolvase gene fusion to *vca1008* (*vca1008*::*tnpR*^{135} [5a]) was reconstructed in a fresh reporter strain background (GOA1245), which harbors the *res-neo-sacB-res* substrate cassette for resolvase, to generate strain GOA1705 (see Table 1). GOA1705 was grown for ca. 17 generations at 37°C with aeration to stationary phase in M9 minimal medium with or without 0.1% D-glucose and in LB broth or was used to infect infant mice for 24 h as described previously (1). Transcriptional induction of *vca1008*::*tnpR*^{135} results in production of resolvase protein (TnpR), which in turn excises the *res-neo-sacB-res* cassette from the genome, yielding a kanamycin-sensitive, sucrose-resistant strain phenotype. Serial dilutions of the final cultures and the mouse small intestinal homogenates were plated on LB agar plus 10 mg of Rif ml^{-1} and on L-agar without NaCl but with 10% sucrose plus 10 mg of Rif ml^{-1}. The percentage of resolved *V. cholerae* cells within each test population was calculated by dividing the number of CFU on sucrose plates by the CFU on the LB plates. The *vca1008*::*tnpR*^{135} fusion was expressed during infection of infant mice but not during growth in vitro in either minimal or rich medium. The resolution levels of the GOA1705 (*vca1008*::*tnpR*^{135} *res-neo-sacB-res*) strain, measured as the percent CFU resolved (see above), were as indicated under the following growth conditions: M9 with glucose, <0.1%; M9 without glucose, <0.1%; LB medium, <0.1%; and in vivo, 75 to 80%. Note that, in vivo, the outputs from four mice were assayed separately, and the range of resolution is shown. The resolution in vivo was significantly greater than during growth in vitro, as determined by the Student two-tailed *t* test.

It is possible that *vca1008* is upregulated in the triple-gene-deletion strain to allow viability and enhanced virulence. This would be consistent with the following observations: (i) we were unable to delete *vca1008* in the triple-gene-deletion strain background (data not shown), (ii) different expression levels of *vca1008* from a plasmid can restore virulence to the *vca1008* chromosomal deletion strain to near or greater than wild-type levels (see complementation analysis above), and (iii) *vca1008* is transcriptionally silent during growth in vitro in the wild-type

TABLE 3. Competition assays in vitro and in infant mouse

Competition	CI[a] for test strain genotype:					
	Δ*vca1008*	Δ*vca1008* Δ*ompU*	Δ*vc0972* Δ*ompT*	Δ*vc0972* Δ*ompT* Δ*ompU*	Δ*vca1008* (pVCA1008-F)	Δ*vca1008* (pVCA1008-R)
In vitro	0.92	0.5*	2.3	2.8	1.9*	1
In vivo	0.025*	0.013*	1.6	13*	4.1*	0.8

[a] The CI were calculated as described in Materials and Methods. Each in vitro competition was done by using two independent LB broth cultures, and each in vivo competition used 10 infant mice. Asterisks indicate significant differences from the parent strain ($P < 0.05$) as determined by the Student's two-tailed *t* test with, as control group, competitions between GOA1264 and GOA6W.

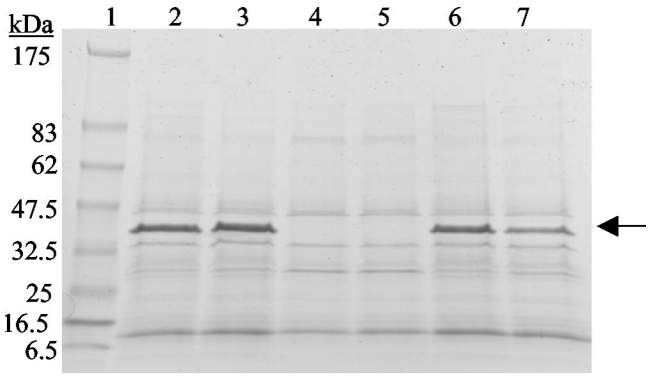

FIG. 2. SDS-PAGE analysis of outer membrane proteins of *V. cholerae* strains. The molecular masses of markers in lane 1 are indicated to the left of the gel. Lane 2, wild-type; lane 3, Δ*vca1008*; lane 4, Δ*ompU*; lane 5, Δ*vca1008* Δ*ompU*; lane 6, Δ*ompT* Δ*vc0972*; lane 7, Δ*ompU* Δ*ompT* Δ*vc0972*. The most intense band in lanes 2, 3 and 6, which runs between the 32.5- and 47.5-kDa markers (marked by arrow), corresponds to OmpU (see the text for discussion).

