Structural features, properties and regulation of the outer-membrane protein W (OmpW) of Vibrio cholerae

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The outer-membrane protein OmpW of Vibrio cholerae was studied with respect to its structure, functional properties and regulation of expression. On SDS-PAGE, the membrane-associated form of OmpW protein (solubilized by either 0·1 % or 2 % SDS at 25 °C) migrated as a monomer of 19 kDa that changed to 21 kDa on boiling. The protein was hyperexpressed in Escherichia coli in the histidine-tagged form and the purified His6-OmpW (heated or unheated) migrated as a 23 kDa protein on SDS-PAGE. Circular dichroism and Fourier-transform infrared spectroscopic analyses of the recombinant protein showed the presence of β-structures (~40 %) with minor amounts (8–15 %) of α-helix. These results were consistent with those obtained by computational analysis of the sequence data of the protein using the secondary structure prediction program Jnet. The recombinant protein did not exhibit any porin-like property in a liposome-swelling assay. An antiserum to the purified protein induced a moderate level (66±6 % and 33±3 % at 1:50 and 1:100 dilutions, respectively) of passive protection against live vibrio challenge in a suckling mouse model. OmpW-deficient mutants of V. cholerae strains were generated by insertion mutagenesis. In a competitive assay in mice, the intestinal colonization activities of these mutants were found to be either only marginally diminished (for O1 strains) or 10-fold less (for an O139 strain) as compared to those of the corresponding wild-type strains. The OmpW protein was expressed in vivo as well as in vitro in liquid culture medium devoid of glucose. Interestingly, the glucose-dependent regulation of OmpW expression was less prominent in a ToxR” mutant of V. cholerae. Further, the expression of OmpW protein was found to be dependent on in vitro cultural conditions such as temperature, salinity, and availability of nutrients or oxygen. These results suggest that the modulation of OmpW expression by environmental factors may be linked to the adaptive response of the organism under stress conditions.

INTRODUCTION

The cell-surface structure of Gram-negative bacteria is important for bacterial physiology as well as communication with the external environment (Nikaido, 1999). The surface envelope of Gram-negative bacteria consists of three essential layers: the cytoplasmic or inner membrane (IM), the outer membrane (OM) and the periplasmic space between the IM and OM. The OM has a highly specialized structure and is usually associated firmly with the underlying peptidoglycan layer predominantly through lipoprotein/matrix protein and linked with cell-surface lipopolysaccharides (LPS) (Lugtenberg & Alphen, 1983). The major components of the OM are phospholipids, LPS and proteins which help it to serve as a physical barrier between the bacterial body and its surroundings and make the organism resistant to host defence factors and toxic materials such as bile salts and antibiotics (Lin et al., 2002; Lugtenberg & Alphen, 1983). Another important function of the OM is to endow the organism with surface hydrophilicity, which is important in bacterial pathogenesis, complement resistance and capacity to avoid specific immune attack. All these functional attributes can be correlated well with the precise molecular organization of cell-surface components such as proteins, LPS and phospholipids (Lerouge & Vanderleyden, 2002; Lin et al., 2002; Lugtenberg & Alphen, 1983; Nikaido, 1999).

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About 50% of the OM mass consists of proteins, in the form of either integral membrane proteins or lipoproteins that are anchored to the membrane by means of N-terminally linked lipids (Koebnik et al., 2000). While a few integral outer-membrane proteins (OMPs) are constitutively expressed, there are other proteins whose synthesis is induced when these are required by the cells. High-resolution structures of OMPs, most of which have become available during the last few years, have established that the majority of these consist of a β-pleated sheet in the form of a barrel that helps the organism to maintain its structural integrity as well as to allow selective permeability of solutes across the membrane (Buchanan, 1999; Koebnik et al., 2000; Schulz, 2000). In addition, OMPs, whose production is often regulated by several global regulators in response to environmental cues, play important roles in bacterial pathogenesis by enhancing the adaptability of pathogens to various environments in vivo and in vitro (Lin et al., 2002; Nikaido, 1999).

The Gram-negative bacterium Vibrio cholerae is the causative agent of cholera, a severe form of diarrhoeal illness in humans with high mortality and morbidity (Kaper et al., 1995). The pathogenic strains of V. cholerae belonging to the serogroups O1 or O139 carry the filamentous CTX prophage (Waldor & Mekalanos, 1996) that encodes cholera toxin (CT), the prime diarrheagenic factor responsible for ‘cholera gravis’ (Kaper et al., 1995), and vibrio pathogenicity island (VPI)-associated genes for the cell-surface exposure of toxin-coregulated pilus (TCP) (Karaolis et al., 1998; Kim et al., 2003), a factor responsible for intestinal colonization of the organism (Taylor et al., 1987). The expression of both CT and TCP is coregulated by the transmembrane protein ToxR (a global regulator) encoded by the toxRS operon (DiRita, 1992). While regulation of most of the genes under ToxR control is mediated through transcriptional activation of tcpH (of the tcpP/H operon) and toxT (the gene encoding another regulatory protein, ToxT), of cytosolic origin (Carroll et al., 1997; Hase & Mekalanos, 1998), the expression of two OMPs, OmpU (38 kDa) and OmpT (40 kDa), of V. cholerae follows a pathway independent of TcpP and/or ToxT (Champion et al., 1997; Miller & Mekalanos, 1988). In fact, ToxR, which is present in V. cholerae strains belonging to both epidemic (O1 or O139) and non-epidemic (non-O1/non-O139) pathogenic serogroups, differentially regulates the expression of these two OMPs by upregulation of OmpU and downregulation of OmpT.

