Multidrug Efflux Systems Play an Important Role in the Invasiveness of Pseudomonas aeruginosa

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Abstract

Pseudomonas aeruginosa is an important opportunistic human pathogen. Certain strains can transmigrate across epithelial cells, and their invasive phenotype is correlated with capacity to cause invasive human disease and fatal sepsisemia in mice. Four multidrug efflux systems have been described in P. aeruginosa, however, their contribution to virulence is unclear. To clarify the role of efflux systems in invasiveness, P. aeruginosa PAO1 wild-type (WT) and its efflux mutants were evaluated in a Madin-Darby canine kidney (MDCK) epithelial cell monolayer system and in a murine model of endogenous septicemia. All efflux mutants except a mexCD-oprJ deletion demonstrated significantly reduced invasiveness compared with WT. In particular, a mexAB-oprM deletion strain was compromised in its capacity to invade or transmigrate across MDCK cells, and could not kill mice, in contrast to WT which was highly invasive (P < 0.0006) and caused fatal infection (P < 0.0001). The other mutants, including mexB and mexXY mutants, were intermediate between WT and the mexAB-oprM mutant in invasiveness and murine virulence. Invasiveness was restored to the mexAB-oprM mutant by complementation with mexAB-oprM or by addition of culture supernatant from MDCK cells infected with WT. We conclude that the P. aeruginosa MexAB-OprM efflux system exports virulence determinants that contribute to bacterial virulence.

Key words: Pseudomonas aeruginosa • bacterial invasion • multidrug efflux system • outer membrane protein • endogenous bacteremia

Introduction

Pseudomonas aeruginosa is a versatile Gram-negative bacterium that is an important opportunistic human pathogen. Although this organism does not cause bloodstream infections in patients with chronic respiratory infections despite long-term colonization, it can be invasive in other patients and cause bacteremia associated with high mortality rates, particularly in those with neutropenia. We have reported that most clinical blood isolates cause lethal endogenous bacteremia in leukopenic mice, whereas human respiratory isolates do not cause bacteremia (1, 2). We have also reported that clinical blood isolates of P. aeruginosa penetrate human intestinal Caco-2 and Madin-Darby canine kidney (MDCK)* epithelial cell monolayers to a greater extent than do respiratory isolates (3, 4). In the MDCK epithelial cell monolayer system, PAO1 and most blood isolates are invasive but lack exoU, which encodes a 70-kD cytotoxic protein ExoU (5, 6), and showed high penetration ability without severe epithelial damage. In contrast, PA103 and

*Abbreviations used in this paper: ANOVA, analysis of variance; LB, Luria-Bertani; MDCK, Madin-Darby canine kidney; MDR, multidrug resistant; OM, outer membrane; RND, resistance-nodulation-division; TER, transmonolayer electrical resistance; WT, wild-type.
Invasiveness of Pseudomonas aeruginosa and Efflux Systems

Materials and Methods

Bacterial Strains and Construction of Mutants. Bacterial strains and plasmids used in this study are summarized in the Table I. P. aeruginosa PAO1 strain K767 (35) was used as the WT, which is invasive and penetrates MDCK cell monolayers by 3 h after infection (4). Strain K767 lacks exoU and did not show any cytotoxicity to MDCK cells within 6 h after infection. To construct P. aeruginosa deletion mutants, the deletions were first made in sab-containing vectors and were subsequently introduced into the chromosome of K767 by replacement (36). The mexAB-oprM deletion strain K1119 (37) and the mexCD-opf deletion strain K1521 (29) were constructed using the mexAB-oprM deletion construct pRS21 and the mexCD-opf deletion construct pRS205, respectively, using the strategy reported previously (38). In these instances, however, the selections of transconjugants carrying a copy of pRS21 or pRS205 were on Luria-Bertani (LB) agar containing 1.5 mg of kanamycin per ml. The mexXY deletion strain K1525 was constructed as described previously (29). To construct the mexB deletion strain K1523, a 4.6-kb KpnI-HindIII fragment from pRS19 (39), containing mexA and part of mexB, was first cloned into pEX18Tc to generate pRS27. Plasmid pRS27 was digested with BamHI and MluI at two unique sites within mexB, 1.8-kb apart, and the 9.1-kb fragment containing the vector was isolated. This fragment was treated with Klenow (New England Biolabs) to facilitate backfilling of the 3' recessed ends and ligated to yield plasmid pRS28. The internal deletion in mexB created in this manner was confirmed to be in-frame by sequencing pRS28 with primer bacmexb1 (5'-ATG TCG AAG TTT TTC ATT GAT AGG-3'). Plasmid pRS28 was mobilized into P. aeruginosa strain K767 and a chromosomal mexB deletion selected using the protocol described previously for the mexXY deletion (29).

