ExoT of Cytotoxic 

Pseudomonas aeruginosa Prevents Uptake by Corneal Epithelial Cells

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The presence of invasion-inhibitory activity that is regulated by the transcriptional activator ExsA of cytotoxic Pseudomonas aeruginosa has previously been proposed. The results of this study show that both ExoT and ExoS, known type III secreted effector proteins of P. aeruginosa that are regulated by ExsA, possess this activity. Invasion was reduced 94.4% by ExoT and 96.0% by ExoS. Invasion-inhibitory activity is not linked to ADP-ribosylation activity, at least for ExoS, since a noncatalytic mutant also inhibits uptake by an epithelial cell line (invasion was reduced 96.0% by ExoSE381A).

The earliest events in most Pseudomonas aeruginosa infections occur at the epithelium. Depending on their interaction with epithelial cells in vitro, clinical isolates of P. aeruginosa may be classed as cytotoxic or invasive, and these phenotypes correspond to distinct genotypes (10). Acute epithelial cell death induced by cytotoxic P. aeruginosa has been demonstrated to be due to the expression of ExoU, delivered via type III secretion, that is regulated by the transcriptional activator ExsA (7). Wild-type cytotoxic strains do not invade epithelial cells. An exoU mutant of P. aeruginosa (PA103exoU::Tn5Tc) is noncytotoxic but remains noninvasive (5, 12). A subsequent mutation in exsA results in an invasive phenotype (5). This result suggests that there is at least one ExsA-regulated gene encoding the inhibition of invasion in cytotoxic P. aeruginosa.

Inhibition of P. aeruginosa uptake by epithelial cells. YopE and YopH, secreted via type III secretion by Yersinia spp., prevent uptake by eukaryotic cells (15). Furthermore, YopH is essential for antiphagocytic function (14). YopH is a protein tyrosine phosphatase (11) that has been demonstrated to prevent uptake of other microorganisms (26).

P. aeruginosa PA103 was used to demonstrate a similar capacity by cytotoxic P. aeruginosa to block uptake of other bacteria. To allow invasion levels to be quantified, a noncytotoxic mutant, PA103exoU (Table 1), was used for these experiments. Invasion of a rabbit corneal epithelial (RCE) cell line was determined as previously described (9). RCE cells were fed with modified SHEM (13), containing bovine pituitary extract (5 μg/ml) in place of cholera toxin. For most assays, cells were cultured on 24-well plates and used between 3 and 6 days after passage. Cells were washed with phosphate-buffered saline (PBS) and inoculated with 200 μl of bacterial suspension (in modified Eagle’s medium with Earle’s salts and l-glutamine, buffered with 1 M HEPES-NaOH [pH 7.6], 0.35 g of NaHCO3 per liter, and 6 g of bovine serum albumin per liter [Sigma, St. Louis, Mo.]). Infected cells were then incubated for 3 h at 37°C in 5% CO2. The bacterial suspension was then carefully aspirated, and the wells were washed with two sequential 0.5-ml aliquots of PBS (Sigma) to remove nonassociated bacteria. Adherent extracellular bacteria were killed by incubation for 1 h after the addition of 1 ml of gentamicin (200 μg/ml). The antibiotic was aspirated, and the excess was removed by one wash of 1 ml of PBS before the cells were lysed with 0.025% Triton X-100. The number of intracellular bacteria was determined by culturing serial dilutions of the lysate.

To demonstrate uptake inhibition by cytotoxic P. aeruginosa, PA103exoU was coincubated with invasive strains of P. aeruginosa. The uptake of invasive strains 6294 and 6487 and an invasive mutant of PA103 (PA103exoUexsA) (Table 1) was compared to the uptake of these strains in the presence of PA103exoU. An inoculum of 10^7 CFU was used for PA103 mutants, and the inoculum for strains 6294 and 6487 was reduced to 2 × 10^6 CFU as these strains are significantly more invasive (10). As a control, the effect of PA103exoUexsA (which lacks invasion-inhibitory activity) on the invasion of 6294 was also examined. Different strains of internalized bacteria were distinguished in viable counts by plating duplicate aliquots on nonselective medium (MacConkey agar) and selective medium (tryptic soy agar [Difco, Detroit, Mich.] with 100 μg of tetracycline per ml or 10 μg of streptomycin per ml [Sigma]).

PA103exoU inhibited uptake of all three invasive P. aeruginosa strains tested (P < 0.001 for all strains) (Fig. 1a to c). In contrast, PA103exoUexsA was not able to inhibit uptake of 6294 (P = 0.062; Mann-Whitney U test) (Fig. 1d).

