The galU Gene of *Pseudomonas aeruginosa* Is Required for Corneal Infection and Efficient Systemic Spread following Pneumonia but Not for Infection Confined to the Lung

Gregory P. Priebe,1,2*, Charles R. Dean,3† Tanweer Zaidi,1 Gloria J. Meluleni,1 Fadie T. Coleman,1 Yamara S. Coutinho,1 Michael J. Noto,1 Teresa A. Urban,3 Gerald B. Pier,1 and Joanna B. Goldberg3

Channing Laboratory, Department of Medicine, Brigham and Women’s Hospital,1 and Departments of Anesthesia (Critical Care) and Medicine (Infectious Diseases), Children’s Hospital,2 Boston, Massachusetts, and Health Sciences Center, University of Virginia, Charlottesville, Virginia3

Received 20 December 2003/Returned for modification 18 February 2004/Accepted 10 March 2004

Acute pneumonias and corneal infections due to *Pseudomonas aeruginosa* are typically caused by lipopolysaccharide (LPS)-smooth strains. In cystic fibrosis patients, however, LPS-rough strains of *P. aeruginosa*, which lack O antigen, can survive in the lung and cause chronic infection. It is not clear whether an LPS-rough phenotype affects cytotoxicity related to the type III secretion system (TTSS). We previously reported that interruption of the galU gene in *P. aeruginosa* results in production of a rough LPS and truncated LPS core. Here we evaluated the role of the galU gene in the pathogenesis of murine lung and eye infections and in cytotoxicity due to the TTSS effector ExoU. We studied galU mutants of strain PA01, of its cytotoxic variant expressing ExoU from a plasmid, and of the inherently cytotoxic strain PA103. The galU mutants were more serum sensitive than the parental strains but remained cytotoxic in vitro. In a corneal infection model, the galU mutants were significantly attenuated. In an acute pneumonia model, the 50% lethal doses of the galU mutants were higher than those of the corresponding wild-type strains, yet these mutants could cause mortality and severe pneumonia, as judged by histology, even with minimal systemic spread. These findings suggest that the galU gene is required for corneal infection and for efficient systemic spread following lung infection but is not required for infection confined to the lung. Host defenses in the lung appear to be insufficient to control infection with LPS-rough *P. aeruginosa* when local bacterial levels are high.

*Pseudomonas aeruginosa* is a major cause of lung and eye infections. In the lung, it is frequently isolated in ventilator-associated pneumonias (39) and in the chronic bronchopneumonia of patients with cystic fibrosis (34). In the eye, it is the most likely pathogen found in ulcerative keratitis in wearers of extended-use contact lenses (1), in whom there is a clinically significant incidence of infection (36). In gram-negative bacteria such as *P. aeruginosa*, lipopolysaccharide (LPS) is a major component of the outer membrane and has three general features: O antigen (also known as O side chain or, for *P. aeruginosa*, the B-band O polysaccharide), core, and lipid A. In LPS-smooth strains of *P. aeruginosa*, 5 to 30% of the LPS cores are substituted with the long, antigenically diverse O side chains that confer serotype identity and complement resistance (41, 55). In contrast, LPS-rough strains contain few, short, or no O side chains and, because of this, are nontypeable and susceptible to in vitro killing by serum complement (18, 55). In general, isolates of *P. aeruginosa* recovered from the environment and from nosocomial infections are LPS-smooth and serum resistant, while those from the lungs of patients with advanced cystic fibrosis are LPS-rough and serum sensitive (18). In addition to being LPS-rough, many *P. aeruginosa* isolates from patients with cystic fibrosis display a mucoid phenotype due to overproduction of the exopolysaccharide alginate (23).

We have shown that the LPS outer core of *P. aeruginosa* is important in the pathogenesis of both lung and corneal infections in that it is the ligand for the cystic fibrosis transmembrane conductance regulator (CFTR) on host cells (35, 46, 59–61). In the lung, CFTR-mediated responses by epithelial cells are a key mechanism of early recognition of infection by the host’s innate immune system (45), promoting clearance of the bacterium by the host (3, 35, 46). In an injured cornea, however, CFTR promotes bacterial ingress into an immune-privileged site and thus exacerbates infection and pathology (59–61).