As is the case with other Gram-negative bacteria, the OMPs of V. cholerae are likely to play an important role in bacterial physiology, contributing to their survival within the host as well as in the wider environment (Reidl & Klose, 2002). However, only limited information is available so far in this regard. OmpU was shown to be important for the survival of vibrios in the intestine by making the organism more resistant to bile salts (Provenzano & Klose, 2000). Another OMP found to be important in the pathogenesis in infant mice is Ir/glA, a 77 kDa protein whose expression is regulated by iron (Goldberg et al., 1990). OMPs of V. cholerae are also expected to stimulate an immune response in the host, which may be relevant to the induction of protective immunity (Das et al., 1998; Sengupta et al., 1992).

The OMP OmpW of V. cholerae has been described as a 22 kDa molecule (Jalajkumari & Manning, 1990). Gene sequence data of V. cholerae have demonstrated the presence of the gene in the chromosome II of the organism (Heidelberger et al., 2000). OmpW appears to be conserved in V. cholerae strains, which led to the development of a PCR-based method for the rapid identification of the organism (Nandi et al., 2000). However, proteins homologous to V. cholerae OmpW have been documented in other bacteria as well (Baldermann et al., 1998; Pilsl et al., 1999). While the preponderance of β-structure in some of these proteins is consistent with their possible localization in the OM (Baldermann et al., 1998), little information is available on their properties or regulation of expression that may be linked to their putative function. Clearly, it is important to obtain such information for V. cholerae, which needs to survive under diverse conditions encountered in the host as well as the wider environment.

In this work, we studied structural and biological properties of the V. cholerae membrane-associated form of OmpW as well as of the recombinant protein, the latter being purified following its hyperexpression in Escherichia coli. OmpW-negative mutants of V. cholerae were generated, and their colonization potential was evaluated and compared with that of the wild-type. Finally, conditions regulating the expression of OmpW in V. cholerae were studied to generate information regarding the putative role of this protein in the bacterial physiology.

**METHODS**

**Bacterial strains and plasmids.** V. cholerae and E. coli strains and plasmids used in this study are shown in Table 1. The V. cholerae strains belong to both the epidemic (O1 and O139) and non-epidemic (O53 and O34) serogroups.

**Growth media and culture conditions.** V. cholerae strains were grown in vitro in AKI medium containing 1·5% (w/v) peptone, 0·4% (w/v) yeast extract, 0·5% (w/v) NaCl, 0·4% (w/v) NaHCO3, pH 7·5. An early exponential-phase seed culture was used as an inoculum and bacteria were grown at 37°C or 28°C for either 6–8 h or 14–16 h. Whenever needed, the medium was further supplemented with simple sugars and/or NaCl to desired concentrations. V. cholerae cells were also grown in Tris-basal salt (TB) medium containing 12·1 g Tris base, 5·8 g NaCl, 3·7 g KCl, 0·15 g CaCl2, 1·35 × 10−2 g FeCl3, 0·1 g MgCl2·6H2O, 0·272 g KH2PO4 and 0·142 g Na2SO4 per litre of medium. The pH was adjusted to 7·4 with dilute HCl. The medium was supplemented with any one or more of the following nutrients as the utilizable source of carbon (glucose, sucrose, galactose), nitrogen (NH4Cl) or both (glutamic acid). E. coli cells were grown in AKI or TB medium or in Luria-broth (LB) medium containing appropriate antibiotics.

**Preparation of the OM.** The method of Filip et al. (1973) was used. Briefly, cells were grown in either AKI (for V. cholerae) or LB (for E. coli) medium for 15–16 h at 37°C with shaking. Harvested bacteria were washed and resuspended in 10 mM HEPES buffer.
The insoluble OM fraction was obtained as a pellet by centrifugation (Sigma) solution to selectively solubilize the inner-membrane part. Designed so as to contain strain O395 and primer pair 5 and 6 (Table 2). Primers were amplified by PCR using a genomic DNA preparation of the lerae amplified by PCR using a genomic DNA preparation of the out the sequence encoding its amino-terminal signal sequence) was *Ap, ampicillin; Km, kanamycin;Sm, streptomycin; Tc, tetracycline.

Cloning and expression of His6-OmpW. The ompW gene (without the sequence encoding its amino-terminal signal sequence) was amplified by PCR using a genomic DNA preparation of the V. cholerae strain O395 and primer pair 5 and 6 (Table 2). Primers were designed so as to contain BamHI and PstI sites at the 5' end of the forward and reverse primers, respectively. The amplicon was separated on 1% (w/v) agarose gel and purified using QIA quick gel purification kit (Qiagen). Next, it was digested with BamHI and PstI and the digested product was ligated to the expression vector pQE32 (Qiagen) (digested with the same enzymes). The resultant recombinant plasmid was used to transform E. coli XL-1 Blue and the transformants were checked for their ability to produce the recombinant protein (His6-OmpW) following induction with 0-25 mM IPTG.

