The \textit{ompU} Parologue \textit{vca1008} Is Required for Virulence of \textit{Vibrio cholerae}

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We made single and combined mutations in \textit{ompU}, \textit{ompT}, and the two putative porin genes \textit{vca1008} and \textit{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 \textit{vca1008} in vitro and during mouse infection. We show that \textit{vca1008} is induced during infection and is necessary and sufficient (in the absence of \textit{ompU}, \textit{ompT}, and \textit{vc0972}) for infection.

\textit{Vibrio cholerae} is a gram-negative bacterium and facultative pathogen that can cause an acute secretory diarrhea known as cholera. When \textit{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. 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 \textit{ompU} transcription is induced, whereas \textit{ompT} transcription is repressed (2, 6, 9).

There are some studies that suggest important functions for \textit{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, a study reported that OmpU does not mediate adherence to rabbit intestinal epithelia (11), and another study reported that \textit{DompU} and \textit{DompT} 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 \textit{V. cholerae} gene \textit{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 \textit{Escherichia coli} maltose-specific LamB and uncharacterized OmpW, respectively. The OmpU and VCA1008 paralogues are more closely related to the \textit{E. coli} nonspecific porins OmpF, OmpC, and PhoE than are the other putative or known porins of \textit{V. cholerae} (Fig. 1). In contrast, \textit{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 \textit{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 \textit{vca1008} 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 \textit{E. coli} SM10\textit{pir} and transferred to \textit{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, \textit{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 \textit{Dvca1008}, double-deletion strains \textit{DompU Dvca1008} and \textit{DompT Dvca1008}, or the triple-deletion strain \textit{DompU DompT Dvc0972} (data not shown). We were unable to construct the quadruple deletion strain, suggesting that the loss of all four porins is lethal to \textit{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 \textit{LacZ} strain GOA6W. Each test strain was grown to mid-exponential phase in LB broth plus 10 \textmu g of rifampin (Rif) ml\textsuperscript{-1} and then mixed 1:1 with the similarly grown GOA6W. Approximately \textit{10}\textsuperscript{5} CFU were inoculated intragastrically into 10 5-day-old mice as pre-
Tests, strain GOA1713 was electroporated with plasmids pMMB67EH-neo, pVCA1008-F, and pVCA1008-R.

As shown in Table 3, strain Δvca1008 was attenuated 40-fold for the infection of infant mice, indicating that this putative porin is necessary for infection. The Δvca1008 ΔompU double-deletion strain was not significantly different from strain Δvca1008, a finding consistent with ompU being dispensable for infection. The Δvca0972 ΔompT 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 ΔompU ΔompT Δvca0972 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 vca0972) for virulence. To test for the occurrence of a spontaneous mutation in the Δvca1008 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 Plac 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 2. Oligonucleotide primers**

<table>
<thead>
<tr>
<th>Primer*</th>
<th>sequence (5' - 3')</th>
<th>Use*</th>
</tr>
</thead>
<tbody>
<tr>
<td>vca1008F1</td>
<td>CAGGAGCGATAGGATGATC</td>
<td>SOE PCR</td>
</tr>
<tr>
<td>vca1008F2</td>
<td>GTAGGAAAATGTAATCAGCCTAAAC</td>
<td>SOE PCR</td>
</tr>
<tr>
<td>vca1008R1</td>
<td>AATGGTAATCATTTTCTACCCATATTAG</td>
<td>SOE PCR</td>
</tr>
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<td>vca1008R2</td>
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</tr>
<tr>
<td>ompU1F</td>
<td>CAGCATGATTTCCGACATT</td>
<td>SOE PCR</td>
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<tr>
<td>ompU1F</td>
<td>ATGGAATACCTCAAGGTCCACAGG</td>
<td>SOE PCR</td>
</tr>
<tr>
<td>ompU1R</td>
<td>GACCTGAAGTATTGTCCATAATTTG</td>
<td>SOE PCR</td>
</tr>
<tr>
<td>ompU2R</td>
<td>CATCAGGTTGCAGGACTTGT</td>
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<tr>
<td>ompT1R</td>
<td>TAGCTTGTATTCGATATCAC</td>
<td>Deletion confirmation</td>
</tr>
<tr>
<td>ompT2R</td>
<td>GATCTTACAACTCTTTGTTTGG</td>
<td>Deletion confirmation</td>
</tr>
<tr>
<td>vca1008</td>
<td>CAAAGGTGTTAAGATCCTAAATTTG</td>
<td>Deletion confirmation</td>
</tr>
<tr>
<td>GOA3</td>
<td>TCTATGAATTTCTGGTGGCAGACATTGATGTG</td>
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</tr>
<tr>
<td>GOA4</td>
<td>TCAATTCTAGAGGTGATGTTTCACCTCATG</td>
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</tr>
<tr>
<td>GOA5</td>
<td>TCAATTCTAGATGCGTGGCAGACATTGATGTG</td>
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</tr>
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<td>GOA6</td>
<td>TCAATTCTAGATGCGTGGCAGACATTGATGTG</td>
<td>vca1008 cloning</td>
</tr>
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</table>

* 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.
* Underlined bases indicate a noncomplementary 5' tail and restriction site.
* SOE PCR refers to splicing-by-overlap-extension PCR used to construct gene deletions.

**TABLE 3. Competition assays in vitro and in infant mouse**

<table>
<thead>
<tr>
<th>Competition</th>
<th>( \Delta \text{vca1008} )</th>
<th>( \Delta \text{vca1008} )</th>
<th>( \Delta \text{vca1008} )</th>
<th>( \Delta \text{vca1008} )</th>
<th>( \Delta \text{vca1008} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>0.6</td>
<td>0.1</td>
<td>2.3</td>
<td>1.9</td>
<td>1.0</td>
</tr>
<tr>
<td>In vivo</td>
<td>0.025*</td>
<td>0.013*</td>
<td>1.6</td>
<td>4.1*</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* 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.
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 Δvca1008 strain or the ΔompT Δvca0972 strain compared to the wild-type (Fig. 2). In contrast, the ΔompU strain and the ΔompU Δvca1008 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 ΔompU ΔompT Δvca0972 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/0 and ompU/0R. 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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REFERENCES
AUTHOR’S CORRECTION

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Volume 186, no. 15, p. 5167–5171, 2004. Due to an illegitimate recombination event, the triple porin mutant (ΔompU ΔompT Δvca0972) 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 ΔompU single and Δvca1008 ΔompU double mutants.