*P. aeruginosa*, like many other gram-negative pathogens, uses a type III secretion system (TTSS) to deliver a number of effector proteins directly into host cells. The *P. aeruginosa* TTSS has in recent years been shown to be linked to increased severity of nosocomial pneumonia in humans (19, 42) and to be important for pathogenesis in murine models of lung (2, 49) and eye (29) infections. The effector proteins of the *P. aeruginosa* TTSS include ExoS and ExoT, which function as both ADP-ribosyltransferases and GTPase-activating proteins (15, 26, 27); ExoY, which is an adenylate cyclase (58); and ExoU, which has recently been shown to be a lipase (13, 44). Interestingly, the exoS and exoU genes appear to be mutually exclusive: *P. aeruginosa* strains have either one or the other (14, 42, 57). *P. aeruginosa* strain PA01, a common laboratory strain

---

1 Corresponding author. Mailing address: Channing Laboratory, 181 Longwood Ave., Boston, MA 02115. Phone: (617) 525-2663. Fax: (617) 525-2510. E-mail: gpriebe@rics.bwh.harvard.edu.

† Present address: Novartis Institutes for Biomedical Research, Cambridge, Mass.
whose genome has been fully sequenced (52), expresses ExoS but not ExoU. On pulmonary and ocular epithelial surfaces, ExoS appears to facilitate bacterial invasion, while ExoU causes rapid cellular cytotoxicity (14). The relationship between LPS phenotype and ExoU-mediated cytotoxicity has not been previously described.

Strains of *P. aeruginosa* with defective LPS outer cores have been shown to be avirulent in a number of studies of animal models of acute infection (9, 11, 30, 37, 51, 53). Many of these models used LPS-rough strains in which the genetic defect responsible for the LPS-rough phenotype was not known (9, 11, 30, 51). In that setting, it is not certain whether the change in virulence can be attributed to the LPS abnormality alone because a genetically undefined mutant might carry other defects that can contribute to altered virulence. Other models utilized algC mutants of *P. aeruginosa* which, while genetically defined, were defective in production of alginate as well as LPS core and O side chain (16, 37, 53).

We previously reported that the galU gene of *P. aeruginosa*, which encodes a UDP-glucose pyrophosphorylase essential for the production of UDP-glucose, is required for the synthesis of a complete LPS core (10). Thus, galU mutants are devoid of O antigen and synthesize a defective LPS core with a electroforetic banding pattern similar to that of the LPS of algC mutants (10). In the current study, we evaluated galU mutants of *P. aeruginosa* for serum sensitivity, in vitro cytotoxicity, and virulence in murine models of corneal infections and pneumonia.

### MATERIALS AND METHODS

**Bacterial strains.** The bacterial strains and plasmids used in these experiments, along with their relevant characteristics and sources, are listed in Table 1. To assess swimming motility, isolated colonies were stabbed into 0.4% L-agar plates. Assessment of growth were performed with standard methods. To calculate the 50% infectious dose (ID₅₀), the maximal corneal pathology scores were recorded. The scoring scheme was as follows: 0, normal cornea; 1, opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the eyeball following inflammatory disease). To calculate the 50% infectious dose (ID₅₀), the maximal corneal pathology achieved in an individual mouse was used. Mice with a pathology grade of 2 or higher were considered infected.