Purification of recombinant OmpW. E. coli XL-1 Blue harbouring pQEBN was cultured in 100 ml LB and expression of the recombinant protein was induced by 0-25 mM IPTG. After 3 h induction at 37°C, cells were harvested, washed and resuspended in buffer A (10 mM Tris/HCl, 200 mM NaCl, pH 7-5), and lysed by sonication. Lysed bacteria were centrifuged (6000 g for 10 min). The pellet consisting of 

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype/relevant characteristics*</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>V. cholerae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O395</td>
<td>O1; classical, Sm'</td>
<td>Mekalanos (1983)</td>
</tr>
<tr>
<td>BN490</td>
<td>O395; ompW::pGP704, Sm' Ap' (OmpW− mutant of O395)</td>
<td>This study</td>
</tr>
<tr>
<td>JJM43</td>
<td>O395; ΔtoxR43</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td>O17</td>
<td>O1; El Tor</td>
<td>Manning et al. (1985)</td>
</tr>
<tr>
<td>Co366</td>
<td>O1; El Tor, Sm'</td>
<td>Mukhopadhyay et al. (2000)</td>
</tr>
<tr>
<td>BN590</td>
<td>Co366; ompW::pGP704, Sm' Ap' (OmpW− mutant of Co366)</td>
<td>This study</td>
</tr>
<tr>
<td>SG25</td>
<td>O139; Sm'</td>
<td>Nandy et al. (1995)</td>
</tr>
<tr>
<td>BN690</td>
<td>SG25; ompW::pGP704, Sm' Ap' (OmpW− mutant of SG25)</td>
<td>This study</td>
</tr>
<tr>
<td>10259</td>
<td>O53</td>
<td>Nandy et al. (1995)</td>
</tr>
<tr>
<td>10325</td>
<td>O34</td>
<td>Nandy et al. (1995)</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XL-1 Blue</td>
<td>Cloning strain for the expression of His₆-OmpW, Tc'</td>
<td>Bullock et al. (1987)</td>
</tr>
<tr>
<td>LE392</td>
<td>Cloning strain</td>
<td>Borck et al. (1976)</td>
</tr>
<tr>
<td>SM10 pipr</td>
<td>thi, thr, leu, tonA, lacA, supE, recA::RP4-Tc::Mu λ pir R6K Km'</td>
<td>Miller &amp; Mekalanos (1988)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pPM440</td>
<td>Contains 2-1 kb of ompW and its flanking regions from the V. cholerae genome, Ap'</td>
<td>Manning et al. (1985)</td>
</tr>
<tr>
<td>pNM505</td>
<td>pBR322 containing ~2 kb EcoRI insert from pPM440; the insert spans the ompW gene and its upstream region necessary for the expression of OmpW, Ap' Tc'</td>
<td>This study</td>
</tr>
<tr>
<td>pBN490</td>
<td>pGP704 derivative containing 220 bp internal fragment of ompW</td>
<td>This study</td>
</tr>
<tr>
<td>pQE32</td>
<td>Expression vector for protein tagged with 6 His residues at the N-terminus</td>
<td>Qiagen</td>
</tr>
<tr>
<td>pQEBN</td>
<td>pQE32 derivative containing gene sequence for OmpW protein without signal sequence</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline.
Circular dichroism (CD) spectra. CD spectra were recorded at 25°C or 45°C by a JASCO spectrophotometer. The protein (300 or 600 μg ml⁻¹) was taken up in buffer A containing either 0-1 % (v/v) Tween 20 or Triton X-100 and the CD spectrum was recorded in the far-UV region (200–250 nm) using a cuvette of 0.1 cm path length, a band width of 2 nm, and a time constant of 4 s. Data were expressed in molar ellipticity values (Greenfield & Fasman, 1969) and plotted against the wavelength scanned. Five spectra were averaged for each sample.

Fourier-transform infrared (FTIR) spectroscopy. The recombinant protein was dialysed against buffer A to allow its precipitation. The precipitated material was resuspended in an appropriate volume of 50 mM sodium phosphate buffer in 99 % (v/v) D₂O (Sigma), pD 7-0 to obtain a suspension of 10 mg protein ml⁻¹. Next, 15 μl of this suspension was taken for FTIR analysis (Nicolet) in the range of 1700 cm⁻¹ to 1600 cm⁻¹ for the amide I band. Secondary structural features of the protein were determined by Fourier self-deconvolution (FSD) analysis (Dong et al., 1996).

Liposome-swelling assay. The methodology of Nikaido et al. (1991) was used, with minor modifications. For this, 3 μmol phosphatidylcholine (type XViE, Sigma) was mixed with 0-2 μmol dicetyl phosphate (Sigma) in chloroform/diethyl ether and dried under a stream of nitrogen and evaporation. The dried lipid film was resuspended in 0-5 ml of an aqueous solution of recombinant OmpW (100 μg ml⁻¹) containing 0-1 % (v/v) Tween 20 (for proteoliposome preparation). Following sonication, samples were dried and reconstituted in 5 mM Tris/HCl buffer, pH 8-0, containing 20 mM stachyose (Sigma). For assay, 20 μl of the proteoliposome suspension was mixed rapidly with 1 ml of isoosmolar solutions of different sugars (arabinose, glucose, sucrose or maltose), and changes in OD₄₅₀ were recorded.

Raising of antiserum. An antiserum to OmpW protein was raised by immunization of rabbits with the protein purified by elution from the gel slices following SDS-PAGE of an OM preparation as described earlier (Sengupta et al., 1992). The OM preparation of E. coli strain LE392 containing the plasmid pPM440 (Manning et al., 1985), which carries the ompW gene of V. cholerae, was used as a source of OmpW protein.