The chromosomal deletion of mexB was confirmed by PCR using primers rpsoligo3 (5'-ATG TCG AAG TTT TTC ATT GAT AGG-3') and rpsoligo4 (5'-CTG TCG TTT TCG ATC AGG-3'). The mexB strain K1536 was obtained after plating strain K767 on LB plates containing 0.4 μg ciprofloxacin (Sigma-Aldrich) per ml. Those overexpressing MexCD-OprJ were confirmed by immunoblotting of cell envelopes with antibodies specific to Opet, as described previously (38). The nslB strain OCR1 has been described previously (35). Plasmids pRK415 (40) and its derivative pRS17 (39), which carries mexAB-oprM, were maintained in strain K1119 (ΔmexAB-oprM) by inclusion of tetracycline (10 μg/ml) in the growth media. To construct a oprM knockout mutant derived from K767 (WT), PCR primers for amplification of the 1.5-kb fragment were syn-

indicating that the four characterized efflux systems described above did not play a significant role in the resistance of P. aeruginosa biofilm to antibiotics (29). However, the role of efflux systems in specific clinical pathogenesis of P. aeruginosa, including invasiveness, has not been determined.

To gain a better understanding of the role of efflux in bacterial pathogenesis, the invasiveness of P. aeruginosa PAO1 and its efflux mutants was evaluated using in vitro MDCK epithelial cell monolayer penetration (4, 30, 31) and gentamicin survival (31, 32) assays, and by electron microscopy. Virulence was also investigated in a murine model of endogenous P. aeruginosa bacteremia, closely mimicking the pathophysiology of septicemia in humans (2, 33, 34).
Table I. Bacterial Strains and Plasmids Used in this Study

| Strain or plasmid | Relevant genotype or phenotype | Source or reference |
|-------------------|--------------------------------|--------------------|
| P. aeruginosa strains |                                 |                    |
| K767              | WT PAO1                        | 35                 |
| K1119             | K767 ΔmelAB-oprM; MexAB-OprM deletion strain | 37                 |
| K1521             | K767 ΔmelCD-oprE; MexCD-OprE deletion strain | 29                 |
| K1523             | K767 ΔmelB; MexB deletion strain | this study         |
| K1525             | K767 ΔmelXY; MexXY deletion strain | 29                 |
| OCR1              | K767 nalB; MexAB-OprM overproducing strain | 35                 |
| K1536             | K767 nfxB; MexCD-OprF overproducing strain | this study         |
| KG4521            | K767 oprM Sm; oprM knockout mutant | this study         |
| Plasmids          |                                 |                    |
| pRK415            | Broad-host-range cloning vector, tetracycline-resistant | 40                 |
| pRSP17            | pRK415:melAB-oprM; melAB-oprM expression plasmid | 39                 |
| pKMM128           | oprM expression plasmid, carbenicillin-resistant | 45                 |

thesized based on nucleotide sequences of the Pseudomonas genome sequencing project database. After amplifying a 1.5-kb region including oprM gene (11) on strain K767 (WT) genomic DNA as a template using #oprM4 (5'-TCATAAGCTTAT-GAAAAACGGTAAGCTTATCC-3') and #oprM3 (5'-TTGAGAATTCAGCTTATCC-3'), a primer pair containing a newly added cutting site (underlined) for restriction nucleases, the region was ligated into the HindIII-EcoRI site in a multicloning site of pMT5059 (41) to yield pMT5059M. The Sall fragment encompassing ΩSm gene from pS1918 (42) was ligated into the Sall site in the oprM gene on pMT5059M to yield pMT5059MSM. Then, the Mob cassette from pMT5071 (43) was cloned into the Nol site, on pMT5059 (41) to yield pMK1. The resulting plasmid was mobilized from the E. coli strain S17–1 to K767 (WT) to introduce the streptomycin-resistant determinant into the oprM gene on the recipient chromosomes to yield KG4521. Disruption of the oprM gene of KG4521 was confirmed by PCR and Western immunoblot analysis using the anti-OprM antibody (44). Plasmid pKMM128 (45) carrying oprM was maintained in KG4521 (ΔoprM) in the presence of 10 μg/ml of carbenicillin. All mutants derived from K767 were motile, resistant to 10% pooled fresh normal human serum, noncytotoxic, and grew in LB broth and MEM as well as their parent strain K767.

