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Pseudomonas aeruginosa outer membrane protein F is an adhesin in bacterial binding to lung epithelial cells in culture

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Adherence to host cells is a crucial step by which bacteria initiate an infection but the bacterial determinants of the process are, as yet, poorly understood. In an effort to identify bacterial adhesins responsible for *Pseudomonas aeruginosa* binding to host cells, we identified porin F (OprF) from the outer membrane of *P. aeruginosa* as adhesin for human alveolar epithelial (A549) cells. Bacterial adhesion assays with ^{35}S-labeled wild type *P. aeruginosa* and its isogenic mutant strain lacking OprF showed that the mutant strain binds 43% less than the wild type to A549 cells ($P < 0.01$). In addition, bacterial binding is significantly reduced ($P < 0.01$) when either A549 cells were pretreated with purified OprF or if bacteria were pre-incubated with a monoclonal antibody to OprF. Finally, ligand binding experiments in which purified OprF protein was added to A549 monolayers showed saturable binding. These data indicate that OprF contributes to bacterial adherence to A549 epithelial cells and could facilitate *Pseudomonas* interactions with the epithelium, including colonization of the airway epithelium or the initiation of pulmonary infection.

Key words: *Pseudomonas* Porin F, bacterial adherence, Lung epithelial cells.

Introduction

Bacterial adherence to host cells is an essential first step by which most pathogens initiate infectious processes [1, 2]. While bacterial factors and host cell receptors that facilitate such interactions are poorly understood, it appears that the factors may vary depending on bacterial strain and cell type. Cell surface appendages and alginate of *Pseudomonas aeruginosa* function as adhesins in bacterial interactions with host cell in culture and, asialylated glycosphingolipids are identified as receptors for both pili and flagella [3–6]. Other investigators have shown

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the existence of several classes of *P. aeruginosa* adhesins that may be involved in bacterial attachment to tracheobronchial mucin and cell surfaces [7, 8]. Outer membrane protein adhesins are also reported in some bacteria, including *E. coli* intimin, *Helicobacter* AlpA and Hope, *Bartonella* Omp43 [9–12]. In *Pseudomonas* a variety of outer membrane adhesins have been described, including fibronectin-binding proteins of 70, 60, 48, and 36 kDa, nitrogen regulated proteins of 75, 62, 89, 38, 28, 18, and 12 kDa, and mucous-binding proteins of 48, 46, 28 and 25 kDa [13–15]. In addition, a root adhesin from the outer membrane of *P. fluorescens* displays a strong homology with *P. aeruginosa* OprF [16]. In this communication, we provide evidence that *P. aeruginosa* OprF is involved in bacterial adherence to epithelial cells derived from the lung.

The outer membrane of *P. aeruginosa* contains a number of membrane-spanning β-barrel proteins called porin [17–19]. These proteins contribute to membrane barrier integrity and can influence passage of substrates and consequently intrinsic resistance to antimicrobial agents [20, 21]. Due to their conserved structure among *P. aeruginosa* serogroups and their antigenic capacity, outer membrane proteins have potential use in the development of vaccines designed to prevent pseudomonal infections [22–25]. We provide new evidence that confirms a role for a specific *P. aeruginosa* outer membrane protein; OprF, in host cell-bacterial interactions.

Results

Adherence of *P. aeruginosa* OprF-deficient mutant strain to A549 cells

Adherence of a wild type *P. aeruginosa* and its isogenic mutant lacking the outer membrane porin F was carried out using ^{35}S-labeled bacteria and A549 cells grown on 24-well tissue culture plates. As shown in Table 1, the wild type *P. aeruginosa* (H103) was more adherent to the target cells than the mutant strain H636. The difference in adhesion between the two strains was statistically significant, demonstrating that OprF contributes to bacterial adherence to A549 cells.

Purified OprF and mAb to OprF reduce bacterial adherence to A549 cells in culture

Pretreatment of the cells with exogenous OprF prior to exposure to ^{35}S-labeled *P. aeruginosa* reduced bacterial binding to the monolayers of A549 cells significantly. OprF reduced bacterial adherence to A549 cells in a concentration dependent fashion, as shown in Figure 1. Pretreatment of bacterial culture with mAb to OprF likewise interfered with bacterial attachment to the cell monolayers in culture.

