Overexpression of MexCD-OprJ Reduces Pseudomonas aeruginosa Virulence by Increasing Its Susceptibility to Complement-Mediated Killing

Pseudomonas aeruginosa is one of the most frequent and severe causes of nosocomial pneumonia as well as the chief cause of morbidity and mortality in chronic lung infections in patients with significant underlying diseases such as cystic fibrosis (CF) and bronchiectasis (1–3). The treatment of these infections is severely compromised by the extraordinary capacity of this pathogen to evade the activity of nearly all available antibiotics through a complex interplay of intrinsic and mutation-driven resistance pathways (4). Among the most relevant resistance pathways are those leading to the overexpression of the chromosomal β-lactamase AmpC along with the inactivation of the carbapenem porin OprD or the overexpression of several efflux pumps encoded in its genome (4–6).

Experimental evidence suggests that acquisition of antimicrobial resistance leads to a reduction of the P. aeruginosa virulence (7–11). It is generally believed that the attenuation of bacterial virulence is the consequence of a nonspecific metabolic burden that would impair bacterial growth in vivo. In this work, we hypothesized that acquisition of antimicrobial resistance could produce specific changes that reduce the ability of P. aeruginosa to evade the immune system effectors, such as complement, which is critical in the host defense against P. aeruginosa (12, 13). To test this hypothesis, we evaluated the resistance to complement-mediated killing of a collection of isogenic P. aeruginosa strains expressing different antimicrobial resistance phenotypes acquired during infection, including overexpression of the chromosomal β-lactamase AmpC, inactivation of OprD, and absence or overexpression of several efflux pumps (Table 1). All strains were previously described except the mutant strain overproducing the MexXY efflux pump (PAOMxZ), which was constructed from...
TABLE 2 Complement sensitivity of P. aeruginosa strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Survival in NHS (%)</th>
<th>C3 binding</th>
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<tbody>
<tr>
<td>PAO1</td>
<td>93 ± 2</td>
<td>0.405 ± 0.039</td>
</tr>
<tr>
<td>PAO1D</td>
<td>88 ± 3</td>
<td>0.478 ± 0.023</td>
</tr>
<tr>
<td>PAAdacB</td>
<td>92 ± 4</td>
<td>0.399 ± 0.021</td>
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<tr>
<td>PAAD</td>
<td>87 ± 1</td>
<td>0.410 ± 0.019</td>
</tr>
<tr>
<td>PAONB</td>
<td>36 ± 2</td>
<td>0.890 ± 0.030</td>
</tr>
<tr>
<td>PAOM</td>
<td>89 ± 4</td>
<td>0.389 ± 0.010</td>
</tr>
<tr>
<td>PAOMxR</td>
<td>88 ± 2</td>
<td>0.450 ± 0.054</td>
</tr>
<tr>
<td>PANB</td>
<td>88 ± 3</td>
<td>0.479 ± 0.021</td>
</tr>
<tr>
<td>PANBMxZ</td>
<td>96 ± 6</td>
<td>0.349 ± 0.013</td>
</tr>
<tr>
<td>JW</td>
<td>74 ± 5</td>
<td>0.650 ± 0.029</td>
</tr>
<tr>
<td>JWNB</td>
<td>32 ± 3</td>
<td>0.910 ± 0.030</td>
</tr>
<tr>
<td>GPP</td>
<td>8 ± 1</td>
<td>1.279 ± 0.050</td>
</tr>
<tr>
<td>GPPNB</td>
<td>1 ± 0.3</td>
<td>1.479 ± 0.051</td>
</tr>
</tbody>
</table>

* Results (means ± SD) are from three independent experiments, each done in duplicate.
* Survival after incubation for 1 h at 37°C in NHS was calculated as the percentage of the bacterial counts obtained with bacteria incubated in HI NHS.
* Arbitrary optical densities at 405 nm.

PAO1 using the cre-lox system for gene deletion and antibiotic marker recycling as done earlier for the other mutants (15–18).

For the serum bactericidal assays, bacteria were grown to an exponential growth phase and resuspended in phosphate-buffered saline (PBS) to a final concentration of 10^8 CFU/ml. Then, 10-μl samples were mixed with 40 μl of PBS and 50 μl of nonimmune human serum (NHS) or heat-inactivated (HI) NHS to give a final concentration of 50% in serum. The mixtures were incubated at 37°C for 60 min and then plated on LB agar plates to determine the number of viable cells. We observed that most of the mutant strains were as resistant as the wild-type strain PAO1 to the bactericidal effect of the complement (Table 2). Among all strains tested, only the nfxB mutant PAONB exhibited a significantly reduced ability to resist complement-mediated killing compared with that of PAO1. To confirm this result, we extended our observation to the clinical isolates JW and GPP. Although both isolates were more susceptible to the complement killing than PAO1, their respective isogenic nfxB mutants, JWNB and GPPNB, were clearly more susceptible than their respective parent strains (Table 2).