**MDCK Cell Monolayer Penetration Assay.** The assay was performed as reported previously (4, 30, 31). In brief, strain 1 MDCK cells in MEM with 10% FBS were seeded at 1.5 × 10⁶ cells per well in Transwell filter units (Costar) containing 0.33-cm² porous filter membranes (3.0-μm pores). Monolayers were incubated at 37°C in 5% CO₂ for 4 d until the transmonolayer electrical resistance (TER) reached the proper range (900–1,200 Ω cm²), as measured with a Millicell-ESR apparatus (Millipore). Monolayers were infected with bacteria by adding 5 μl (3.5 × 10⁶ CFU) freshly grown bacteria cultured in LB broth overnight at 37°C with shaking at 150 rpm. In some experiments, TER was monitored at several time intervals after infection to assess damage to monolayers. The assay was performed in triplicate, and results are expressed as an average ± SD. Each assay was repeated at least three times to confirm the reproducibility.

**Bacterial Association and Invasion Assay.** Gentamicin survival assays were performed to quantify the bacteria which invaded MDCK cells (31, 32). After a 3-h infection, monolayers were washed six times with PBS and then incubated a further 2 h in fresh medium containing 200 μg/ml of gentamicin to kill extracellular bacteria only. Monolayers were washed three times with PBS and then lysed with a 1% Triton X-100 (Sigma-Aldrich) for 15 min. Appropriate dilutions were spread onto agar plates, incubated at 37°C overnight, and CFUs counted to quantify bacteria surviving intracellularly. Associated bacteria (including both adherent and invading bacteria) were measured by the addition of a 1% Triton X-100 to the monolayers 3 h after infection followed by six washes without gentamicin treatment. As a control, filters units with medium only and without MDCK cells were inoculated with bacteria to determine baseline adherence to plastic. The baseline values were subtracted from those obtained by incubation with epithelial cells as described above.

**Electron Microscopy.** For transmission electron microscopy (TEM), monolayers infected with bacteria were washed seven times with PBS at 37°C and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate containing 2 mM CaCl₂ (cacodylate buffer) for 60 min. After washing with cacodylate buffer, samples were postfixed in cold 1% OsO₄ in the same buffer for 30 min, and then stained with cold 2% uranyl acetate for 30 min. Samples were dehydrated in a series of ethanol and embedded in Epon 812 resin, and were sectioned and stained with uranyl acetate and lead citrate before examination in a JEOL JEM-1210 transmission electron microscope. For scanning electron microscopy (SEM), the monolayers were fixed as described above, dehydrated in a critical point apparatus, and were examined with a JEOL JSM-35C/LaB₃-III–A scanning electron microscope, after a gold sputter coating.

**Evaluation of Virulence of the Efflux Mutants in a Marine Model of Endogenous Bacteria.** The animal studies were approved by the Animal Care and Use Committee of Nagasaki University, and were conducted in accordance to the Guidelines for Animal Ex-
performation, Nagasaki University. Specific-pathogen-free male BALB/c mice (Japan S.L.C. Co., Ltd.) weighing 20–24 g were used in these experiments. Fecal specimens were cultured before the study to ensure the absence of *P. aeruginosa*. Endogenous bacteremia was induced as described previously (2, 33, 34). In brief, a suspension of 10⁷ CFU/ml *P. aeruginosa* in sterile 0.45% saline was given in the drinking water between days 1 and 4. The normal intestinal flora of the mice was disturbed by administration of sodium ampicillin (Meiji Seika Kaisha, Ltd.) by intraperitoneal injection on days 5, 7, and 9. These doses of cyclophosphamide induce leukopenia (<1,000/mm³) in mice from days 8 to 14 without lethality in the absence of oral bacterial inoculation (33, 34).

**Effect of Monolayer Supernates on Penetration of *P. aeruginosa* Strains.** The supernates of monolayers infected with strains K767 (WT) or K1119 (ΔmexAB-oprM) were collected 3 h or overnight after infection of MDCK cells, and diluted appropriately. The medium of the MDCK cell monolayers on day 4 was replaced with these supernates and strains K767 or K1119 were added 1 h later as described above. As controls, the supernates from MDCK cell-free MEM or LB broth inoculated with the strains were also tested.