**Murine corneal infection model.** We used our well-described model for *P. aeruginosa* corneal infection (37). Scratch-injured eyes (one eye per animal) of C3H/HeN mice (five mice per group) were infected with 5 μl containing the *P. aeruginosa* challenge strain. Mice were then monitored daily for 1 week, and corneal pathology scores were recorded. The scoring scheme was as follows: 0, macroscopically identical to the infected contralateral control eye; 1, faint opacity partially covering the pupil; 2, dense opacity covering the pupil; 3, dense opacity covering the entire anterior segment; 4, perforation of the cornea and/or phthisis bulbi (shrinkage of the eyeball following inflammatory disease). To calculate the 50% infectious dose (ID₅₀), the maximal corneal pathology achieved in an individual mouse was used. Mice with a pathology grade of 2 or higher were considered infected.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. aeruginosa</strong> strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type strain, LPS-smooth, noncytotoxic, serogroup O2/O5</td>
<td>M. Vasil (10)</td>
</tr>
<tr>
<td>PAO1 galU</td>
<td>galU mutant of PAO1 in which the galU gene is disrupted by an accCl cassette; Gm'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47, 48</td>
</tr>
<tr>
<td>PAO1(pUCP19exoUspcU)</td>
<td>galU mutant of PAO1 carrying plasmid pUCP19exoUspcU; Cb'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>PAO1(pUCP19exoUspcU)</td>
<td>galU mutant of PAO1 carrying plasmid pUCP19exoUspcU; Gm'&lt;sup&gt;a&lt;/sup&gt; and Cb'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1 galU</td>
<td>galU mutant of PAO1 in which the galU gene is disrupted by an accCl cassette; Gm'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>PAO1(pUCP19exoUspcU)</td>
<td>galU mutant of PAO1 carrying plasmid pUCP19exoUspcU; Gm'&lt;sup&gt;a&lt;/sup&gt; and Cb'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22</td>
</tr>
<tr>
<td>PAO1(pUCP19exoUspcU)</td>
<td>galU mutant of PAO1 carrying plasmid pUCP19exoUspcU; Gm'&lt;sup&gt;a&lt;/sup&gt; and Cb'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
</tr>
<tr>
<td>AK1012</td>
<td>LPS-defective derivative of PAO1 (absent O antigen, incomplete core)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUCP20</td>
<td>Broad-host-range shuttle vector; Ap'&lt;sup&gt;a&lt;/sup&gt; Cb'&lt;sup&gt;a&lt;/sup&gt;</td>
<td>47, 48</td>
</tr>
<tr>
<td>pUCP19exoUspcU</td>
<td>pUCP19-based plasmid carrying the exoU gene and its chaperone gene, spcU</td>
<td>2</td>
</tr>
<tr>
<td>pUCP18Ω-Tc</td>
<td>pUCP18 harboring a 2.1-kb Smal Ω-Tc interposon fragment derived from pH45-Ω-Tc inserted into the ScaI site within the bio gene; Tc'</td>
<td>10</td>
</tr>
<tr>
<td>pCD204</td>
<td>pUCP18ΩTc carrying a 1.036-bp PCR fragment encompassing galU from PAO1 in the same orientation as the lac promoter; Tc'</td>
<td>10</td>
</tr>
</tbody>
</table>

* Abbreviations: Ap, ampicillin; Gm, gentamicin; Cb, carbenicillin; Tc, tetracycline.
garding the use of animals in research. We used our previously described (2) model of acute fatal pneumonia following intranasal application of P. aeruginosa in mice sedated with ketamine and xylazine (0.2 ml of a mixture of 6.7 mg/ml ketamine and 1.3 mg/ml xylazine in 0.9% saline injected intraperitoneally). For quantification of CFU in lungs and spleens, mice were sacrificed with CO2 at the indicated time points, and then organs were harvested, weighed, and homogenized in water containing 1% proteose peptone. Homogenates were diluted in PBS containing 1% fetal calf serum and then plated on TSA for enumeration of CFU after overnight growth at 37°C. The limit of detection was 1 CFU in 100 μl of the undiluted tissue homogenate, which corresponded to approximately 100 CFU per g for the spleens or lungs.

Histopathology was performed as previously described with lungs fixed in 1% paraformaldehyde in PBS instilled via the trachea after euthanasia (38). For survival analyses, mice were monitored daily for 10 days to assess mortality. Moribund animals were sacrificed and considered nonsurvivors. In some experiments, mice were treated with doxycycline hydrochloride (Sigma, St. Louis, Mo.), 30 mg/kg given intraperitoneally once a day starting 1 day prior to challenge to promote retention of complementing plasmids. For intranasal 50% lethal dose (LD50) experiments, groups of four or five mice were inoculated nasally with various doses of each P. aeruginosa strain and monitored to day 10 for mortality, with most mortality occurring by day 3 for the wild-type strains and day 5 for the galU mutants. LD50s were calculated by probit or logit analysis with natural-log-transformed bacterial doses and the Systat software program (Systat Software Inc., Richmond, Calif.).

