Evidence for the location of OprM in the *Pseudomonas aeruginosa* outer membrane

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**Abstract:** OprM with a *M*ₐ of 49 K is associated with the multidrug resistance of *Pseudomonas aeruginosa*. Detergent fractionation of bacterial cells has demonstrated that OprM is located in the outer membrane from which it sediments with the other major outer membrane proteins. In this study we have determined the location of OprM as the *P. aeruginosa* outer membrane. Western immunoblots of cell fractions, obtained by sucrose density gradient centrifugation of whole cell lysates, were probed with an OprM-specific murine polyclonal antiserum.

**Key words:** OprM; *Pseudomonas aeruginosa*

**Introduction**

*Pseudomonas aeruginosa*, a causative agent of opportunistic infections, exhibits intrinsically high resistance to various antimicrobial agents. The investigation of the mechanisms of antibiotic resistance in this bacterium might be crucial for overcoming the problems posed by this resistance in bacterial infections. Wild-type strains of *P. aeruginosa* normally express OprM (*M*₁, 49 K); however, in NalB-type multidrug-resistant strains the level of OprM is greatly increased [1,2]. Comparison of characteristics of the wild-type strains and the NalB-type mutants indicated that the nalB mutation induces alterations in outer membrane permeability, resulting in decreased accumulation of antibiotics, which led to the expectation that overproduction of OprM would be associated with multidrug resistance in the nalB mutants [1]. However, no direct evidence for this association has been provided. More recently, we demonstrated from the characterization of OprM-deficient mutants that OprM plays an important role in the expression of both intrinsic and acquired resistance in *P. aeruginosa* [2]. Sedimentation of OprM, with other major outer membrane proteins such as OprF [3,4], after solubilization of total membrane proteins with sodium *N*-lauroyl sarcosinate, identified OprM as a protein component of the outer membrane [1]. However, there is little further information confirming the location within the *P. aeruginosa* outer membrane.
Materials and Methods

The wild-type strain, PAO1, and the OprM-overexpressed nalB mutant, PAO6006 [5], of *P. aeruginosa* were used in this study. The cells were grown aerobically until the late-exponential phase at 37°C in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl), and were collected by centrifugation. Cells were then fractionated by twostep sucrose density gradient centrifugation, using the following sucrose solutions prepared in Tris·HCl as described by Hancock and Nikaido [6]: 70% (w/v) sucrose and 15% (w/v) sucrose solutions for the first gradient, and 70% (w/v), 64% (w/v), 58% (w/v) and 52% (w/v) sucrose solutions for the second gradient. Centrifugation was performed at 145,000 × g for 12 h and 20 h for the first and the second gradient, respectively. After the first centrifugation, the top layer (cytoplasmic contents) and the middle layer (membranes) were recovered. The membranes were further fractionated in the second gradient centrifugation step and the fractionated membranes were collected from the bottom of the centrifuge tube. The collected fractions were characterized by determinations of succinate dehydrogenase activity [7], protein content [8], and by sodium dodecylsulfate-polyacrylamide gel electrophoresis [9] followed by Western immunoblot assay. In one blot, we used a murine monoclonal IgG₁ antibody, F001, specific to *P. aeruginosa* OprF (Mr, 37 K), as the first antibody. This monoclonal antibody was generated by cell fusion between the myeloma cell line SP2/O-Agl4 and splenocytes from Balb/C female mice immunized with OprF, which was chromatographically purified from strain PAO1 according to the method described by Yoshimura and Nikaido [10]. Specificity of this antibody was established by the following immunoblots: visualization of a 37-kDa protein band corresponding to OprF in the sarcosinate-insoluble protein preparation from *P. aeruginosa* PAO1 and loss of the band in the preparation from an OprF-deficient PAO strain, KG1079 [11,12] (Fig. 1). In another blot, we probed for OprM by using an OprM-specific murine polyclonal antiserum [2] as the first antibody. The immunization, cell fusion, immunoblot assay and other immunological techniques were performed according to the laboratory method described in the manual of Harlow and Lane [13].

Results and Discussion

From the second sucrose gradient centrifugation of membranes obtained from whole cell lysates of strain PAO6006, three fractions were revealed; two translucent white (peaks A and B in Fig. 2) and one deep red (peak C in Fig. 2). Of the three fraction bands, A and B were identified as outer membrane fractions, and C as an inner membrane fractions, as determined by the activity of the inner membrane marker enzyme, succinate dehydrogenase (Fig. 2) and by the protein profiles (Fig. 3A). Furthermore, identification of peaks A and B as outer membrane fractions was supported by the visualization (Fig. 3C) of high signal intensities at the position of OprF (Mr, 36 K) in preparations of fractions A and B subjected to immunoblot assay using the murine monoclonal antibody, F001 (Fig. 1); specifying to OprF which is known to exist in the outer membrane

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**Fig. 1.** Specificity of murine monoclonal antibody, F001. Outer membrane proteins released from *P. aeruginosa* PAO1 cells by brief sonication were fractionated by SDS-PAGE (A) and analyzed by Western immunoblot assay (B). Lane 1, PAO1; lane 2, KG1079. Mobilities of standard proteins and the calculated molecular masses of relevant proteins are shown at the left and the right of the gels, respectively.
Fractionation of total membranes of *P. aeruginosa* PAO6006 by the second sucrose density gradient centrifugation. (●) protein concentration; (○) succinate dehydrogenase activity.

Detection of OprM by Western immunoblot assay using the OprM-specific murine polyclonal antiserum showed that the protein was distributed in fractions A and B, but not in fraction C (Fig. 3B). Moreover, immunoblot assays using these antibodies with the cytoplasmic contents did not exhibit any signal (data not shown). These results confirm the location of OprM within the *P. aeruginosa* outer membrane.

Several investigators [1,14,15] have previously concluded that the *nalB* mutation endows quinolone and β-lactam resistance resulting from a decreased permeability of the outer membrane which prevents the accumulation of the agents in resistant *P. aeruginosa* cells. We have shown, through the characterization of OprM-deficient mutants, that the expression of OprM is associated with both acquired and intrinsic antibiotic resistance in *P. aeruginosa* [2]. However, the precise mechanism of action is, as yet, unknown. We believe that the confirmation of the location of OprM in the outer membrane is an important first step towards the investigation of this mechanism.

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