**Statistics.** Analysis of variance (ANOVA) was used for the comparisons among three or more groups. Student’s *t* test was used to compare means between the two groups. Chi-square test was used to compare survival rates. A level of 5% was accepted as statistically significant.

**Results**

**Penetration of Strain K767 (WT) and Its Efflux Mutants through MDCK Cell Monolayers.** Both the parent WT strain K767 and strain K1521 (ΔmexCD-oprF) penetrated MDCK monolayers by 3 h, whereas K1119 (ΔmexAB-oprM) was not detected in the basolateral medium until 6 h after infection (Fig. 1 A). At all time points, the penetration of both K767 (WT) and K1521 (ΔmexCD-oprF) was significantly greater than that for K1119 (ΔmexAB-oprM) (*P < 0.0006, ANOVA*). Compared with the WT, relative capacities of the mutants to penetrate monolayers at 3 and 6 h after infection are summarized in Fig. 1 B. Strains K1523 (ΔmexB) and K1525 (ΔmexXY) were also compromised for invasion, but to lesser extents than the mutant with total deletion of *mexAB-oprM* (K1119). The invasive capacities of strains OCR1 (nalB) and K1536 (nfxB), hyperexpressing efflux systems, were also intermediate between strains K767 (WT) and K1119 (ΔmexAB-oprM).

**Electron Microscopy.** When observed by scanning electron microscopy, most of the bacteria, including strain K767 (WT) and its efflux mutants, adhered near the edge (cell-to-cell junctions) of MDCK cells (Fig. 4 A), whereas significant level of invasion (86.7 ± 9.6 vs. 1.18 ± 0.15 × 10⁵ CFU, *P = 0.0002*, Fig. 3 B). Fig. 3 C shows the time courses of invasion for the strains K767 (WT) and K1119 (ΔmexAB-oprM) by the gentamicin survival assay. Significantly greater numbers of K767 (WT) than K1119 (ΔmexAB-oprM) invaded the epithelial cells (*P < 0.0065*, at each time point). Strain K767 (WT), which penetrated monolayers by 3 h (Fig. 1 A), entered the cells within 30 min, and the number of intracellular bacteria per monolayer increased in a time-dependent manner. The small number of K1119 (ΔmexAB-oprM) which had transversed the monolayers by 6 h (Fig. 1 A), was reflected in by the low level of invasion by 60 min. The number of intracellular K1119 (ΔmexAB-oprM) bacteria also increased in a time-dependent manner, but the value at 360 min was comparable to K767 (WT) only 60 min after infection (200 ± 45 vs. 130 ± 17 CFU, *P = 0.0653*).
K767 (WT) showed an additional adherence pattern, adherence around the center of the epithelial cells and seemed to be in the process of invading (Fig. 4 B). The microvilli of MDCK cells, to which \textit{P. aeruginosa} isolates adhered, were grossly unaltered. Transmission electron microscopy showed that K767 (WT) contacted with the epithelial cell surface (Fig. 4 C) and a bacterium was internalized (Fig. 4 D) 15 min after infection. At later time points bacteria invaded into the cells and reached the layer of the polycarbonate filter within 3 h after infection, as reported elsewhere (46, 47) but without appearance of cell injury. In contrast, K1119 \((\Delta \text{mexAB-ompM})\) did not penetrate at all by 30 min, and only a small number of bacteria invaded cells by 3 h after infection (data not shown).

**Evaluation of the Virulence of the Efflux Mutants in a Murine Model.** The animal model used in this study reflects the differences in the important pathophysiological steps in authentic human infections, including bacterial colonization and invasion. All strains of \textit{P. aeruginosa} tested survived equally well in the drinking water; all were recovered at equal titer at the end of the oral bacterial challenge. Furthermore, on day 8, equivalent numbers of \textit{P. aeruginosa} were recovered from the stools of animals challenged with each different bacterial strain. Strains K767 (WT) and K1521 \((\Delta \text{mexCD-oppJ})\) induced lethal endogenous septicemia in mice, whereas strain K1119 \((\Delta \text{mexAB-ompM})\) failed to kill any mice \((P < 0.0001, \text{Fig. 5 A})\). The mortality rates of mice given K1523 \((\Delta \text{mexB}, 20\% \text{ mortality rate}, P < \text{Fig. 5 A})\).