An antiserum to the purified recombinant protein His₆-OmpW was also raised by immunizing rabbits intramuscularly with 500 μg purified protein emulsified with Freund’s complete adjuvant. The first injection was followed by three similar injections of the purified protein (in incomplete adjuvant) at weekly intervals. Serum was collected by vein puncture and stored at −20°C.

SDS-PAGE and immunoblotting. SDS-PAGE analysis of test samples was carried out in 12-5 % (w/v) polyacrylamide gels. Test samples consisted of purified protein (~2–10 μg), OM preparation (~20–30 μg protein) or lysates of bacterial whole cells (about 10⁷ cell equivalent). The OM preparation or purified protein or cell lysates of bacteria were usually solubilized (if not mentioned otherwise) at 100°C for 10 min in sample buffer containing 2 % (w/v) SDS and 5 % (v/v) β-mercaptoethanol. Following electrophoresis, resolved bands were visualized either by Coomassie brilliant blue or by silver staining. For immunoblotting, electrophoresed proteins were transferred to nitrocellulose membrane by electroblotting. Immunodetection of OmpW protein was carried out by using appropriate rabbit antiserum to OmpW protein and peroxidase-conjugated goat anti-rabbit IgG (Genet) as the secondary antibody.

Generation of ompW mutants of V. cholerae strains. Mutants of V. cholerae strains (belonging to the O1 and O139 serogroups) were generated by insertional inactivation of the ompW gene in the V. cholerae chromosome. For this, an internal fragment (220 bp) of ompW was obtained by HindIII and Hpal digestion of plasmid pPM440. The fragment was ligated to pBluescript, previously digested with HindIII and Smal. The recombinant plasmid was then digested with SalI and XbaI to generate a 253 bp fragment that was cloned into the suicide vector pGP704 (digested with the same enzymes). The resulting plasmid pBN490 was transformed into E. coli SM10 zipir (Km) and subsequently mobilized to wild-type V. cholerae strains (Sm*) by conjugation. The transconjugants were selected on LB plates containing appropriate antibiotics and transconjugants were checked for insertional inactivation of ompW by PCR [using different combinations of primers 1–4 (Table 2)] and by immunoblotting experiments (using anti-OmpW serum). Complementation of the ompW mutant strain was carried out by transforming the strain with the recombinant plasmid pNM505 through electroporation and the transformants were selected on LA plates containing tetracycline. Selected transformants were checked for the restoration of OmpW expression by immunoblotting experiments.

In vitro and in vivo growth properties of V. cholerae strains. In vitro growth properties of ompW mutant strains were compared with those of the corresponding wild-type strains in a competitive assay. For this, 50 ml AKI medium was inoculated with equal amounts (about 10⁷ c.f.u.) of the mutant and wild-type strains. The organisms were allowed to grow at 37°C for 15–16 h with mild shaking and the number of organisms in the mixture was enumerated by dilution plating on LB plates with or without appropriate antibiotics.

In vivo growth properties of wild-type V. cholerae strains and their ompW mutants were determined by intestinal colonization experiments in suckling mice. Colonization properties were determined by a competition assay (Taylor et al., 1987) where groups of mice were inoculated with a 1:1 mixture of the wild-type and mutant strains. After 18 h of challenge, animals were killed and the number of viable organisms was determined by plating on LB plates as described above.

Table 2. Primers

<table>
<thead>
<tr>
<th>Primer no.</th>
<th>Target gene</th>
<th>Sequence (size)</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ompW (sense)</td>
<td>5'-CAACAAAGAGGTGACTTTATTGTG-3' (24-mer)</td>
<td>Nandi et al. (2000)</td>
</tr>
<tr>
<td>2</td>
<td>ompW (antisense)</td>
<td>5'-GAACCTTAACCCCGGGC-3' (19-mer)</td>
<td>Nandi et al. (2000)</td>
</tr>
<tr>
<td>3</td>
<td>ompW (sense)</td>
<td>5'-CCACCTACCCGTTGACT-3' (23-mer)</td>
<td>Nandi et al. (2000)</td>
</tr>
<tr>
<td>4</td>
<td>ompW (antisense)</td>
<td>5'-GGTTTGTGGAATTTAGATTCC-3' (22-mer)</td>
<td>Nandi et al. (2000)</td>
</tr>
<tr>
<td>5</td>
<td>ompW (sense) with a BamHII site*</td>
<td>5'-ACGAGGATCCACAAAGAGGTGACTTTATTGTG-3' (33-mer)</td>
<td>This study</td>
</tr>
<tr>
<td>6</td>
<td>ompW (antisense) with a PstI site*</td>
<td>5'-ATCCCTGCAGGTTGACCTATAACCCCGGG-3' (34-mer)</td>
<td>This study</td>
</tr>
</tbody>
</table>

*Restriction sites for primers 5 and 6 are underlined.
**Protection experiments in vivo.** The protective ability of the antiserum to the recombinant OmpW protein was determined by passive protection experiments in a suckling mice model (Sengupta et al., 1992). Results were expressed as the percentage protection by comparing the number of survivors after 24 h of vibrio challenge in the experimental (with antiserum) against control (without antiserum) groups.