We determined the binding of the complement component C3 to this collection of isogenic strains. For this purpose, bacterial cells were opsonized for 30 min in NHS or HI NHS, washed three times with PBS, and resuspended in 50 mM carbonate-bicarbonate buffer (pH 9.0) containing 1 M NH₄OH to disrupt the ester bonds between the C3 fragments and the bacterial surface. After 2 h at 37°C, bacteria were removed by centrifugation, and C3 was quantified by an enzyme-linked immunosorbent assay (ELISA) as previously described (19). All mutant strains bound amounts of C3 similar to those bound by the parent strain PAO1, except for the nfxB mutant, which bound approximately 2-fold more C3 than PAO1 (Table 2). Altogether, these results indicate that mutation of nfxB increases the binding of C3 to P. aeruginosa and, consequently, impairs its ability to resist the lytic effect of the complement.

Mutation of nfxB leads to overproduction of the efflux pump MexCD-OprJ and, consequently, increased resistance to the fluoroquinolone antibiotics. However, it has been reported that mutation of nfxB also causes global changes in the physiology and metabolism of P. aeruginosa (20). To study the specific effects of the overproduction of MexCD-OprJ on the reduced ability to resist complement-mediated killing observed in the nfxB-deficient mutant, we determined the survival rate in NHS of the double mutant PANBMxD. This mutant harbors deletions in both nfxB and mexD. MexCD-OprJ knockdown in PAONB abolished OprJ expression (18) and increased the resistance to complement up to the levels seen with PAO1 (Fig. 1A). Moreover, the double mutant bound levels of human C3 similar to those observed in PAO1 (Fig. 1B), suggesting that complement sensitivity in the nfxB mutant is due to inappropriate expression of MexCD-OprJ. Immunoblot binding assays using purified human C3 labeled with the infrared dye 800CW demonstrated that OprJ failed to bind C3 (data not shown), suggesting that the increased binding of C3 in the nfxB mutant is due to increased expression of other C3-binding surface proteins.
molecules or due to changes in the accessibility of C3 to these molecules. This claim is consistent with recent results showing that MexCD-OprJ overexpression produces major changes in membrane physiology (20).

Our results using purified human complement C3 indicated that the nfxB mutant bound more C3 than PAO1. However, a prerequisite for complement having a role in respiratory tract infections is the ability of the human lung fluids to opsonize bacteria. To determine whether the local complement system in the human lung has a role in the opsonization of the bacteria, we incubated the bacteria with human bronchoalveolar lavage fluid (BALF) and determined the binding of C3 as previously described (19). Incubation of P. aeruginosa with BALF led to the activation of complement and binding of C3 on the bacterial surface (Fig. 2). As occurred using human serum, the nfxB mutant bound almost 2-fold more C3 than the parent strain PAO1 and the double mutant PANBMxD. All together, the data of these experiments show that the local complement system present in human lung fluids is sufficient to opsonize bacteria and suggest that the nfxB mutant will be cleared from the lung more efficiently and rapidly than the wild-type strain.

To test this hypothesis, BALB/c mice were infected intranasally with 2 × 10⁷ CFU of the wild-type strain PAO1, PAONB, or PANBMxD, and after 12 h, the numbers of viable P. aeruginosa isolates in the lung homogenates were quantified (Fig. 3). The bacterial loads in the lungs of the animals infected with PAO1 or PANBMxD were higher than the loads in those infected with PAONB, suggesting that the increased susceptibility to complement due to overexpression of the MexCD-OprJ efflux pump facilitates clearance of P. aeruginosa from the lung.

The results of this work demonstrate that a reduction in virulence, commonly associated with antimicrobial resistance, may in some cases be due to specific changes that reduce the ability of the pathogen to evade the host immune system rather than to nonspecific burdens reflected in bacterial growth impairment.

According to this result and given the crucial role of the complement in the host defense against P. aeruginosa infections (12), nfxB mutants will emerge rarely and mainly in patients with compromised complement levels. This notion is supported by the low number of natural nfxB mutant strains isolated in clinical settings (21–24) and is in agreement with the results of the experiments performed by Hirakata et al. (10), who showed that an nfxB mutant was less virulent that the parent strain in a mouse model of bacteremia. In contrast, nfxB mutants are highly prevalent among P. aeruginosa isolates from cystic fibrosis patients with chronic respiratory infections (25). Indeed, mutation of nfxB is involved in the early adaptation to the chronic setting (26), perhaps by promoting biofilm growth. It is likely that the combination of treatment with antibiotics along with the protection conferred by the biofilm against the host humoral immune effectors such as complement (27) promotes the selection of this type of mutant.

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REFERENCES