Levels of bacterial association with RCE cells (i.e., the sum of adherent and invasive bacteria) were determined for both PA103exoU and PA103exoUexsA as previously described (9). Bacteria were incubated with RCE as described above, and then cells were washed vigorously three times with PBS before the cells were lysed with Triton X-100 and bacteria were enumerated. There was no significant difference between the abilities of PA103exoU and PA103exoUexsA to associate with cells (mean ± standard error, [1.40 ± 0.32] × 10^6 CFU/well compared to [1.19 ± 0.24] × 10^6 CFU/well, respectively; P = 0.1489 by the Mann-Whitney U test). This suggested that uptake inhibition by PA103exoU was not due to an inability to associate with the host cell membrane.

A likely candidate for an invasion inhibitor might be a Pseudomonas homolog to YopH, a type III effector that inhibits uptake of Yersinia. A TBLASTN 2.0.8 search of the published genome of P. aeruginosa failed to find a region homologous to YopH (1) or to the catalytic region that is invariantly conserved among protein tyrosine phosphatases such as YopH (11).

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Of the type III secreted effector proteins that have been reported for *P. aeruginosa* (19, 20), only ExoU and ExoT are produced by the cytotoxic strain PA103. ExoU has been shown to be essential for acute cytotoxicity; ExoT is not required (7). ExoT and ExoS possess invasion inhibitory activity. To investigate the role of ExoT in uptake inhibition, the ability of a noncytotoxic mutant of PA103 with a second mutation in *exoT* (PA103*exoUexoT*) (Table 1) to invade corneal epithelial cells was compared to that of PA103*exoU*. PA103*exoUexoT* invaded epithelial cells 40-fold more efficiently than PA103*exoU* (Fig. 2). When *exoT* was complemented back in *trans* (Table 1), inhibition of invasion was restored (Fig. 2).

ExoT and ExoS belong to the ADP-ribosyltransferase family. Although they have 75% amino acid identity, ExoT possesses only 0.2% enzymatic activity compared to ExoS (18). Interestingly, both exoS and a noncatalytic version of *exoS* (Table 1) complemented the invasion-inhibitory activity that was lost by mutating *exoT* in PA103*exoU* (Fig. 2), suggesting that ADP-ribosylating activity is not required for the inhibition of uptake, at least by ExoS.

Actin microfilaments are involved in the ability of epithelial cells to take up *P. aeruginosa* (8). It was recently reported by Vallis and coworkers (17) that ExoT, ExoS, and the noncatalytic mutant ExoSE381A caused morphological changes to CHO cells without apparent membrane damage. In the present study, RCE cells were also found to display rounded morphology, but not trypan blue staining, after infection with bacteria expressing these proteins (data not shown). These changes to

### TABLE 1. Strains of *P. aeruginosa* used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristic(s)</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PA103<em>exoU</em>:Tc</td>
<td>PA103<em>exoU</em>: transposon mutant of cytotoxic strain; lacks ExoU</td>
<td>7</td>
</tr>
<tr>
<td>PA103<em>exoU</em>:Tc:exsA::Ω</td>
<td>PA103*exoUexsA::Ω; lacks entire ExsA-regulated system</td>
<td>5</td>
</tr>
<tr>
<td>6294</td>
<td>Clinical isolate; invasive phenotype</td>
<td>0</td>
</tr>
<tr>
<td>6487</td>
<td>Clinical isolate; invasive phenotype</td>
<td>0</td>
</tr>
<tr>
<td>PA103<em>exoUexoT</em>:Tc pUCP</td>
<td>PA103<em>exoUexoT</em>:Tc; lacks ExoU and ExoT; contains control plasmid encoding Cb'</td>
<td>17</td>
</tr>
<tr>
<td>PA103<em>exoUexoT</em>:Tc</td>
<td>ExoT expressed in <em>trans</em></td>
<td>17</td>
</tr>
<tr>
<td>pUCPexoT</td>
<td>ExoT expressed in <em>trans</em></td>
<td>17</td>
</tr>
<tr>
<td>PA103<em>exoUexoT</em>:Tc</td>
<td>ExoS expressed in <em>trans</em></td>
<td>17</td>
</tr>
<tr>
<td>pUCPexoS</td>
<td>ExoS expressed in <em>trans</em></td>
<td>17</td>
</tr>
<tr>
<td>PA103<em>exoUexoT</em>:Tc</td>
<td>Noncatalytic mutant ExoS, expressed in <em>trans</em></td>
<td>17</td>
</tr>
<tr>
<td>pUCPexoSE381A</td>
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</table>

FIG. 1. PA103*exoU* inhibits uptake of invasive strains of *P. aeruginosa*. PA103*exoU* was coincubated with invasive strains of *P. aeruginosa* during infection of RCE cells, and uptake was quantified. PA103*exoU* (□) inhibited the invasion of all strains assayed when coincubated with 6294 (■) (a), 6487 (□) (b), and PA103*exoUexsA* (□) (c). PA103*exoUexsA* (■) did not inhibit invasion when coincubated with 6294 (■) (d).
host cell morphology suggest that the cytoskeleton may be affected by these proteins, and this might explain the cells’ loss of ability to take up bacteria.