**RESULTS**

**Detergent solubility, heat modifiability and trypsin sensitivity of the membrane-associated form of OmpW**

Detergent solubility of the membrane-associated form of OmpW protein was studied by treatment of the OM suspension with either 2% (w/v) SDS or 2% (v/v) Triton X-100 at 25°C for 30 min and subsequent centrifugation of the treated material at 100,000 g to obtain the supernatant (detergent-soluble) and pellet (insoluble) fractions. Further analysis of both the fractions revealed that the membrane-associated OmpW protein could be fully solubilized by the SDS treatment while Triton X-100 treatment led to its partial solubilization.

Heat modifiability of membrane-associated OmpW was studied by solubilizing of the OM preparation by treatment with 2% SDS for 10 min at 25°C or 100°C and subsequent SDS-PAGE analysis of the material. Fig. 1(a) shows the SDS-PAGE profiles of the native (unheated) and heated preparations of OM derived from different V. cholerae strains. An upward shift (by about 2 to 3 kDa) in the migration of the OmpW protein was noted in the heated OM preparations of the organisms belonging to O1 as well as non-O1/non-O139 serogroups. Similar results were obtained in immunoblotting experiments with these preparations. A representative result is shown in Fig. 1(b).

To study the electrophoretic properties of the OmpW protein solubilized under mild conditions, the OM preparation was treated with 0.1% or 0.5% SDS at 25°C. Following centrifugation (10,000 g), the supernatant (unheated or heated to 100°C) was subjected to SDS-PAGE analysis using 0.1% (w/v) SDS and developed by immunoblotting. The results obtained again demonstrated heat-modifiable properties of OmpW extracted with a lower concentration (0.1%) of SDS, as the unheated protein migrated with subunit molecular masses of 19 and 21 kDa, which, however, changed exclusively to 21 kDa on heating (data not shown). The data did not produce evidence for the existence of any oligomeric form of the protein isolated under these mild conditions.

The OmpW protein in its membrane-associated form was found to be partially sensitive to trypsin: treatment of the OM preparation with the enzyme resulted in only partial reduction in the intensity of the band as compared to that of the untreated control (data not shown).

**Characterization of the recombinant protein (His$_6$-OmpW)**

The recombinant (His$_6$-OmpW) protein was isolated by solubilization and purification in the presence of 8 M urea and subsequent removal of urea by stepwise dialysis in the presence of 0.1% Tween 20 or Triton X-100. The purity of the renatured protein thus obtained was checked by SDS-PAGE analysis followed by either Coomassie blue or silver staining of the protein bands (Fig. 2a). As expected, the recombinant protein migrated with a subunit molecular mass of 23 kDa, that is about 2 kDa higher than that of the membrane-associated form of OmpW (21 kDa). Serological relatedness between the 23 and 21 kDa proteins was

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**Fig. 1.** Heat-modifiable property of membrane-associated OmpW. (a) SDS-PAGE profiles of OM preparations of V. cholerae strains grown in AKI medium. Samples were run without heat treatment (25°C) or after heating (100°C) for 10 min. (b) Immunoblot detection of OmpW bands in the SDS-PAGE profiles of OM preparation of V. cholerae O17. An antiserum to the OmpW protein was used to develop the blot. Bands are shown by arrows.

**Fig. 2.** SDS-PAGE profiles of purified His$_6$-OmpW (10 µg per lane) in 0.1% Tween 20. (a) Gels were developed with silver stain (lane 1) or Coomassie blue (2). The OM preparation of V. cholerae O395 (3) was also run for comparison. (b) Immunoblot reactivity of purified His$_6$-OmpW (lanes 1 and 3) and the membrane-associated form of OmpW (2 and 4). The blot was developed with antisera to the purified His$_6$-OmpW (1 and 2) or to the OmpW protein gel-purified from OM (3 and 4). Arrows show the OmpW bands.
established through immunoblot data generated with two different antisera raised separately against the gel-purified OmpW (21 kDa) and the purified recombinant 23 kDa protein (Fig. 2b).

The recombinant protein, purified in the presence of 0.1% Tween 20, was subjected to CD spectroscopic analysis (Fig. 3). The spectra determined at 25 °C showed a negative peak around 215–217 nm characteristic of β-structures in proteins. The intensity of this peak increased marginally at 45 °C, although further heating of the protein to 60 °C resulted in its precipitation, leading to loss of the signal. Comparable results were also obtained when the spectroscopic measurements was carried out with the protein purified in the presence of 0.1% Triton X-100 (data not presented). Molar ellipticity values at 217 nm, calculated from the CD spectra obtained in Tween 20 as well as Triton X-100, are presented in Table 3. The data were analysed by the CDNN program (Bohm et al., 1992) to obtain information about the secondary structural features of the recombinant OmpW protein. The analysis predicts the predominance of β-structure (39.5%) with minor amounts of α-helix (15.3%) in the protein in 0.1% Tween 20 at 25 °C. The rest (24.9%) was predicted to consist of random coil structures. The coil structure of the protein increased to 34.7% with a concomitant decrease in the α-helical content (8.0%) when the data generated in 0.1% Triton X-100 were analysed. However, the β-structure content remained essentially unchanged (38.7%). No significant difference between the secondary structure contents of the protein at 25 °C and 45 °C could be noted on the basis of this analysis (Table 3).

The recombinant protein purified in the presence of 0.1% Tween 20 was subjected to FTIR analysis (for the amide I band); the results are presented in Fig. 4. While the normal spectrum (curve A) does not show any sharp peak features, the Fourier self-deconvolution spectrum (curve B) reveals the presence of several peaks at the wave numbers indicated.