**Figure 2.** Changes of TER after infection with \textit{P. aeruginosa} strain K767 (WT of PAO1) and its efflux mutants. TER was measured in triplicate, and results are expressed as an average \(\pm\) SD of area multiplied by resistance.

**Figure 3.** Bacterial association with (A) and invasion into (B and C) MDCK epithelial cells of \textit{P. aeruginosa} strain K767 (WT of PAO1) and its efflux mutant K1119 \((\Delta \text{mexAB-ompM})\). Bacterial association was evaluated by lysis of the epithelial cells with Triton X-100, 3 h after infection. Bacterial invasion was assessed at indicated times similarly but after the treatment with 200 \(\mu\text{g/ml}\) of gentamicin for 2 h to kill extracellular bacteria. The assay was performed in triplicate, and results are expressed as mean \(\pm\) SD.

**Figure 4.** Scanning (A and B) and transmission (C and D) electron micrographs of MDCK epithelial cell monolayers infected with \textit{P. aeruginosa} strain K767 (WT of PAO1) and its efflux mutants K1119 \((\Delta \text{mexAB-ompM})\). Bacteria adhere near the edge of the epithelial cells without loss of microvilli (A; K1119, 3 h after infection). Bacteria adhere around the center of the epithelial cells and some bacteria appear to be invading into the MDCK cells, without loss of microvilli (B; K767, 1 h after infection). Bacteria are contacting the epithelial cell surface (C; K767) and a bacterium is internalized into a MDCK cell (D; K767) 15 min after infection. Original magnifications, 4,800\(\times\) (A and B) and 10,000\(\times\) (C and D). Bar, 1 \(\mu\text{m}\).
0.0001 compared with K767), K1525 (ΔmexXY, 55%, \( P = 0.00066 \)), OCR1 (nalB, 80%, \( P = 0.035 \)), and K1536 (nfxB, 70%, \( P = 0.0079 \)) were intermediate between those for strains K767 (100%) and K1119 (0%) (Fig. 5 B).

**Influence of Complementation of K1119 (ΔmexAB-oprM) with mexAB-oprM on Its Invasiveness.** To confirm the role of mexAB-oprM in the expression of invasiveness, the ΔmexAB-oprM deletion strain K1119 was complemented with mexAB-oprM by introducing plasmid pRSP17 carrying the genes and examined with strains K767 (WT), K1119 (ΔmexAB-oprM), and K1119/pRK415 (plasmid control). K1119/pRSP17 (mexAB-oprM complemented strain) showed the invasiveness equivalent to WT in both in vitro (Fig. 6 A) and the animal model (Fig. 6 B), while plasmid control strain K1119/pRK415 had the same phenotype, which was compromised in its capacity to penetrate MDCK monolayer (Fig. 6 A, \( P < 0.0001 \)) and to kill leukopenic mice (Fig. 6 B, \( P < 0.0001 \)) as K1119 (ΔmexAB-oprM).

**Influence of a Single Mutation in oprM on the Invasiveness of P. aeruginosa.** A oprM knockout mutant KG4521 was examined with K767 (WT) and KG4521/pKMM128 (oprM complemented strain) to clarify the role of OprM in the loss of virulence in P. aeruginosa. Strain KG4521 (oprM knockout) was significantly less virulent in the MDCK epithelial monolayer system (Fig. 6 A, \( P = 0.0466 \)) and in the animal model (Fig. 6 B, \( P = 0.02703 \)). However, the loss of virulence was modest compared with the complete mexAB-oprM deletion mutant K1119. Complementation of KG4521 with oprM restored the virulence entirely (Fig. 6).

