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Cloning and Characterization of the Gene Encoding the OmpU Outer Membrane Protein of *Vibrio cholerae*

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The OmpU outer membrane protein is a member of the ToxR regulon of *Vibrio cholerae* and has recently been shown to be a potential adherence factor for this species. Using PCR and degenerate oligonucleotide primers based on internal peptide sequences of purified OmpU, we have cloned and sequenced the gene encoding OmpU. The *ompU* gene is predicted to encode a 36,646-molecular-weight protein which is present in both cholera toxin-positive and -negative *V. cholerae* O1 and O139 strains.

The ToxR regulon is essential for virulence in *Vibrio cholerae* (7, 9). Originally described as a positive regulator of *ctx* genes which encode cholera toxin (15), ToxR was also found to positively regulate expression of an essential intestinal colonization factor, TCP (23). ToxR also regulates a 38-kDa outer membrane protein called OmpU (16), which we have recently shown to be a putative adherence factor of *V. cholerae* (22). A survey using Tn*phoA* found at least 17 ToxR-regulated genes in *V. cholerae*, many of which have been subsequently cloned and sequenced (18). However, despite the prominence of OmpU in outer membrane profiles of *V. cholerae* and the success in cloning a variety of ToxR-regulated genes, cloning the gene encoding OmpU has proven to be difficult. In light of the potential importance of OmpU as an adherence factor of *V. cholerae*, we pursued the cloning of the gene encoding OmpU by PCR technique.

We previously reported the amino-terminal sequence of the purified OmpU protein from *V. cholerae* 395 (22). The amino-terminal sequence was used to design a degenerate oligonucleotide probe (K227) with the following sequence: 5′-GAC (T)GGC(T)ATC(T)AACCAGT(A)CC(T)GGT(C)GAC(T)A AA(G)GC-3′, which was labeled with γ-^{32}P and used to screen a genomic library of *V. cholerae* classical Ogawa strain 395 constructed in the cosmid vector pHC79 (12) in *Escherichia coli* HB101. Even under stringent hybridization conditions, this probe gave nonspecific reactions and was of no use in detecting cloned *ompU* DNA. We then turned to a PCR procedure utilizing the K227 primer and a second primer based on an internal peptide sequence of OmpU. Purified OmpU was sent to the Protein and Nucleic Acid Facility, Beckman Center, Stanford University, where it was digested with cyanogen bromide. The resulting peptides were purified by high-performance liquid chromatography, and the amino-terminal sequences of several of the peptides, designated Pep1 through Pep7, were determined by automated Edman degradation. A degenerate oligonucleotide was designed from Pep4, which was chosen because it yielded the lowest number of codon degeneracies compared to the other peptides. This oligonucleotide primer (K281) consisted of the following sequence: 5′-A ACAGA(G)CCA(C,T)GCA(G)AAA(G)TACAGGTTC(T) TCCAT-3′. Primers K227 and K281 were used in a PCR reaction containing 200 ng of purified genomic *V. cholerae* 395 DNA, 400 ng of each primer, 200 μM deoxynucleoside triphosphates (dNTPs), 2 U of *Taq* polymerase, and 2 mM $MgCl_2$ in *Taq* polymerase buffer (Life Technologies, Gaithersburg, Md.). After an initial cycle of 94°C for 4 min, 65°C for 1 min, and 72°C for 3 min, a total of 30 cycles were performed at 94°C for 1 min, 65°C for 1 min, and 72°C for 3 min. The resulting 0.7-kb PCR product was electrophoresed and excised from a 1% agarose gel, purified with an Ultrafree MC (Millipore) column, treated with T4 DNA polymerase (2 μg of DNA, 50 mM Tris-Cl pH 8.0, 5 mM $MgCl_2$, 5 mM dithiothreitol, 100 μM dNTP mix, 50 μg of bovine serum albumin per ml, 10 U of T4 DNA polymerase, 5 min on ice) to repair the 3′ overhang, and cloned into the *Sma*I site of pBluescriptIISK$^-$ (Stratagene) to create pVS11.

The nucleotide sequence of the PCR product cloned into pVS11 was determined by using the Ready Reaction Dye Dideoxy Terminator Cycle Sequencing Kit (Applied Biosystems) and an Applied Biosystems model 373A automated sequencer. The predicted amino acid sequence encoded by the 686-bp fragment was compared to the peptide sequences determined for OmpU. Nearly perfect matches were seen with the N-terminal sequence of OmpU and the Pep4 sequence, from which the PCR primers were designed (data not shown). In addition, matches were also seen with the sequences determined for Pep7, Pep2, and Pep6, demonstrating that we had cloned at least part of the *ompU* gene. The cloned insert of pVS11 was purified, labeled with γ-^{32}P-dATP by random priming, and used as a probe to detect the *ompU* gene sequences from the cosmid library of *V. cholerae* 395 as previously mentioned. Surprisingly, of the ca. 1,000 colonies screened, none reacted with the *ompU* probe. The integrity of this genomic library was demonstrated by the ready isolation of clones hybridizing with *ctxA*, *tcpA*, and *nanH* probes (data not shown). Southern hybridization analysis of strain 395 chromosomal DNA revealed single fragments (1-kb *Pst*I, 13-kb *Bgl*II, 3-kb *Hin*dIII, 11-kb *Cla*I, 10.5-kb *Pvu*II, 14-kb *Eco*RI, and 14-kb *Ava*I) that hybridized with the *ompU* probe (data not shown). Several attempts were made to generate partial chromosomal