Table 1. Adherence of wild type *P. aeruginosa* and its isogenic OprF mutant to lung epithelial cells in culture

P. aeruginosa strain	% Adherence[a]	
	Primary type II pneumocytes	A549 cells
Wild Type (H103)	13.6 ± 1	18.5 ± 5
OprF – mutant (H636)	6.5 ± 2[b]	9.5 ± 1.5[b]

[a] Mean ± SD for triplicate wells in 3 independent assays.
[b] Significant ($P < 0.01$).

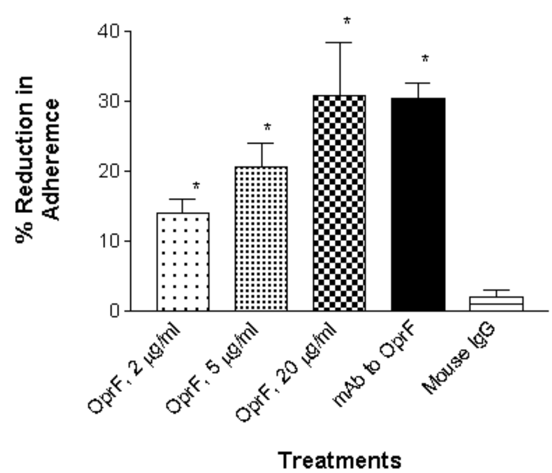

Figure 1. Competition of purified OprF with binding of ^{35}S-labeled *P. aeruginosa* to A549 cells. Pretreatment of A549 cells with purified OprF reduced bacterial adherence to the cells grown in 24 well plates. Likewise, pretreatment of bacteria with monoclonal antibody to OprF reduced bacterial adhesion. Reduction in adherence by non-immune mouse IgG was not statistically significant. Values in each case represent means ± SD of triplicate observations in at least 3 independent experiments. Asterisks indicate difference from control ($P < 0.05$).

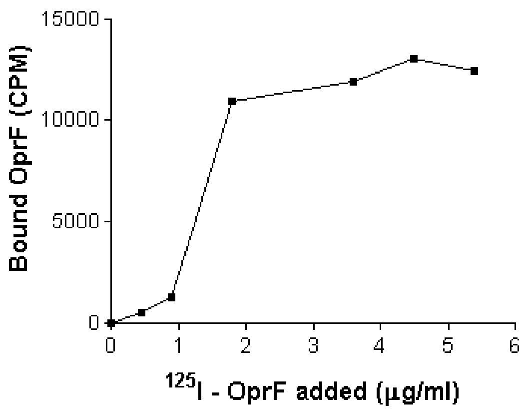

Figure 2. Binding of purified OprF to A549 cells grown in 96 well culture plates. Iodinated OprF bound to the cells in a concentration related manner and achieved a plateau at a concentration of 2.6 µM.

Non-immune mouse IgG, however, did not alter bacterial adherence to the cells. These data demonstrate that the interaction of OprF with binding sites on the A549 cell surface exhibits specificity.

Binding of ^{125}I-OprF protein to A549 cells

Iodinated OprF protein was next used to study the binding properties of OprF to cell surface receptor(s) on the A549 epithelial cells in RPMI medium containing 0.5% BSA. We found that OprF binds to A549 cells in a concentration-dependent manner and that the binding begins to plateau at a concentration of 2.6 µM OprF (Fig. 2). OprF in a concentration of greater than 2.6 µM (100 µg/ml) caused cell detachment, thereby hindering competition assays with excess amounts of unlabeled OprF. Because a relatively high concentration of OprF appears to influence cellular adhesion to plastic matrices, we were unable to construct a specific binding curve in order to calculate ligand-receptor binding parameters.