Statistical analyses. Serum sensitivity (percent surviving) and percent cytotoxicity were assessed for significance by analysis of variance with Fisher’s protected least significant difference (PLSD) test used for pairwise comparisons with the Statview software program (SAS Institute, Cary, N.C.). Where appropriate, Bonferroni correction for multiple comparisons was performed. Nonparametric data were evaluated by Mann-Whitney U test with Statview. Survival data were analyzed by Fisher’s exact test or by survival analysis with the Kaplan-Meier method, also with Statview.

RESULTS

Motility and growth characteristics. The swimming and twitching motilities of the galU mutant of PAO1 were not significantly different from those of its wild-type counterpart (data not shown). Both PA103 and its galU mutant displayed no swimming motility, as expected, but they had similar twitching motilities (data not shown). Growth curves were also similar, and there were no obvious differences in lag time or onset of stationary phase. Doubling times in Luria broth were 34 and 37 min for PAO1 and its galU mutant, respectively, and 34 and 38 min for PA103 and its galU mutant, respectively. In a minimal salts medium containing 0.5% glucose as the sole carbon source (33), doubling times were 66 and 68 min for PAO1 and its galU mutant, respectively, and 71 and 80 min for PA103 and its galU mutant, respectively.

Serum sensitivity. With unadsorbed pooled human serum, we found that the galU mutant of PAO1 was significantly more serum sensitive than the wild-type strain PAO1 at serum concentrations of 10 and 20% (Fig. 1). The wild-type strain of PA103 was more serum sensitive than a typical LPS-smooth strain, as has been noted previously (G. B. Pier, unpublished observations). Because of this, the difference seen with the galU mutant of PA103 was not as pronounced and only tended significantly more toward statistical significance. As expected, a known LPS-mediated cytotoxicity variant of strain PAO1, ExoU+ PAO1, which expresses the ExoU cytotoxin from a plasmid, was highly cytotoxic even at an MOI as low as 0.5:1 (Fig. 2A).

The galU mutant of ExoU+ PAO1 and ExoU+ PAO1 showed similar levels of cytotoxicity. Controls with PAO1 carrying the empty vector pUCP19 and its galU mutant carrying this vector showed the same low levels of cytotoxicity as did the wild-type strain PAO1 (data not shown). At two of the three MOIs tested, the galU mutant of the inherently cytotoxic strain PA103 was more cytotoxic than the wild-type strain (Fig. 2B). While these differences were statistically significant, they were modest and of unclear biological significance. Taken together, these data suggest that an intact LPS is not required for ExoU-mediated cytotoxicity. It is also noteworthy that ExoU+ PAO1 has greater cytotoxicity than PAO1 at lower MOIs. This may relate to the facts that ExoU+ PAO1 is a recombinant strain expressing ExoU from a high-copy-number plasmid and/or that PA103 does not have the exoS gene, while PAO1 does (14).

Virulence in murine corneal infection. In our model of corneal infection after scratch injury (37), the galU mutants of PAO1 and PA103 were highly attenuated for virulence (Fig. 3). After challenge with PAO1 at 106 CFU per eye, pathology with scores of ≥2 developed in all mice by day 3, and the scores were significantly higher than those produced from PAO1 galU at 106 CFU per eye (P < 0.01 by Mann-Whitney U test).