**Table 3.** Estimation of secondary structure contents of the recombinant OmpW based on the analysis of its CD spectra

<table>
<thead>
<tr>
<th>Detergent present (0.1%, v/v)</th>
<th>Temp. (°C)</th>
<th>Molar ellipticity (deg cm² dmol⁻¹) at 217 nm</th>
<th>Secondary structure content (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>α-Helix</td>
</tr>
<tr>
<td>Tween 20</td>
<td>25</td>
<td>8.5 × 10³</td>
<td>15-3</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>9.4 × 10³</td>
<td>15-9</td>
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<td>Triton X-100</td>
<td>25</td>
<td>8.3 × 10³</td>
<td>8-0</td>
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<tr>
<td></td>
<td>45</td>
<td>8.4 × 10³</td>
<td>7-9</td>
</tr>
</tbody>
</table>

Fig. 3. CD spectra of the recombinant OmpW recorded in the UV region. Protein sample (14.7 μM) was taken up in 10 mM Tris/HCl buffer (pH 7.5) containing 200 mM NaCl and 0.1% Tween 20. Spectral measurements were made at 25 °C (■) and 45 °C (○). Data are expressed in ellipticity values after solvent spectra correction.

Fig. 4. Normal FTIR (A) and Fourier self-deconvolution (B) spectra of the amide I band of the recombinant OmpW. The protein was precipitated from a solution containing 0.1% Tween 20, resuspended in 50 mM phosphate buffer, pH 7.0, in 99.9% D₂O and spectra were recorded at 25 °C. Peak positions indicative of α- and β-structures are shown by arrows.
in the figure. Some of these peaks are characteristics of protein secondary structural features that are indicated by arrows. While the majority of the peak positions suggest the presence of $\beta$-structural features (both parallel and anti-parallel) in the protein, peak positions associated with $\alpha$ or random coil structures are also discernible. Similar conclusions could be drawn from the FTIR data (not shown) obtained by using the recombinant protein purified in the presence of 0-1% Triton X-100.

Porin-like activity of the recombinant OmpW protein was tested by suspending its proteoliposome preparation in iso-osmolar concentration (20 mM) of different mono-(glucose and arabinose) and disaccharides (sucrose and maltose). However, the preparation did not exhibit any porin-like property, being non-permeable to all the sugars tested (data not presented).

Hydropathicity analysis and prediction of secondary structural features of OmpW protein

The full-length OmpW protein was subjected to hydropathicity analysis by the Kyte and Doolittle program (Kyte & Doolittle, 1982). Fig. 5 shows the plot generated with the window size 6. Stretches of $\alpha$-helical and $\beta$-structures in the protein, as predicted by the secondary structure analysis program Jnet (Cuff & Barton, 2000) and PHD (Rost & Sander, 1993) are also shown in the figure for comparison. Computer-based analysis predicts high hydrophobicity for the leader peptide (signal sequence) region, which is likely to consist of a long stretch of helical structures. The analysis also shows that the mature protein contains periodic hydrophobic and hydrophilic regions. While the PHD program predicts the occurrence of stretches of $\beta$-structures in the hydrophobic regions, the Jnet program predicts shorter stretches of both $\alpha$- and $\beta$-structures for the corresponding regions (Fig. 5). Incidentally, the C-terminal end of the OmpW protein is shown to contain a hydrophobic region with $\beta$-structures.

Regulation of OmpW expression in V. cholerae cells grown in vitro and in vivo

Regulation of OmpW protein expression in vitro was studied by growing cells under different cultural conditions and determining the protein level in whole-cell lysates by immunoblotting experiments using specific antisera.

The effect of simple sugar supplementation (as an easily metabolizable carbon source) of AKI medium upon the expression of OmpW protein was studied. Glucose, mannose, fructose and maltose considerably inhibited the expression of OmpW by V. cholerae, but arabinose and galactose did not exhibit any such inhibitory effect (Fig. 6a).

AKI medium contains about 0.5% (w/v) NaCl. To ascertain whether further increase in NaCl concentration has any effect on the OmpW expression, V. cholerae cells were grown in AKI medium containing 0.5% and higher (4%) salt concentrations. Immunoblot data (Fig. 6b) demonstrate considerable reduction in the OmpW band intensity in cells grown under higher salt concentrations. Further, the expression of OmpW protein in cells grown in AKI medium was found to be decreased at 42°C as compared to that observable in cells grown at 37°C (Fig. 6c). Similar results

![Fig. 5. Hydropathicity plot of the OmpW protein with its signal sequence (—including). Stretches of $\alpha$-helical (grey bars) and $\beta$-structures (black bars), as predicted by the Jnet and PHD programs, are also shown.](#)
were obtained with cells grown in the biochemically defined TB medium. Thus, *V. cholerae* grown in TB medium supplemented with glutamic acid as the carbon and nitrogen source supported the expression of OmpW while additional supplementation with glucose (as an easily metabolizable carbon source) resulted in considerable reduction of its expression (Fig. 7). However, the suppressive effect of glucose was found to be dependent on its concentration in the medium. It is noteworthy that supplementation of TB medium (containing glutamic acid) with galactose (as additional carbon source) or NH₄Cl (as easily metabolizable nitrogen source) did not suppress OmpW expression.