Discussion

Adherence to host cell surfaces is an important determinant of pathophysiologic bacterial functions. This includes bacterial colonization and cellular invasion that lead to onset of infection and microbial dissemination to distant organs [26, 27]. Bacterial adhesion is a multistep process that requires cellular factors and bacterial components such as pili, flagella, and exoproducts [1, 2]. The cellular components integral to this process are poorly understood at this time. Since proteins in the outer envelope of other bacteria mediate microbial attachment to host cells [28, 29], we reasoned that *P. aeruginosa* outer membrane proteins might likewise be involved in adherence of this organism to lung epithelial cells. The present data demonstrate, for the first time, that OprF, an outer membrane protein of *P. aeruginosa* contributes to bacterial adherence to lung epithelial cells. Pseudomonal OprF thus appears to function as an adhesin similar to the major outer membrane protein of *Legionella pneumophila* or cell wall components of *Streptococcus pneumonia*, as previously reported [28, 29].

Binding of radiolabeled OprF to A549 cell monolayers shows clearly that this protein binds to the cell surfaces in a concentration-dependent manner. Although we were not able to perform a direct competition assay with excess amount of unlabeled OprF, a consistent saturable binding pattern where an average of greater than 15% of the added protein bound to the cells was always observed. In addition, the functional assay data reported here confirm a competition phenomenon between OprF and adhering bacteria to the target cells.

Other reports also indicate that porin proteins from *P. aeruginosa* and other microorganisms could be toxic and cause cell injury and apoptosis [30]. Non-toxic concentrations of porins, however, cause production and release of proinflammatory cytokines by a variety of cells including endothelium, epithelia, and leukocytes [17]. While we can not be certain of the mechanism by which high concentrations of OprF effects cellular dissociation from a plastic matrix, we speculate that the known effects of OprF on cellular toxicity and signaling are likely responsible for the observed effects. We must emphasize that the endotoxin contamination of the OprF was less than 10 pg/ml as assayed by *Limulus* amebocyte tests, so the antiadhesive effect was not likely attributable to endotoxin. Our data, however, clearly implicate OprF in *Pseudomonas*-epithelial cell adhesion and suggest that this adhesin may carry broader functionality that could influence receptor-mediated alterations in epithelial cell interactions with their microenvironment.

To confirm our finding that OprF functions as an adhesin in bacterial binding to A549 cells, we used a bacterial adhesion assay that measures binding of radiolabeled bacteria to cell monolayers in tissues culture plates. Mutant bacterial strains lacking OprF protein adhered less to the cells than the wild type strain indicating that OprF contributes, in part, to bacterial adherence. Furthermore, purified OprF and a monoclonal antibody to an extracellular domain of OprF [31] reduced binding of the wild type *P. aeruginosa* to A549 cell monolayers. Similar findings have been reported with other porin proteins from *L. pneumophila* and cell wall components of *S. pneumonias* [28, 29]. The fact that OprF alone does not completely block bacterial adhesion suggests that other proteins, perhaps including bacterial components such as flagella and pili, participate in bacterial binding as previously reported by other investigators [6, 32]. We are in the process of determining the relative contribution of each of these factors to bacterial adherence using appropriate mutant strains lacking two or more of these elements.

In conclusion, our findings demonstrate that OprF contributes to bacterial adherence to lung epithelial cells in culture. Consistent with this, an OprF homologue has been reported to be an adhesin in *P. fluorescens* for attachment to plant roots [16]. Our findings demonstrate a novel mechanism that promotes binding of *P. aeruginosa* to the epithelium and suggest the potential importance of these interactions to colonization or initial stages of infections of the airway epithelium with *P. aeruginosa*.

Materials and Methods

Cell culture and bacterial strains

We utilized established lines of human lung epithelial cells (A549) from ATCC (Rockville, MD, U.S.A.) as well as our own primary cultures of rabbit lung type II epithelial cells in these studies. Alveolar type II pneumocytes were isolated from pathogen-free rabbits according to our published method [33] and seeded on 24-well culture plates in a serum-free medium (LHC-9; Biofluids, Rockville, MD, U.S.A.) until use. A549 cells were routinely cultured on T-75 tissue culture flasks (Costar, Cambridge, MA, U.S.A.) in RMPI-1640 supplemented with 2 mM L-glutamine, 10% fetal calf serum, and 1% antibiotic mixture (penicillin, 100 U/ml; streptomycin, 100 U/ml; and fungizone, 250 ng/ml) from Sigma. For selected experiments, A549 cells were grown to confluence on 24-well tissue culture plates. All cells were maintained in a humidified incubator at 37°C in 5% CO_2.