strain background and thus would presumably require upregulation in the triple-gene-deletion background from which is cannot be deleted. To examine whether VCA1008 (or other porins) are upregulated in the triple-gene-deletion strain, we analyzed the outer membrane protein profiles of this and other strains generated in the present study by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Outer membrane proteins were purified from mid-exponentially growing cells in LB broth at 37°C with aeration as described previously (15). Proteins were separated on 4 to 12% gradient SDS-PAGE gels and stained with Coomassie brilliant blue. There were no detectable changes in the outer membrane protein profiles for the Δ*vca1008* strain or the Δ*ompT* Δ*vc0972* strain compared to the wild-type (Fig. 2). In contrast, the Δ*ompU* strain and the Δ*ompU* Δ*vca1008* double-deletion strain were both missing the band corresponding to OmpU. This was confirmed by Western blotting with anti-OmpU polyclonal antiserum (data not shown). Analysis of the Δ*ompU* Δ*ompT* Δ*vc0972* strain revealed the appearance of a new band migrating at roughly the same position as OmpU but at a slightly lower intensity (Fig. 2, lane 7). This new band also cross-reacted with the anti-OmpU serum upon Western blotting (data not shown). We hypothesize that this new protein species represents VCA1008, which has been upregulated in the triple-gene-deletion background. VCA1008 has 33% identity and 55% similarity to OmpU and has an estimated molecular mass nearly identical to that of OmpU. To confirm that *ompU* was deleted in this strain, we isolated genomic DNA and PCR amplified a DNA fragment predicted to span the *ompU* deletion junction by using the primers *ompU*F0 and *ompU*R0. These primers hybridize to sequences outside the regions cloned for *ompU* deletion construction by SOE. A PCR product of the size expected for the deletion was obtained, and sequencing of the PCR product revealed the expected deletion junction (data not shown). Thus, *ompU* has been deleted from this strain.

Despite the sequence similarity of VCA1008 to OmpU and its apparent cross-reactivity with anti-OmpU antibodies, VCA1008 and OmpU are not functionally redundant, as we had originally speculated. The *vca1008* gene is the only one of the four encoding known or putative porins tested in the present study that is necessary for infection of infant mice. Thus, either the activity of VCA1008 or its pattern of expression during infection, or both, are different from that of OmpU. It is known that OmpU porin increases the resistance to bile and anionic detergents (13, 14) and has a role in resistance to organic acids (7). Perhaps VCA1008 is more efficient in one or more of these functions or, alternatively, fulfills another, unknown role during infection.

The transcriptional induction of *vca1008* was investigated and was shown to be induced during infection but not during growth in minimal or rich media. These results indicate that the induction observed in vivo is not a result of a general stress response to nutrient deprivation, as might occur within the small intestine, but rather a kind of specific response to intestinal infection. This result is unexpected, given that Xu et al. (19) reported *vca1008* to be repressed during infection of rabbit ligated ileal loops. These conflicting results may be due to the use of different animal hosts, the use of ligated ileal loops as opposed to an unrestricted intestinal tract, or the use of transcriptional profiling, which provides an average value of gene expression.

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AUTHOR'S CORRECTION

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Carlos G. Osorio, Hector Martinez-Wilson, and Andrew Camilli

Tufts University Medical School, Tufts University, Boston, Massachusetts 02111, and Programa de Microbiología y Micología, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Volume 186, no. 15, p. 5167–5171, 2004. Due to an illegitimate recombination event, the triple porin mutant (Δ*ompU* Δ*ompT* Δ*vc0972*) we reported is still UmpU$^+$. The correct strain has been constructed and exhibits different phenotypes than what was reported. It grows in LB broth with a similar doubling time and final cell density as the parent strain, GOA1264 (OmpU$^+$ OmpT$^+$ VC0972$^+$). However, it is outcompeted by GOA1264 ~200-fold in a competition in LB broth at 37°C after 16 generations and is outcompeted 3-fold in competition in infant mice. In addition, the outer membrane protein profile of the triple mutant is indistinguishable from that of the Δ*ompU* single and Δ*vca1008* Δ*ompU* double mutants.