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Refolding and functional assembly of the *Vibrio cholerae* porin OmpU recombinantly expressed in the cytoplasm of *Escherichia coli*

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ABSTRACT

OmpU is one of the major outer membrane porins of *Vibrio cholerae*. OmpU has been biochemically characterized previously for its 'porin'-property. However, previous studies have used the OmpU protein extracted from the bacterial outer membrane envelope fractions. Such method of isolation imposes limitations on the availability of the protein reagent, and also enhances the possibility of the OmpU preparation being contaminated with lipid molecules of bacterial outer membrane origin, especially lipopolysaccharides (LPS). Here we report a strategy of purifying the *V. cholerae* OmpU protein recombinantly overexpressed in heterologous protein expression system in *Escherichia coli*, without its being incorporated into the bacterial membrane fraction. In our strategy, the majority of the protein was expressed as insoluble inclusion body in the *E. coli* cytoplasm, the protein was dissolved by denaturation in 8 M urea, refolded, and purified to homogeneity in presence of detergent. Our strategy allowed isolation of the recombinant OmpU protein with significantly enhanced yield as compared to that of the wild type protein extracted from the *V. cholerae* membrane fraction. The recombinant *V. cholerae* OmpU protein generated in our study displayed functional channel-forming property in the synthetic liposome membrane, thus confirming its 'porin'-property. To the best of our knowledge, this is the first report showing an efficient refolding and functional assembly of the *V. cholerae* OmpU porin recombinantly expressed as inclusion body in the cytoplasm of a heterologous host *E. coli*.

Introduction

Porins are one of the major outer membrane proteins of the Gram-negative bacteria. In general, they form non-specific, transmembrane β-barrel channels that allow free diffusion of hydrophilic molecules [1,2]. Although the primary function of the bacterial outer membrane porins is to form diffusion channels in the bacterial outer membrane [3,4], they also act to serve other functions [5–9]. For example, many Gram-negative bacterial porins have been shown to be involved in the survival mechanisms of the bacteria against bactericidal agents, adhesion/colonization process, and modulation of the host immunity during bacterial infections.

OmpU is one of the major outer membrane porins of *Vibrio cholerae* (Fig. 1) [10–14], the causal organism for the disease cholera. In *V. cholerae*, OmpU expression is positively regulated by ToxR, the transmembrane transcriptional activator protein, which also regulates expression of the critical virulence factors including cholera toxin and the toxin-coregulated pilus [15,16]. *V. cholerae* OmpU has been shown to provide bile resistance in *V. cholerae*, as well as resistance of the organism against the antibacterial peptides [17,18]. Studies have also indicated involvement of the OmpU protein in the adhesion process of the bacteria during *V. cholerae* infection [14]. Altogether, these data strongly suggest that the *V. cholerae* porin OmpU could be critically involved in the pathogenesis process of the organism. *V. cholerae* OmpU has been biochemically characterized previously for its 'porin'-property [18,19]. However, this protein has not yet been studied, particularly at the molecular level, in terms of exploring its ability to evoke cellular responses that would have critical implications for the host-pathogen interaction mechanisms.

Previous studies have characterized the 'porin'-property of the OmpU protein extracted from the *V. cholerae* membrane fractions. Such method of protein extraction allows recovery of only limiting amount of protein reagent. Also, it enhances the possibility of the protein reagent being contaminated with bacterial membrane components like lipopolysaccharides[1] (LPS) [19]. In the present report, we describe purification of the recombinant form of *V. cholerae* OmpU protein overexpressed in the cytoplasm of *Escherichia coli*. In our strategy, the nucleotide sequence encoding the OmpU protein,

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[1] *Abbreviations used:* LPS, lipopolysaccharides; IPTG, isopropylthiogalactoside; LDAO, Lauryldimethylamine N-oxide; BHI, Brain Heart Infusion; n-octyl POE, n-Octylpolyoxyethylene; PC, phosphatidylcholine; MTCC, Microbial Type Culture Collection; PDB, Protein Data Bank; CD, circular dichroism

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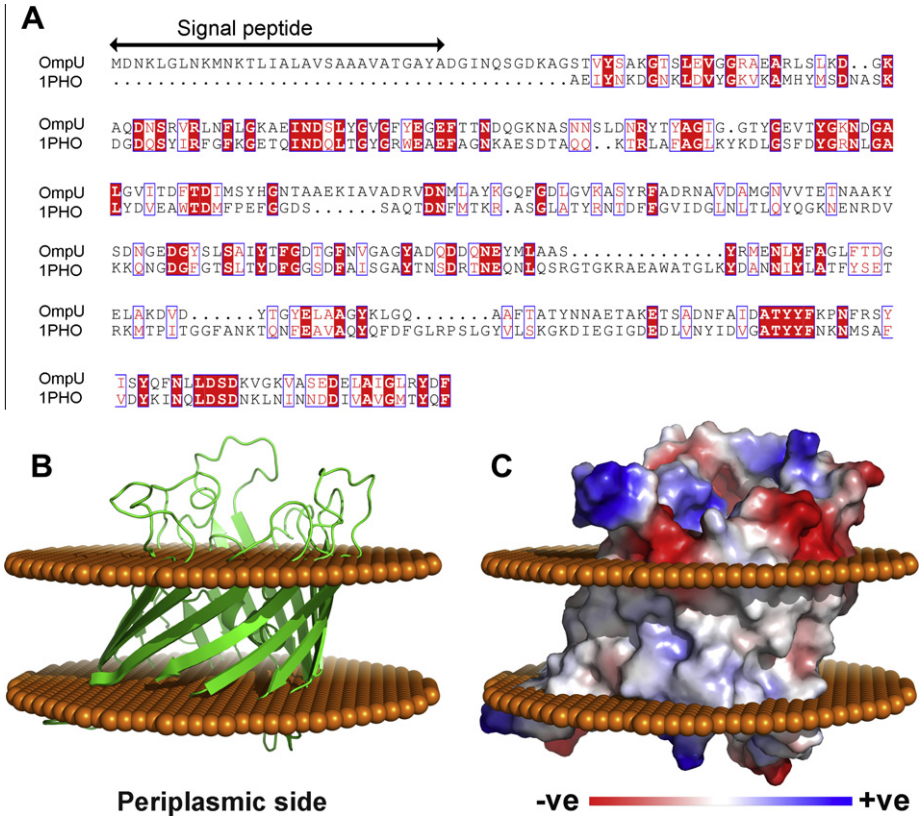