**Effect of Monolayer Supernates on Penetration of P. aeruginosa Strains.** Experiments were performed to determine if soluble factors enhanced the penetration of P. aeruginosa across MDCK cells. Neither supernates of K767 (WT) or K1119 (ΔmexAB-oprM) cultured overnight in cell-free LB broth, nor K1119 (ΔmexAB-oprM) cultured overnight in MEM in the presence of MDCK cells, when readded to MDCK cells, influenced the penetration of K767 (WT). On the other hand, the supernate from MDCK cells incubated overnight together with K767 (WT), caused a slight but significant enhancement of the penetration of strain K767 (WT) itself into MDCK monolayers (\( P = 0.0475 \), ANOVA). Supernates from MDCK cell monolayers infected overnight with K767 (WT) dramatically and significantly enhanced the penetration of K1119 (ΔmexAB-oprM) into MDCK cells (\( P < 0.0001 \), ANOVA). The K1119 (ΔmexAB-oprM) supernate also significantly enhanced the penetration of K1119 (ΔmexAB-oprM) (\( P < 0.05 \)), but the...
effect was nearly 10-fold less than that observed with the K767 (WT) supernate (245 ± 128 vs. 2320 ± 385, P = 0.0081) (Fig. 7 A). The supernates of K767 (WT) incubated in MEM in the absence of MDCK cells showed a very low level of enhancement of invasion of the K1119 (ΔmexAB-oprM) cells (unpublished data). The supernates of strain K767 (WT) incubated with MDCK cells overnight, enhanced the penetration of strain K1119 (ΔmexAB-oprM) through monolayers in a concentration-dependent manner (Fig. 7 B). In contrast, the supernates obtained 3 h after infection demonstrated a relatively weak enhancement effect compared with those from overnight cultures.

Discussion

This study using MDCK epithelial cell monolayer system demonstrates an association between bacterial efflux mechanisms and invasion of eukaryotic cells. The ΔmexAB-oprM deletion strain K1119 was the least invasive among the efflux mutants tested, while K1521, the mexcCD-oprJ deletion strain, was as invasive as WT. The loss of only mexB (K1523) or mexXY (K1525) decreased invasion but in a less dramatic fashion than for the deletion of the entire mexAB-oprM operon (K1119). Data from gentamicin survival assays, electron microscopy studies, and the animal studies supported these findings. The current in vitro and in vivo animal models represent intestinal epithelium and gut–derived endogenous bacteremia, respectively, but not respiratory epithelium nor pneumonia models. Mex and OM proteins may influence the bacterial susceptibility to antibody independent complement killing or opsonic killing by neutrophils; however, all mutants in this study were serum-resistant as the WT, suggesting that the mutations did not affect their susceptibility to antibody-independent complement killing. This study did not include phagocytes in the in vitro system and our animal model was leukopenic. Therefore, we do not believe that the changes we observed in the mutants influenced their interactions with phagocytic cells.

Our data suggest that deletion of all three genes in the MexAB–OprM efflux system is necessary to yield the maximum loss of invasiveness. The complementation of ΔmexAB-oprM deletion strain K1119 with mexAB-oprM restored the invasiveness to the level of WT, and consequently confirmed that the genes, mexAB-oprM, actually played an important role in the loss of invasiveness. The MexXY–OprM and MexAB–OprM systems share OprM as a channel-tunnel (16, 17), and only the deletion of genes in these two efflux systems decreased the level of invasiveness modestly. However, the mexB deletion strain K1523 displayed a more similar phenotype to the complete mexAB-oprM mutant K1119 than the mexXY mutant K1525. The difference in virulence between the ΔmexB mutant and the ΔmexXY mutant may depend on the expression of each efflux system; MexAB–OprM is expressed constitutively. We expected that OprM is a critical determinant of invasiveness in P. aeruginosa and that the invasive determinant(s) are exported through OprM only; however, the loss of invasiveness in the oprM knockout mutant, KG4521 was modest, suggesting the possibility that OM proteins other than OprM also could work with MexAB.

The efflux system–overproducing strains such as nalB and nfxB, also demonstrated somewhat reduced invasiveness, akin to the efflux deletion strains (Fig. 1 B). These paradoxical findings may be explained by recent reports, which showed that nalB type mutants, hyperexpressing the MexAB–OprM (23–25) and nfxC type mutants, overexpressing the MexEF–OprN (26, 27), produce lower level of virulence factors because of their influence on cell-to-cell signaling regulated by quorum-sensing systems. Therefore, it appears that normal efflux levels are necessary for P. aeruginosa to express maximal invasiveness.

