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Functional characterization of *Pseudomonas fluorescens* OprE and OprQ membrane proteins

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Abstract

Outer membrane (OM) proteins of the OprD family may enable bacteria of the genus *Pseudomonas* to adapt to various environments by modulating OM permeability. The OprE and OprQ porins from *P. fluorescens* strain MF0 were purified and identified by MALDI-TOF mass spectrometry and N-terminal and internal microsequencing. These proteins, when reconstituted in an artificial planar lipid bilayer, induced similar ion channels with low single-conductance values. Secondary structure prediction of both proteins showed similar folding patterns into a 16 transmembrane β-strands barrel but a highly variable amino-acid composition and length for their putative external loops implicated in porin function. Both proteins were overexpressed under poor oxygenation conditions, but not by using several amino acids as sole carbon source, indicating a different specificity for these proteins compared to the paradigm of this protein family, OprD.
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Keywords: *Pseudomonas*; Porins; OprD; OprE; OprQ; Single channel conductance value; Expression

Bacteria from the genus *Pseudomonas* are present in all major natural environments, including water, soil, the plant rhizosphere, and the human body. Some of these environments are subjected to rapid variations, necessitating high levels of physiologic and genetic adaptability in bacteria living in such hostile habitats [1]. This adaptability involves many modifications, some of which affect outer membrane (OM) permeability. This permeability essentially depends on channel-forming proteins, which can be divided into three classes: general porins, specific porins, and highly substrate-specific gated channels translocating their ligands in an energy-dependent manner [2]. OM permeability is modified by changes to porins in two main ways: (1) modulation of porin channel size, as reported for OprF, the major non-specific porin of psychrotrophic and mesophilic *Pseudomonas* species [3], and (2) differential expression of porins with narrow channels to decrease OM permeability [4], which seems to be particularly common for specific porins, such as OprD [5–7].

This porin OprD, which binds basic amino acids, is the paradigm for a 19-member family of OM proteins in *P. aeruginosa* [8]. These proteins, displaying growth condition-regulated production, seem to be specific for different classes of substrates, but may also act as non-specific porins for small substrates [7,9]. Member of this family, OprE, has been reported to be induced in anaerobic conditions in *P. aeruginosa* [10] and to form pores *in vitro* excluding molecules larger in size than di- or trisaccharides [11]. Little is known about OprQ, which may allow saccharide diffusion [12]. In the psychrotrophic *P. fluorescens* strain MF0, OprE, and OprQ were found to be overexpressed in an adaptive OprF-deficient mutant, presumably to compensate for the loss of this major non-specific porin [13]. This species, closely related to *P. aeruginosa*, colonizes many

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cold environments and was recently shown to be a potential opportunistic pathogen in humans, as it can adhere to nerve cells and trigger apoptosis [14,15]. OprE and OprQ have been shown to bind fibronectin, suggesting that they may be involved in virulence [16]. We therefore characterized these two proteins, investigating their structures and functions in *P. fluorescens*.

Materials and methods

Bacterial strains and growth conditions. Pseudomonas fluorescens MF0 was grown in nutrient broth (Difco), or on nutrient broth agar (1.5% w/v) plates, at 28 °C, under normal (200 mL culture in 2 L erlenmeyer) or poor (500 mL in 2 L) oxygenation. Cells were harvested in the late exponential growth phase by centrifugation at 8000*g* for 10 min at 4 °C.

OM protein purification and characterization. OMs were isolated by the spheroplast procedure described in Dé et al. [17]. OprE and OprQ were purified according to Jaouen et al. [3]. The purity of the preparations was checked by SDS–PAGE and silver staining. Purified porins were concentrated by trichloroacetic acid/acetone precipitation and dissolved in 0.25 M Tris–HCl, 6 M guanidine. Samples were reduced by adding 10 mM dithiothreitol and alkylated with 50 mM iodoacetamide, and then were digested overnight at 37 °C with trypsin to a final protease/protein ratio of 1:5. The resulting peptides were pooled by lyophilization. The peptide mixture was dissolved in trifluoroacetic acid/acetonitrile/water (1:20:79, v/v/v), separated by capillary reverse-phase HPLC (Applied Biosystems), and subjected to Edman degradation in an Applied Biosystems 492 automated protein sequencer. For proteins' N-terminal sequences, porins were blotted onto Prosorb (Applied Biosystems) before Edman degradation. Mass spectra were recorded on an Autoflex (Bruker) MALDI time-of-flight mass spectrometer in the positive-ion mode. The acceleration voltage was set to 20 kV. Samples were prepared according to El Hamel et al. [18].

Reconstitution in planar lipid bilayers. Virtually solvent-free planar lipid bilayers were formed by the Montal and Muller technique [19], as described by Jaouen et al. [3]. Macroscopic current and selectivity experiments were carried out as described in Dé et al. [20]. A palmitoyloleoylphosphatidylcholine/dioleoylphosphoethanolamine solution (POPC/DOPE, 7/3, Lipid Products) at 0.5% (w/v) in hexane was used as lipid.