The role of the ToxR protein in the expression of OmpW was studied in a ToxR-negative mutant of *V. cholerae* strain O395. Immunoblot data presented in Fig. 8 demonstrate that the glucose-mediated suppression of OmpW expression was considerably less pronounced in the mutant strain as compared to that observable with the corresponding wild-type strain.

In *vivo* expression of OmpW protein by *V. cholerae* was studied by growing the bacteria in ligated ileal loops of rabbits. Immunoblot results demonstrated that the OmpW protein was expressed equally well by different *V. cholerae* strains grown either *in vivo* or *in vitro* (data not shown).

**In vitro and in vivo growth properties of ompW insertion mutants of V. cholerae**

Growth properties of three *V. cholerae* wild-type strains and their mutants were determined by competitive growth experiments *in vitro* as well as *in vivo* in mouse intestine (Table 4). The *ompW* mutant strains did not exhibit any major deficiency in their *in vitro* growth properties in AKI medium as compared to those of their respective wild-type strains. The competitive growth indices ranged between 0·74 and 0·93 for all the three *V. cholerae* strains tested.

The *in vivo* growth properties of *ompW* mutants were determined through a competitive colonization assay in mouse intestine. Results obtained with a *V. cholerae* O1 classical strain (O395) demonstrate only marginal (about 1·2-fold) reduction of the *in vivo* colonization ability of the mutant strain as compared to that of the wild-type strain (Table 4). The *ompW* mutant of El Tor biotype strain Co366 also

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**Table 4.** Competitive growth *in vitro* and colonization properties *in vivo* of wild-type and *ompW* mutants of *V. cholerae* strains

<table>
<thead>
<tr>
<th>Competing strains (characteristics)</th>
<th><em>In vitro competition</em></th>
<th><em>In vivo competition</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Inoculum ratio†</td>
<td>Recovery ratio‡</td>
</tr>
<tr>
<td>BN490 (mutant), O395 (O1, wild-type)</td>
<td>0·83</td>
<td>0·77</td>
</tr>
<tr>
<td>BN590 (mutant), Co366 (O1, wild-type)</td>
<td>0·9</td>
<td>0·67</td>
</tr>
<tr>
<td>BN690 (mutant), SG25 (O139, wild-type)</td>
<td>1·1</td>
<td>0·91</td>
</tr>
<tr>
<td>BN690 (mutant, pNM505), SG25 (O139, wild-type)</td>
<td>0·81</td>
<td>0·65</td>
</tr>
</tbody>
</table>

†Ratio of the number of organisms of the mutant strains to that of the corresponding wild-type strains in the inoculum.

‡Ratio of the number of organisms of the mutant strain to that of the wild-type strain recovered.

§Recovery ratio/inoculum ratio.
Results presented above (Fig. 2b) demonstrate that the antiserum possessed moderate protective ability against the OmpW protein. Arrows indicate the position of the OmpW protein.

**DISCUSSION**

The OM-associated protein OmpW, solubilized in the monomeric form by mild detergent treatment, exhibited a heat-modifiable character, a feature common to several other OMPs of Gram-negative bacteria (Beher et al., 1980). A heat-modifiable character (from 19 kDa to 22-5 kDa) was also observed with the Omp21 protein of *Comamonas acidovorans* (and its homologues), which exhibits considerable sequence homology (about 48 %) with that of OmpW (Baldermann et al., 1998). The shift in the electrophoretic mobility of the native form of OMPs on heating in the presence of bound SDS is attributed to structural transitions from β-sheet to α-helix through an irreversible denaturation (unfolding) process that is influenced by the state of their association with LPS and/or peptidoglycan (Lugtenberg & Alphen, 1983; Nakamura & Mizushima, 1976). Detergent solubility and trypsin sensitivity profiles of OmpW suggest that the protein is not tightly associated with the peptidoglycans, and this may account for the relatively small shift (about 2 to 3 kDa) of its electrophoretic mobility on heating.

Bacterial OMPs are usually characterized by β-barrel structures of 8–20 strands inserted in the membrane (Koebnik et al., 2000). The Omp21 homologues were proposed to be the members of the 8-stranded β-sheet family of proteins (Baldermann et al., 1998). Our spectroscopic data on OmpW predict the predominance of β-structures (Figs 3 and 4, Table 3) that are in agreement with the computational analysis (Fig. 5). The analysis also predicts the presence of α-helical segments with high hydrophobicity indices in the leader peptide region, a feature common to transmembrane signal sequences of many bacterial OMPs (Zhai & Saier, 2002). The significance of the small (8–15 %) amount of helical structures in the mature form of the protein is difficult to predict at this stage. These structures could be associated with the loop portion of the protein that may be present on either side (periplasmic and extracellular) of the OM (Stathopoulos, 1999). Susceptibility, though partial, of the membrane-associated OmpW to trypsin digestion also predicts exposure of parts of the protein that are sensitive to the enzyme.

Liposome-swelling data from this study did not demonstrate porin-like activity in the purified recombinant.
Table 5. Determination of the protective efficacy of anti-OmpW serum against V. cholerae challenge

Anti-OmpW serum was raised by immunizing a rabbit with the purified recombinant protein and the serum was absorbed with whole cells of V. cholerae BN490 (OmpW-deficient mutant).