**ExoT inhibits uptake of an invasive strain of *P. aeruginosa***. Unlike PA103exoU, PA103exoUexoT was not able to block uptake of the invasive strain 6294 by RCE cells (Table 2). Complementation with exoT in trans restored the ability of PA103exoUexoT to block RCE cell uptake of 6294. This result demonstrated that ExoT in a cytotoxic strain can function to block invasion of invasive *P. aeruginosa*.

The results of this study demonstrated that both ExoT and ExoS can inhibit the uptake of a cytotoxic strain of *P. aeruginosa* by epithelial cells. Interestingly, invasive *P. aeruginosa* encodes and secretes both of these proteins, yet these strains invade efficiently. Furthermore, mutation of exsA in an invasive strain (PAO1) does not significantly affect its ability to invade (10). There are a number of possible explanations for this. *P. aeruginosa* produces exoenzyme S as a heterologous aggregate of ExoS and ExoT (17). Possibly, the interaction of these two effectors results in the loss of their invasion-inhibitory activity. Alternatively, invasive strains might also encode a suppressor of ExoT and ExoS or an effector that has a more dominant and positive effect on invasion than ExoT or ExoS.

A third possibility is that the ExsA-regulated type III secretion system is not activated by cell contact with corneal epithelial cells in invasive *P. aeruginosa*. Vallis and coworkers (16) have shown that there are differences in stimulation of the ExsA-regulated system between low-calcium conditions and the presence of serum or cell contact with CHO cells. Although ExoT and ExoS are secreted by invasive *P. aeruginosa* under conditions inducing growth (such as low calcium levels), they may not necessarily be secreted upon contact with corneal epithelial cells in culture.

What is the role of inhibition of invasion by ExoT in the pathogenesis of cytotoxic *P. aeruginosa*? There are considerable similarities between the type III secretion systems of *P. aeruginosa* and those of *Yersinia*. In this study, we have shown that ExoT inhibits uptake of *P. aeruginosa*, similar to the function of YopH of *Yersinia* spp. A strain of *Yersinia pseudotuberculosis* lacking YopH remains cytotoxic (14), and an exoT mutant of *P. aeruginosa* also remains cytotoxic (7). However, a yopH deletion mutant of *Y. pseudotuberculosis* is avirulent in a murine model of intraperitoneal infection, while an exoT mutant of *P. aeruginosa* remains virulent in an acute lung infection model (3, 7). The presence of ExoU, which is associated with acute cytotoxicity, may mask any effect of ExoT in this model. It is clear, however, that ExsA-regulated proteins other than ExoU are involved in virulence. An exsA mutant of invasive *P. aeruginosa* is avirulent or less virulent in acute infection models (4, 7). Invasive *P. aeruginosa* does not encode ExoU, indicating an important role for other effectors, at least in this class of *P. aeruginosa* strains at some stage of the infectious process.

ExoT is the only type III effector protein reported to date that is encoded by both invasive and cytotoxic *P. aeruginosa* strains. The conservation of this gene across strains would suggest an important role in survival; this may involve the control of phagocytosis by eukaryotic cells.

**TABLE 2. Uptake of 6294 during coincubation with PA103 mutants**

<table>
<thead>
<tr>
<th>Coincubation of 6294 with:</th>
<th>% Relative uptake of 6294 ± SD*</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA103exoU</td>
<td>2.54 ± 1.20</td>
</tr>
<tr>
<td>PA103exoU/exsA</td>
<td>107.63 ± 12.63</td>
</tr>
<tr>
<td>PA103exoU/exoT</td>
<td>95.65 ± 23.01</td>
</tr>
<tr>
<td>PA103exoU/exoT with exoT in trans</td>
<td>1.09 ± 0.38</td>
</tr>
</tbody>
</table>

* Uptake of 6294 when coincubated with PA103 mutant strains expressed as a percentage relative to uptake of 6294 incubated alone.

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