General DNA procedures. Standard methods were used for general DNA procedures [21]. The porin genes were amplified with the following oligonucleotide primers: *oprE*, 5′ (5′-CTGCAGGGACCAAATCAACT-3′), 3′ (5′-GAAGGCCTTGAGAGCTGTA-3′); *oprQ*, 5′ (5′-CGATTCACGAAATTGACACG-3′), 3′ (5′-CAGAACACGTTGATCGGGTA-3′), designed on the basis of a ClustalW alignment of the putative *oprQ* and *oprE* genes from the sequenced *P. fluorescens* PF5, SBW25, and PF0 genomes. Polymerase chain reaction (PCR) was carried out with 10 ng of purified chromosome and 1 U *Taq* DNA polymerase (Roche): after a hot start at 94 °C for 5 min, the DNA was subjected to 30 cycles of denaturation for 1 min at 94 °C, primer annealing for 1 min at 60 °C, and elongation for 2 min at 72 °C, followed by a final 10-min elongation step. Sequences of OprE and OprQ, deposited in the EMBL database under Accession Nos. AJ866545 and AJ866544, respectively, were analyzed with BlastP and ClustalW for identification and multiple protein sequence alignments (http://www.infobiogen.fr).

Total RNAs were extracted using the RNA isolation kit (Roche Diagnostics) as described by the manufacturer. RNA integrity was checked by gel electrophoresis and RNA concentration was determined by OD_{260}. RT-PCR experiments were achieved on 10 ng of total RNAs using the one-step RT-PCR procedure (Roche Diagnostics). Absence of contaminating DNA was checked by making a PCR directly on the RNA extracts. Primers used in RT-PCR experiments were designed using a ClustalW alignment of OprD, OprE, and OprQ, ensuring an amplification of the only searched gene. The following primers: *oprQ*5′, ACAAGAACGGCAAGCAAGAC; *oprQ*3′, CGGAACTGATCACCGTACT; *oprE*5′, GCCGGTGGTGACTACAAGAT; *oprE*3′, AGGCCGCTGTACAGATTGTT; 16*S*5′, GGGGGTAGAATTCCAGGTGT; and 16*S*3′, CGGCAGTCTCCTTTGAGTTC were used in the same conditions as described above.

Results and discussion

Pseudomonas fluorescens MF0 is a psychrotrophic bacterium and potential opportunistic pathogen [15]. As OprE and OprQ have been reported to bind fibronectin [16], we characterized these two proteins, which are candidates for involvement in virulence.

Purification and identification of OprE and OprQ

Pseudomonas fluorescens MF0 was grown at 28 °C in conditions of poor oxygenation to induce overexpression of OprE [10]. Omps were isolated by the spheroplast procedure and analyzed by SDS–PAGE (Fig. 1-1). In these growth conditions, two major Omps are localized in the 45–46 kDa range (A, B in Fig. 1-1) which may include members of the large OprD family in *P. aeruginosa*, such as OprE and OprQ [8]. These two proteins were purified by preparative electrophoresis followed by electroelution in the non-ionic detergent Triton X-100 (Fig. 1-2), and their molecular masses (45,641 Da for A and 46884 Da for B) were determined by MALDI-TOF spectrometry (Fig. 1-3).

The proteins were identified by Edman degradation sequencing of the N-terminus of the entire protein and of internal trypsin digestion peptides. In all cases, homogeneous sequencing results free of contaminating sequences were obtained, confirming the purity of our samples. BlastP searches gave high similarity scores with proteins of the large OprD family. The N-terminal sequence of the 45 kDa (A) protein (NDQDQSKGFIEDSH) was similar to those of OprQ and OprD from *P. aeruginosa* PAO1 (93% and 86%, respectively), *P. fluorescens* strain PF5 (79% and 93%), strain SBW25 (100% and 79%), and strain PF0 (86% and 71%). We then sequenced the N-terminal region of three internal peptides obtained by trypsin digestion (YGDQFPAVPV, LESGFTQGTV, and NAGGIGDGGN), to confirm the identity of this protein. These three peptides were 70–100%, 80–100%, and 50–100% similar to OprQ and only 40–60%, 60–90%, and 30–50% similar to OprD from *P. aeruginosa* PAO1, *P. fluorescens* strains PF5, SBW25, and PF0, respectively. The same procedure was applied to the 46 kDa protein. The N-terminal sequences of the entire 46 kDa protein (AGFVEDSKATLGLR) and of the three internal peptides (LPVIITNDGR, DLTLVGGQIEK, and VGVPGLTAPV) were clearly similar to those of the OprE protein (93%, 80%, 91%, and 80% similarity) of *P. aeruginosa*, *P. fluorescens* strains PF5 (100%, 80%, 82%, and 90%), SBW25 (100%, 100%, 91%, and 90%), and PF0 (100%, 100%, 91%, and 90%). Thus, the 45 kDa (A) protein was clearly identified as the homolog of OprQ, and the 46 kDa (B) protein as the homolog of OprE in *P. fluorescens* MF0.

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