<table>
<thead>
<tr>
<th>Challenge strain (serogroup/biotype)</th>
<th>Challenge dose* (c.f.u.)</th>
<th>Preincubated with†</th>
<th>Dilution of antiserum</th>
<th>No. of survivors/no. challenged (% protection)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>O395 (O1, Classical)</td>
<td>5 × 10⁷</td>
<td>Normal saline</td>
<td>1:50</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preimmune serum</td>
<td>1:50</td>
<td>1/6 (16-6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-OmpW serum</td>
<td>1:100</td>
<td>4/6 (66-6)</td>
</tr>
<tr>
<td>Co366 (O1, El Tor)</td>
<td>3.3 × 10⁷</td>
<td>Normal saline</td>
<td></td>
<td>0/6 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preimmune serum</td>
<td>1:50</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-OmpW serum</td>
<td>1:100</td>
<td>3/6 (50)</td>
</tr>
<tr>
<td>10325 (O34)</td>
<td>9.5 × 10⁷</td>
<td>Normal saline</td>
<td>1:50</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preimmune serum</td>
<td>1:50</td>
<td>1/6 (16-6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anti-OmpW serum</td>
<td>1:100</td>
<td>4/6 (66-6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3/6 (50-0)</td>
</tr>
</tbody>
</table>

*Each mouse was challenged with about 10 LD₅₀ dose of bacteria in 0.1 ml.
†Bacterial suspensions were incubated at 37°C for 30 min with saline or with preimmune or immune serum of appropriate dilutions before challenge.
‡Six mice were included in each group and protection was expressed as percentage of survivors in each group.

OmpW protein. This may not be surprising since OmpU and OmpT proteins appear to be the predominant porins in V. cholerae (Chakrabarty et al., 1996; Simonet et al., 2003), capable of allowing more than 90% diffusion of the β-lactam antibiotic cephaloridine (Wibbenmeyer et al., 2002). That OmpW is likely to be devoid of porin activity is also supported by the observation that the protein does not exist in the oligomeric or tightly peptidoglycan-associated forms that are usually the characteristics of porins. It is pertinent to mention that the Omp21 of C. acidovorans was also found to be devoid of any porin activity by ion conductance assay (Baldermann & Engelhardt, 2000).

A database search reveals that proteins with sequence homologies to OmpW of V. cholerae are present in a large number of bacteria (Nandi, 2003). For example, the homology is around 59% for E. coli OmpW. Interestingly, an antiserum raised against V. cholerae OmpW failed to recognize the homologous protein in the whole-cell lysate of E. coli. Thus, despite sharing considerable sequence similarity amongst themselves, the OmpW homologues may possess structural features diverse enough to perform host-specific functions (Nandi, 2003). These include their role in survival through stabilization of the OM against cell-surface factors of pilus or non-pilus origin. Our findings that the OmpW protein is immunogenic and expressed by V. cholerae in vitro are indicative of its protective potential.

Bacterial OMPs are known to play an important role in the cells’ adaptive response to environmental conditions (Lin et al., 2002; Nikaido, 1999). Evidently, in vitro expression of OmpW by V. cholerae is also governed by these conditions. Thus, the expression of the protein in AKI medium was enhanced under nutrient-limiting conditions generated either by exhaustion of the available nutrients at the later stage of growth or even at an earlier stage by serial dilution of the medium (Nandi, 2003). Our results also demonstrate modulation of expression of OmpW protein in V. cholerae cells grown under stress conditions such as elevated temperature, high salt concentration and low aeration. Interestingly, the effect was more pronounced in non-O1/non-O139 V. cholerae that are usually non-pathogenic, although ubiquitously present in the environment.

The addition of glucose (as an easily metabolizable carbon source) and some other simple saccharides had a suppressive effect on the expression of OmpW in V. cholerae (Figs 6a and 7). Expression of several other proteins of V. cholerae, e.g. haemagglutinin/protease or Hap (Silva et al., 2003), mannose-sensitive haemagglutinin or Msha (Lang et al., 1994) and OmpS (Lang & Palva, 1993), showed glucose-dependent regulation involving cAMP/CRP. The CAMP/CRP pathway also plays a role in the expression of microagglutination or immobilization of vibrios, thereby interfering with the colonization process that is mediated by cell-surface factors of pilus or non-pilus origin. Our findings that the OmpW protein is immunogenic and expressed by V. cholerae in vivo are indicative of its protective potential.
OmpT (Li et al., 2002) as well as that of CT and TCP (Skorupski & Taylor, 1997), which are under the control of the ToxR regulon. Results obtained so far demonstrate that the glucose-mediated regulation of OmpW becomes less prominent in a toxR mutant of V. cholerae as compared to that seen in the toxR wild-type strain (Fig. 8). This finding is also supported by the recent demonstration that the transcriptional activation of ompW could be influenced by TcpP/H or ToxR/S regulon (Bina et al., 2003). Thus, it is quite possible that regulation of OmpW expression by nutrient and other environmental conditions (temperature, osmolarity) may require the participation of one or more of these regulons, which are known to respond to environmental cues.

The ompW gene is located in the small chromosome (Chr II) of V. cholerae, which contains genes involved in metabolic and regulatory pathways (Heidelberg et al., 2000; Schoolnik & Yildiz, 2000). Several ORFs have been identified in the regions upstream and downstream of ompW with putative functions important for nutrient utilization and other metabolic processes. A proposed role for the small chromosome is its involvement in adaptation to nutritional and other stress conditions in the intestine as well as the extraintestinal milieu (Schoolnik & Yildiz, 2000; Xu et al., 2003). The modulation of OmpW expression may therefore be linked to such adaptive response under stress conditions.

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