![Graph](http://iai.asm.org/)

**FIG. 1.** Serum sensitivity of P. aeruginosa PAO1 and PA103 and their corresponding galU mutants in comparison to the LPS-rough P. aeruginosa strain AK1012. Bacteria were incubated for 1 h with pooled human serum. Bars represent the mean of three separate experiments, and error bars represent the standard error of the mean. *P < 0.01 in comparison to PAO1 at similar percent serum by analysis of variance with Fisher’s PLSD (significant after correction for multiple comparisons).
suggesting ID50s for PAO1 and PAO1 galU of \(10^6\) CFU and \(10^8\) CFU, respectively. We previously reported (37) that the ID50 of PAO1 in this model, defined by the proportion of mice achieving a pathology score of \(\geq 2\), was \(10^3\) CFU per eye, with \(10^4\) CFU as the upper limit of the 95% confidence interval. After challenge with the cytotoxic strain PA103 at \(10^4\) CFU per eye, pathology scores of \(\geq 2\) developed in all mice by day 3 and were significantly higher than with PA103 galU at \(10^8\) CFU per eye (\(P = 0.02\) by Mann-Whitney U test). These data suggest ID50s for achieving a pathology score of \(\geq 2\) for strains PA103 and PA103 galU are \(<10^4\) CFU and \(>10^6\) CFU, respectively, signifying at least a 4 log10 difference in ID50.

**Virulence in murine acute lethal pneumonia.** With our previously described murine model of acute pneumonia following intranasal administration of *P. aeruginosa* to anesthetized adult mice (2), we found that the galU mutant of PAO1 was significantly attenuated, possessing an LD50 about 1 log10 above that of PAO1 (Fig. 4). For the cytotoxic strains, the degree of attenuation of virulence was more pronounced. The galU mutant of ExoU+ PAO1 had an LD50 2 log10 higher than that of ExoU+ PAO1, while the galU mutant of PA103 had an LD50 1.4 log10 higher than that of the wild-type strain (Fig. 4). Interestingly, however, the galU mutant of the highly virulent PA103 strain was still more lethal than the wild-type, noncytotoxic PAO1 strain.

We next assessed the viable counts of the galU mutants in comparison to their wild-type counterparts in lungs and spleens following intranasal inoculation. We previously reported that death in this acute pneumonia model correlates with systemic bacterial dissemination, as measured by CFU in the spleen or liver (2). These prior experiments (2) used LPS-smooth strains of *P. aeruginosa*, both ExoU expressing (cyto-
toxic) and ExoU deficient (noncytotoxic). As shown in Fig. 5A, in comparison to mice challenged with a similar dose of PAO1, there were twofold lower CFU/g of lung in mice challenged with the galU mutant of PAO1 after 1 h (P < 0.01 in comparison to PAO1 by Mann-Whitney U test); and this difference increased to 3 log_{10} by 20 h (P < 0.01 in comparison to PAO1 by Mann-Whitney U test). At 20 h, there was minimal dissemination to the spleen in mice challenged with the galU mutant, with median levels about 3 log_{10} lower than those in mice challenged with PAO1 (P < 0.01 in comparison to PAO1 by Mann-Whitney U test). Interestingly, by day 4 after challenge with the galU mutant of PAO1, at which time three of six mice had died and the remainder appeared sick, the viable counts in the lungs had fallen dramatically compared with the 20-h time point (2.5 log_{10} lower, with a median of 5.1 × 10^4 CFU per g, 25th percentile of 2.8 × 10^4, and 75th percentile 6.4 × 10^4); and there were still no CFU detected in the spleens, even of the mice that had died. Thus, even when the galU mutant of PAO1 caused mortality from pneumonia after intranasal inoculation, there were relatively few bacteria in the lung and no systemic spread of bacteria.