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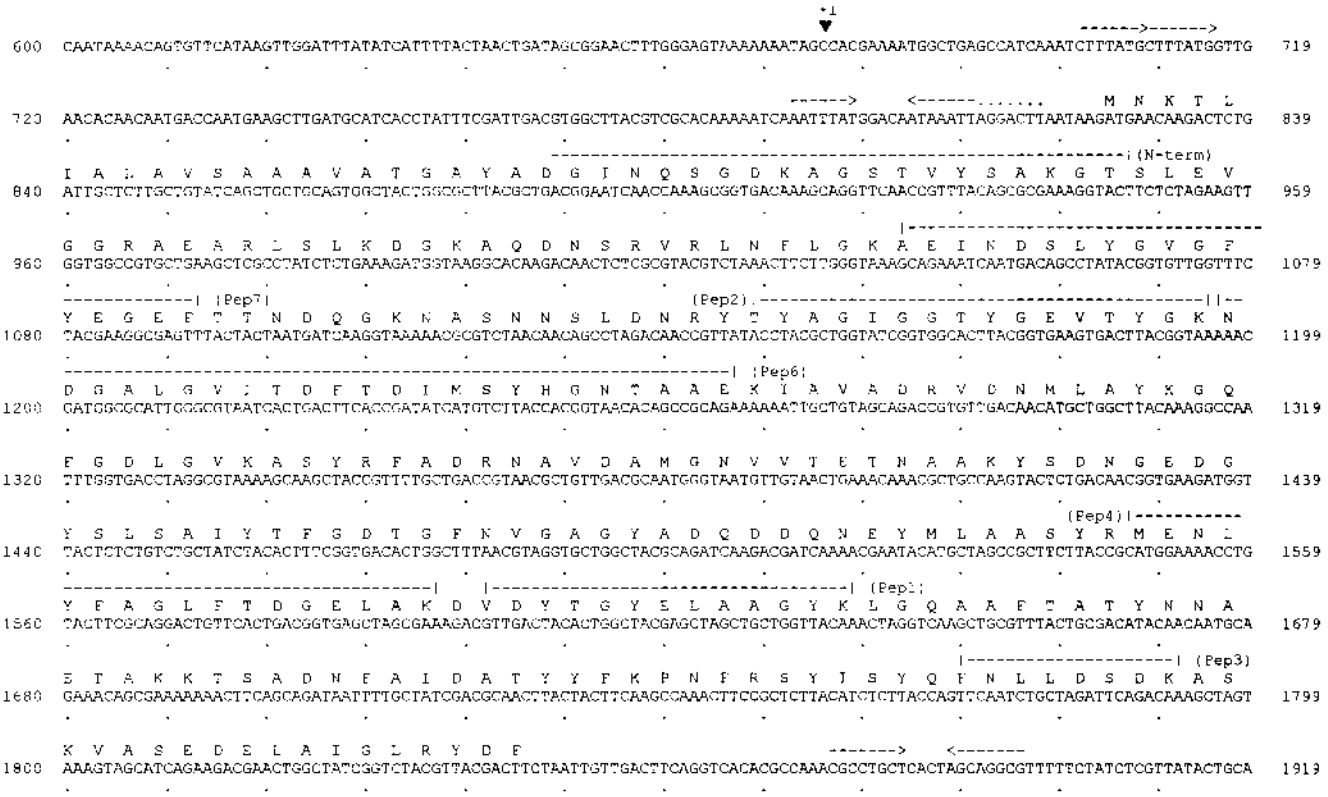

FIG. 1. Nucleotide sequence and predicted amino acid sequence of the *ompU* gene from *V. cholerae* 395. Positions of the various internal peptides are indicated above the corresponding sequence. A potential ribosomal binding site immediately upstream of the start codon is indicated by dots. Arrows indicate a tandem direct repeat sequence of CTTTTATG present upstream of *ompU*, an inverted repeat located near the Shine-Dalgarno sequence, and a region of dyad symmetry characteristic of a Rho-independent terminal located downstream of the termination codon. The start site of the transcript is indicated at nucleotide 676. The sequence has been submitted to GenBank (accession number U73751).