We used a wild type *P. aeruginosa* (H103) and its isogenic protein F deficient mutant strains (H636) for comparison. The mutant strain H603 was constructed by Ω-cartridge insertion mutagenesis from the wild type H103 strain [34]. Cultures were grown to late log phase in LB broth with high or low salt content (Gibco RBL, Rockville, MD, U.S.A.) at 37°C.

Isolation and purification of OprF

Pseudomonas aeruginosa PAO1 were grown to an OD_{600} of 0.8, harvested, and resuspended in 20 ml cold 20% sucrose in 10 mM Tris pH 8.0 (ICN Biomedicals, Inc. Aurora, OH, U.S.A.), and 50 μg/ml deoxyribonuclease 1 (Amersham Pharmacia biotech, Baie d'Urfe, Quebec, Canada). The bacterial suspension was incubated at room temperature for 15 min prior to breaking the cells with a French press at 15,000 psi. The outer membrane was prepared by a 2-step sucrose gradient of 50 and 60% sucrose. The outer membrane was collected and resuspended in Tris buffer containing 3.0% Octyl-POE (BACHEM, King of Prussia, PA, U.S.A.). The mixture was incubated for 1 h at 37°C prior to spinning at 41,000 rpm for 1 h. We saved the supernatant while resuspended the pellet in Tris buffer with 3.0% Octyl-POE and 10 mM EDTA disodium salt pH 8.0 (Fisher Scientific, Vancouver, B.C. Canada and processed as outlined above. The supernatant was collected and resolved by a 12% SDS-PAGE (BioRad Laboratories, Mississauga, ON, Canada) to confirm the presence of OprF. The supernatant was then dialyzed in 0.6% Octyl-POE, 10 mM Tris pH 8.0 and 10 mM EDTA disodium salt pH 8.0. The supernatant was run on a Pharmacia FPLC LCC 500 system (Amersham Pharmacia Biotech, Baie d'Urfe, Quebec, Canada) with MonoQ column. Proteins were eluted with a NaCl gradient with OprF eluting at 28% NaCl. The purity of the final preparation was confirmed by SDS-PAGE analysis of the fractions.

We used monoclonal antibodies specific to surface epitopes of OprF as described earlier [35].

Bacterial adherence to epithelial cells monolayers in culture

We studied the adherence of wild type and different phenotypic mutants of *P. aeruginosa* to monolayers of epithelial cells on a 24-well culture plates according to our previously published method [36]. For competition assays, either A549 cells were pretreated with purified OprF or bacteria were pre-incubated with a monoclonal antibody to OprF prior to adhesion assay. Cultures of *P. aeruginosa* maintained for 18 hr in LB broth containing ^{35}S-methionine (10 μCi/ml) were microfuged at 10,000 rpm for 2 min and rinsed 3 times in order to remove unbound label. The bacterial concentration was measured by a spectrophotometer at OD_{600} and necessary dilutions were made to obtain an OD_{600} reading of 0.08. This dilution strategy results in about 2–3 $\times 10^7$ bacteria/ml as determined by colony counting on an agar plate. Binding of *P. aeruginosa* to lung epithelial cells was carried out on a 24-well culture plate in an atmosphere of 5% CO_2 and humidified air for 2 h. Nonadherent bacteria were removed and the cells were washed 3 times before they were lysed with a mixture of SDS (2%) and NaOH (0.1%). Samples were diluted in 5 ml Ecolite (ICN) and radioactivity was then measured in a radiation counter.

Binding of purified OprF to epithelial cells in culture

We radio-iodinated the OprF protein with IODO-BEADS (Pierce, Rockford, IL, U.S.A.) according to our previously published method [26]. For binding assays, varying concentrations of ^{125}I-OprF were added to confluent cell monolayers of A549 cells grown on 96-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ, U.S.A.). Binding was carried out for 90 min at 37°C. Cells were then washed 3 times to remove unbound proteins before adding lysis buffer. The samples were then removed and the radioactivity of the bound protein was measured in a Gamma counter (Micromedia systems, Horsham, PA, U.S.A.).

Statistical analyses

Bacterial adhesion data were analyzed using ANOVA followed by an unpaired student's *t*-test. A P value < 0.05 was accepted to indicate statistical significance.

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