Data from gentamicin survival assays (Fig. 3 A) and electron microscopy studies (Fig. 4 A) also confirmed that the differences between WT and the ΔmexAB-oprM mutant in
bacterial penetration through MDCK epithelial cell monolayers, were correlated with differences in their invasiveness; the strains adhered to the epithelial cells equally well. Electron microscopy studies showed a very similar invasion pattern to that reported in the study using tracheal epithelial cells (48). Interestingly, in contrast to the observation of microvilli loss when MDCK cells are infected with Salmonella isolates for at least 2 h (31), microvilli appeared to remain intact when such cells were infected with P. aeruginosa strains for at least 3 h. The difference between the two bacterial species is consistent with the observation that P. aeruginosa frequently colonizes the intestine of normal human subjects (49) without producing any symptoms, and shares characteristics with normal enteric microbial flora. Conversely, Salmonella species are not part of the normal flora and have the capacity to cause invasive disease in normal hosts. The molecular mechanisms accounting for differences in the pathology of disease in the gastrointestinal tract remains unexplained in spite of the reported use of the cystic fibrosis transmembrane conductance regulator by both Salmonella typhi and P. aeruginosa to enter epithelial cells (50–52). Recently, it has been reported that S. typhi LPS is altered by contact with epithelial cells, exposing the bacterial ligand for cystic fibrosis transmembrane conductance regulator on the LPS (53). Similar findings in P. aeruginosa–epithelial cell interaction could further clarify the molecular mechanisms of invasion for this bacterial species.

Our previous study demonstrated that noncytotoxic isolates of P. aeruginosa, including PAO1, expressed minimal epithelial cell damage; PAO1 did not induce LDH release from MDCK cells or the formation of plaques as detected by staining with trypan blue at 6 h after infection; cytotoxic isolates showed clear evidence of cell damage within 3 h (4). In this study, changes in TER of monolayers infected with the efflux mutants, in particular the mexAB-oprM deletion strain, were substantially less than for the WT (Fig. 2). These findings may mean that bacterial invasion may disrupt the tight junctions between epithelial cells even though they do not induce demonstrable cytotoxicity.

The supernates from MDCK cells infected overnight with WT restored the capacity of the ΔmexAB-oprM strain K1119 to penetrate monolayers in a concentration-dependent manner (Fig. 7). When the WT strain was added from the basolateral side, neither the basolateral nor apical supernates showed any enhancing effect (data not shown). The enhancement of invasion by the K1119 (ΔmexAB-oprM) supernate was modest, and much less than by the WT supernate, suggesting that other efflux systems may export virulence determinants, albeit to a lesser degree than for MexAB–OprM. These findings strongly indicate that WT exports invasion determinant(s) into the extracellular environment, using predominantly MexAB–OprM (with lesser contributions by MexXY–OprM or other undetermined systems). It remains to be determined if the putative determinant(s) of invasiveness act directly as invasive factor(s) or as regulatory factor(s). Although it is still possible that mammalian products could be involved in the findings, studies are currently underway to purify and determine the molecular nature of the determinant(s) in the bacterial supernate which confer invasiveness.

The recovery of invasiveness of the mexAB-oprM deletion strain, mediated by the supernate of WT, required cell–to-cell contact between the bacterium and MDCK cells (Fig. 7). These data suggest that the type III protein secretion systems (for a review, see reference 54) may be involved in the expression of invasiveness in P. aeruginosa. Type III secretion systems require close contact between bacterium and eukaryotic cell in order to deliver virulent bacterial proteins directly into the cytoplasm of the cell. ExoU and some other exoenzymes are recognized type III effectors in P. aeruginosa (55). In other bacterial species such as Salmonella, the bacteria modulate actin organization to induce their own uptake by nonphagocytic cells (56, 57). Efflux protein homologues can secrete proteins since type I secretion systems are RND homologues (58). However, the relation of efflux systems to the type III protein secretion system has not been demonstrated to date, and proteins secreted by the type III systems are likely too large for export via MDR efflux systems. Therefore, it is possible that contact of bacteria with the epithelial cells may be a trigger to stimulate the efflux of invasiveness determinant(s).

Our findings in this study strongly suggest that invasion determinant(s) are predominantly exported by P. aeruginosa via MexAB–OprM. Hence, MDR efflux systems in P. aeruginosa might be critical for the efflux of virulence factors, in addition to their established role of exporting harmful substances such as antibiotics or detergents. It seems practical for bacteria to utilize efflux systems to export virulence determinants and physiological products, as a physiological process. In fact, recent reports suggest that E. coli expels bacterial cellular metabolites such as indole and bile acids using the efflux systems AcrEF (59) and AcrAB (60), respectively. Our findings are consistent with a novel physiological role for such “multidrug” efflux systems, namely in enhancing bacterial invasiveness in P. aeruginosa infections. Furthermore, efflux inhibitors, which are under development to depress MDR by efflux systems (61), may also serve to reduce bacterial invasiveness in P. aeruginosa infections.

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