Histopathological analysis of the lungs of mice that received intranasal PAO1 galU (7 × 10^8 CFU) showed moderate to severe pneumonia 20 h after inoculation, with alveoli and airways filled predominantly with neutrophils and, to a lesser extent, edema, hemorrhage, and bacterial microcolonies (Fig. 6). We previously reported similar pathology following infection with the wild-type strain PAO1 (38). As another marker of lung injury, we also assessed the weights of the lungs of mice infected with the galU mutants compared with those of mice infected with the wild-type strains. Although the mean weight of lungs (± standard deviation) after infection with PAO1 (0.260 ± 0.015 mg) was significantly higher than that after infection with PAO1 galU (0.189 ± 0.024 mg), the weights of the PAO1 galU-infected lungs were still significantly higher than those of uninfected age-matched mice (0.139 ± 0.019 mg), with P = 0.002 for PAO1 galU-infected versus uninfected lungs by analysis of variance with Fisher’s PLSD (P < 0.017 is considered significant after correction for multiple comparisons). Lung weights after infection with the cytotoxic strains were higher, and here the weights of the lungs infected with the galU mutants were nearly identically as high as those of the lungs infected with the corresponding wild-type strains: 0.306 ± 0.026 mg and 0.305 ± 0.025 mg for lungs infected with PA103 and PA103 galU, respectively, and 0.241 ± 0.012 mg and 0.237 ± 0.035 mg for lungs infected with ExoU⁺ PAO1 and ExoU⁺ PAO1 galU, respectively. These observations suggest that significant lung injury can occur even in the absence of a complete LPS caused by the galU mutation.

In separate experiments to verify that the attenuation in virulence was due only to a defective galU gene, mice were challenged with the complemented galU mutant of PAO1 (strain PAO1 galU[pCD204]) or the galU mutant carrying the empty cloning vector (strain PAO1 galU[pUCP182-Tc]) (Fig. 5B and 5C). This complementing plasmid was previously shown to repair the LPS defect (10). In these studies, it was apparent that carrying the tetracycline resistance plasmid conferred a survival impairment for the bacteria, since viable counts in the lung were about 2 log_{10} lower than those of PAO1 and PAO1 galU at a similar time point despite inocula that were only about twofold lower (compare Fig. 5A and 5B). This impairment was also seen when the mice were treated with daily intraperitoneal doxycycline starting the day prior to infection to select for retention of the plasmid (data not shown). Despite these limitations, as shown in Fig. 5B, the bacterial burdens in the lung were significantly lower in mice challenged with the galU mutant carrying the empty vector compared with the complemented mutant (P < 0.01 by Mann-Whitney U test), and the levels in the spleen of the mutant.
carrying the empty vector were also marginally lower ($P = 0.068$ by Mann-Whitney U test). Complementation of the $galU$ mutant of PAO1 did restore lethality following intranasal inoculation of $2 \times 10^8$ CFU in mice treated with doxycycline (Fig. 5C), and this was statistically significant.

We also assessed viable counts in the lungs and spleen six hours after inoculation with the cytotoxic strains ExoU$^+$ PAO1 (Fig. 7A and 7B) or PA103 (Fig. 7C) and their corresponding $galU$ mutants. Compared to bacterial levels achieved by parental strains, viable counts in the lungs were significantly lower in mice challenged with the $galU$ mutant of ExoU$^+$ PAO1 ($P < 0.01$ in comparison with ExoU$^+$ PAO1 by Mann-Whitney U test), whereas the lung bacterial counts were similar to parental levels in mice challenged with the $galU$ mutant of PA103. For these cytotoxic strains, we also observed minimal dissemination of the $galU$ mutants to the spleen, while the wild-type strains disseminated significantly more. The difference in splenic CFU between ExoU$^+$ PAO1 and its $galU$ mutant was more pronounced after challenge with a higher dose (Fig. 7A and 7B). These experiments with the higher dose of ExoU$^+$ PAO1 verified that even when levels in the lung of the $galU$ mutant were as high as those of ExoU$^+$ PAO1 that are associated with dissemination, the $galU$ mutant was still not detected in the spleen.

**DISCUSSION**

In the present study, we investigated the role of the LPS O antigen and outer core of *P. aeruginosa* in the pathogenesis of murine corneal infections and acute lethal pneumonia with $galU$ mutants, which are genetically defined LPS-rough mutants. In prior studies, we found that the LPS of these $galU$ mutants is devoid of O antigen and has a truncated outer core, running similarly on sodium dodecyl sulfate-polyacrylamide gel electrophoresis as the LPS of *P. aeruginosa* algC mutants (10). While the $galU$ gene product is responsible for synthesis of UDP-glucose from glucose-1-phosphate and UTP, the algC gene encodes a bifunctional enzyme with both a phosphoglucomutase activity, which interconverts glucose-6-phosphate and glucose-1-phosphate, and a phosphomannomutase activity (7, 62). Since $galU$ and algC mutants are both unable to synthesize UDP-glucose, it follows that the defect in the LPS core would be similar.