Fig. 1. Homology modeling of *V. cholerae* OmpU showed the archetypical β-barrel architecture of the porin proteins. Polypeptide sequence corresponding to the *V. cholerae* OmpU [20] was subjected to BLAST search in the NCBI server for finding the suitable template with experimentally determined three-dimensional structure. The most suitable template found in the Protein Data Bank (PDB) [22] by BLASTP [23] was the crystal structure of an *E. coli* porin (PDB ID:1PHO). Amino acid sequence alignment of *V. cholerae* OmpU and *E. coli* porin 1PHO was generated using the Biology Workbench server available online at http://workbench.sdsc.edu [24]. The sequence alignment was visualized using ESPript server (http://espript.ibcp.fr/ESPript/ESPript/) [25]. The *V. cholerae* OmpU protein structure was modeled based on the structure of 1PHO with the SWISS-MODEL server (http://www.expasy.org/spdbv/) [26]. Orientation of the *V. cholerae* OmpU structure model in the membrane lipid bilayer was generated in the OPM server found online (http://opm.phar.umich.edu/server.php). Protein structure models were represented using PyMOL [DeLano WL, The PyMOL Molecular Graphics System (2002) found online (http://pymol.org)]. Surface electrostatics on the OmpU structural model were calculated within PyMOL. (A) Amino acid sequence alignment of *V. cholerae* OmpU and *E. coli* porin 1PHO. The Signal peptide region of OmpU is marked. Notably, *V. cholerae* OmpU does not contain any cysteine and tryptophan residue. (B) Cartoon representation of the homology-based structural model of *V. cholerae* porin OmpU. (C) Surface representation showing the distribution of the electrostatic potential on the surface of the *V. cholerae* porin OmpU structural model. Proposed orientation of the structural model in the membrane lipid bilayer is shown in both the representations. Lipid molecules are represented as orange spheres.

lacking the N-terminal signal peptide, was cloned into the overexpression system in *E. coli*. As a result, majority of the protein was expressed as insoluble inclusion body in the *E. coli* cytoplasm. The inclusion body was solubilized in 8 M urea, and subsequently the recombinant OmpU protein was refolded and purified to homogeneity in presence of detergent. The 'porin'-property of the recombinant OmpU protein was confirmed by monitoring its ability to form functional transmembrane channel in the membrane lipid bilayer of the synthetic liposome systems. Altogether, our strategy established an efficient method of producing recombinant *V. cholerae* OmpU porin in its functional form with a significantly improved yield, as compared to that of the wild type OmpU protein isolated from the *V. cholerae* membrane fractions.

Material and methods

Bacterial strains

The *V. cholerae* El Tor O1 strain was obtained from the Microbial Type Culture Collection and Gene bank facility (MTCC Code 3905) of the Institute of Microbial Technology, Chandigarh, India. The *E. coli* TOP10 cells were from Invitrogen, and *E. coli* Origami B cells were from Novagen.

Overexpression, purification, and refolding of the recombinant form of the V. cholerae OmpU protein

The nucleotide sequence encoding the *V. cholerae* OmpU porin protein [20], omitting the region for the N-terminal signal peptide, was PCR amplified with the gene specific primers using the *V. cholerae* genomic DNA as the template. Primers were designed based on the available *V. cholerae* OmpU gene sequence (found online at http://blast.ncbi.nlm.nih.gov). The forward and reverse primers were modified so as to incorporate the *Nde*I and *Bam*H1 restriction endonuclease sites at the 5′- and 3′-ends of the amplified gene products, respectively. The reverse primer also introduced a stop codon at the 3′-end of the OmpU coding sequence, upstream to the *Bam*H1 site.

Forward Primer:
AATGTTcatatgGACGGAATCAACCAAAGCGGTGACA
Reverse Primer:
ATTCAAggatccTTAGAAGTCGTAACGTAGACCGATAGCC

The amplified PCR product was cloned into pCR2.1-TA cloning vector (Invitrogen), and transformed into *E. coli* TOP10 cells (Invitrogen). Transformed cells were screened for the positive plasmid