libraries with different restriction fragments in the appropriate size ranges by using both high- and low-copy-number plasmid vectors, but no colonies were recovered that hybridized with the *ompU* probe. These results suggest that the complete *ompU* gene is either unstable in plasmid vectors or that the protein product of the cloned gene may be toxic to the host *E. coli* strains. To overcome this possibility, we screened a genomic library (kindly donated by Michelle Trucksis) of *V. cholerae* 395 constructed in the λZAPII vector (Stratagene, La Jolla, Calif.) and recovered a phage clone that hybridized with the *ompU* probe. The phage clone contained a 6-kb insert with the *ompU* sequences being located approximately in the middle of the insert. The complete nucleotide sequence of both strands of the central 2.1-kb region of this clone was determined by generating PCR products from the phage clone by using primers directed to internal *ompU* sequences and the multiple cloning site of the λZAP vector. The PCR fragments were purified by using Wizard PCR kits (Promega) and directly sequenced as before.

The sequence of the *ompU* gene is shown in Fig. 1. The predicted protein product is a 341-residue protein with a molecular weight of 36,646. The experimentally determined N-terminal amino acid sequence of the purified protein starts at residue 22, thereby predicting a 21-amino-acid signal peptide which closely resembles typical signal peptides (20). The processed protein would therefore have a predicted molecular weight of 34,656 compared to the molecular mass of 38 kDa estimated from polyacrylamide gel electrophoresis analysis (16, 22). An excellent ribosomal binding site is located upstream of the predicted start codon. Another in-frame ATG is located 27 bp upstream of the start codon shown in Fig. 1. However, no obvious ribosomal binding site is located upstream of this codon, and the use of this codon as the start would yield a poor signal peptide that would be unusual for an outer membrane protein. Sequences corresponding to the internal peptides were located in the predicted protein sequence (Fig. 1) except Pep5, which was a minor sequence found in the preparation containing Pep4. In all, 36% (115 residues) of the 320 residue-processed OmpU protein were determined by protein sequencing. Comparison of the determined peptide sequences with the predicted protein sequence shows 93% agreement.

Primer extension mapping of the *ompU* message revealed a transcript from wild-type strain 395 that was barely detectable in mRNA from JJM43, a *toxR* mutant derived from 395 that does not produce OmpU (Fig. 2). The 5′ end of this primer extension product maps to nucleotide 676 on the sequence shown in Fig. 1, just downstream of a potential prokaryotic-10 promoter element having the sequence TAAAAA, and we conclude that the primer extension product represents the major transcription initiation site. Results of transcription studies using *lacZ* gene fusions support the mRNA data in suggesting that the *ompU* promoter is ToxR regulated (5). Transcription of *ompU-lacZ* is not dependent on the ToxT transcription activator, the direct activator for several other ToxR-regulated virulence genes in *V. cholerae* (5), which is consistent with the finding that a *V. cholerae toxT* mutant continues to produce OmpU in wild-type amounts (4).

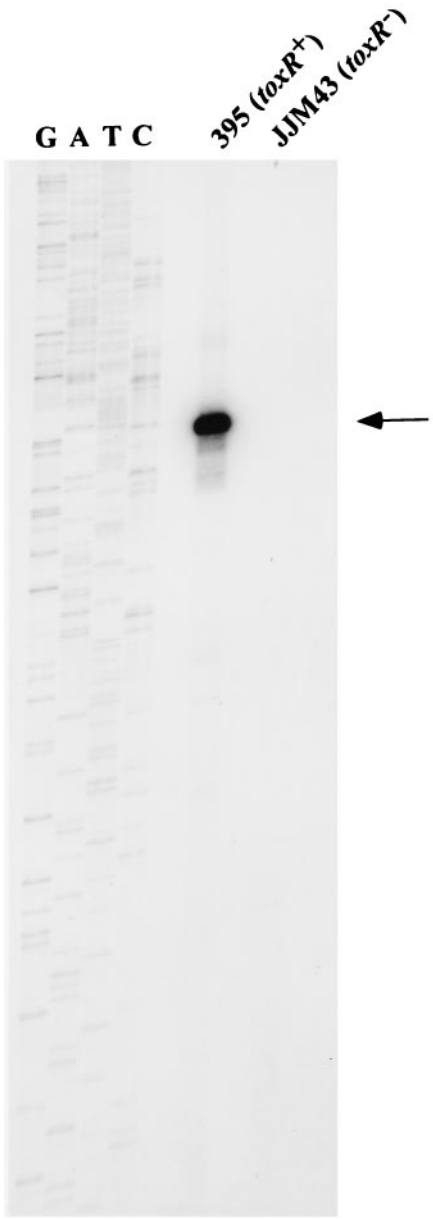

FIG. 2. Primer extension of *ompU* mRNA. RNA was isolated from wild type classical strain 395 and *toxR* mutant JJM43 and primer extension were performed as described previously (10) by using 10 μg of total RNA. The primer used has the sequence 5′-AGCAAGAGCAATCAGAGTCTTGTTCAT-3′ and is homologous to the first nine codons of the *ompU* open reading frame. The arrow indicates the major product representing the 5′ end of the *ompU* message. The nucleotide at which transcription initiates was determined by dideoxy sequence analysis (lanes G A T C) of an *ompU* clone by using the same primer as that used in the RNA analysis.

The sequences preceding the transcription start site for *ompU* give no clue as to the mechanism of its regulation by ToxR. There are no elements with significant homology to elements found in the *ctxA* and *toxT* promoters, two other promoters that require ToxR for activation and to which ToxR binds (11, 19). In the former, a directly repeated heptad (TT TTGAT) and associated downstream sequences are required for ToxR binding, while in the latter, ToxR dependence requires an inverted repeat element with no homology to the TTTTGAT repeat (11, 19). Preliminary data show that ToxR binds to the *ompU* promoter, but the specific sequences required for this binding have not been determined (5).

The position of the transcription start site relative to translation initiation suggests that there is an untranslated leader of 159 nucleotides in the *ompU* mRNA. Such leaders have been observed in other outer membrane proteins, including the *pagC* gene of *Salmonella typhimurium* (21), the *inv* gene of *Yersinia enterotocolitica* (17), and the *ompA* gene of *E. coli* (8). The *ompA* leader is an mRNA stability determinant (8), but the significance of the untranslated leaders of the *pagC* and *inv* genes has not been determined. The *ompU* untranslated leader is not predicted to have the extensive structure found in the *ompA* leader, although there is an inverted repeat element within the sequence just upstream of the putative ribosome binding site (Fig. 1) which perhaps plays a regulatory role in OmpU production.

Alignment of OmpU with other *E. coli* porins using the GAP program (Genetics Computer Group, University of Wisconsin [6]) revealed identities/similarities with OmpC (28%/55%), OmpF (26%/51%), and PhoE (28%/51%). The porin-like nature of OmpU has recently been demonstrated by Chakrabarti et al. (3). We have reported the cross-reactivity of *E. coli* OmpA with antisera raised against *V. cholerae* OmpU (22). Alignment of the predicted OmpU sequence with OmpA reveals 23% identity and 45% similarity; however, OmpU doesn't share consensus motifs with other members of the OmpA family of proteins (13). A search of the GenBank database by using the BLAST program also revealed similarities with a number of bacterial adhesins, which is noteworthy in light of our previous work demonstrating that OmpU is a putative adhesin of *V. cholerae* (22). Regions of identity/similarity were seen with *E. coli* K88 (57%/80% over 21 residues), *E. coli* AIDA (33%/50% over 48 residues), *Salmonella* SEF14 fimbriae (40%/60% over 30 residues), *Bordetella pertussis* pertactin (31%/48% over 47 residues), *Neisseria meningitidis* class I Omp (25%/44% over 79 residues), and *Haemophilus influenzae* HMW1 (34%/48% over 43 residues). Several of these adhesins have hemagglutinating activity, and it is noteworthy that the hemagglutinating activity of OmpU has also recently been demonstrated (3). However, the exact residues necessary for adhesion in OmpU or the majority of these other adhesins have not been localized, so whether these sequence similarities have any relevance to adherence remains to be determined.

The previous difficulty encountered by us and other investigators in cloning the *ompU* gene is consistent with the difficulty encountered in cloning genes encoding porins from other species such as *Neisseria gonorrhoeae* into *E. coli* (2). Although other members of the ToxR regulon were identified and cloned by using Tn*phoA* mutagenesis (18, 23), we were unable to recover any Tn*phoA* that disrupted production of the OmpU protein (data not shown). This result suggests that gross mutation of *ompU* is a lethal event, which is consistent with our failure to date to construct a viable *ompU* mutation in *V. cholerae* (1).

The cloning and sequencing of *ompU* will allow further investigations on several fronts. First, the role of OmpU as an adhesin can now be tested by constructing more subtle mutations which can inactivate the adhesive function while maintaining viability. Using the cloned *ompU* gene as a probe, we found homologous sequences in all O1 and O139 *V. cholerae* strains tested, even cholera toxin-negative sewage isolates such as 1074-78, which was incapable of colonizing volunteers (14). Comparison of *ompU* sequence differences in these various strains could yield insights into specific residues involved in adherence. Second, the role of OmpU as a porin and detailed structure-function studies related to this activity can now be

pursued. Third, the ToxR-mediated regulation of *ompU* expression can now be studied in great detail, which will undoubtedly yield important insights into the ToxR regulon that is crucial for virulence of *V. cholerae.*

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