Many of the prior reports evaluating the virulence of LPS-rough strains of *P. aeruginosa* in animal models have used the murine burn wound infection model, which primarily evaluates bacteremia and survival after direct inoculation into the burn wound (9, 16, 30). The only one of these studies that used a genetically defined LPS-rough strain was that evaluating the algC mutant of PAO1 (16). Of note is the fact that the algC
The general limitation of the burned-mouse model is that the pathogen in the current study had growth patterns and doubling correspondingly, with ExoU from one mouse. (A and B) Low- and high-dose challenges, respectively, with ExoU+ PAO1 and its galU mutant at the indicated inocula; (C) challenge with PA103 (2.0 × 10^7 CFU for the wild-type strain and 1.3 × 10^7 CFU for its galU mutant). *, P < 0.01 in comparison to the wild-type strain by Mann-Whitney U test; #, P < 0.03 in comparison to the wild-type strain by Mann-Whitney U test.

FIG. 7. Viable counts of cytotoxic P. aeruginosa strains and their corresponding galU mutants in the lungs and spleens of C3H/HeN mice 6 h following intranasal inoculation. Each point marks the result from one mouse. (A and B) Low- and high-dose challenges, respectively, with ExoU+ PAO1 and its galU mutant at the indicated inocula; (C) challenge with PA103 (2.0 × 10^7 CFU for the wild-type strain and 1.3 × 10^7 CFU for its galU mutant). *, P < 0.01 in comparison to the wild-type strain by Mann-Whitney U test; #, P < 0.03 in comparison to the wild-type strain by Mann-Whitney U test.

The algC mutant of PAO1 was also shown to be avirulent in a neonatal mouse model of pneumonia (53) and in a murine corneal infection model (37), although the role of alginate in those models was not tested. Production of the alginate capsule should not be affected by the absence of the galU gene product, given its lack of direct involvement in the alginate biosynthetic pathway. Indeed, the galU mutants of P. aeruginosa described in this study can be made phenotypically mucoid by transfection with a cloned algT gene (17), which counteracts the usual inhibition of alginate synthesis (J. B. Goldberg, unpublished observations). The P. aeruginosa alginate capsule plays a significant role in the chronic lung infection seen in patients with cystic fibrosis (34) and has recently been shown to be a virulence factor for oropharyngeal colonization of transgenic mice with cystic fibrosis, even when expressed at a low level by a typical nonmucoid strain (5). While alginate has not traditionally been thought to play a role in acute pneumonia, there are suggestions in recent literature that it might (50, 56).

A prior report evaluating the LPS-rough P. aeruginosa strain AK1012 in an acute murine pneumonia model found that the rough strain was cleared more efficiently than a smooth strain 4 h after administration of a relatively low inoculum (31). Interestingly, the rough strain elicited almost threefold higher numbers of polymorphonuclear leukocytes in the bronchoalveolar lavage fluid. While the reasons for the increased polymorphonuclear leukocyte influx were not evaluated, it is possible that the inability of epithelial cells to take up the rough strain via CFTR might lead to overexuberant inflammation initiated by the resident macrophages and other antigen-presenting and phagocytic cells. This speculation would also fit with our own observation that the mice dying after challenge with the galU mutants did not die from continued bacterial growth in the lungs or from dissemination of bacteria. It is likely that these mice died from severe lung injury, as suggested by the lung weights and the findings on histological analysis at the earlier time point. We are currently investigating the character and degree of inflammation in pneumonia due to the galU mutants. This model is highly relevant to the study of lung injury due to pneumonia in humans because in most human cases of pneumonia, bacterial levels can be controlled by antibiotic therapy, even when exuberant inflammation is seen.

The algC mutant of PAO1 was also shown to be avirulent in a neonatal mouse model of pneumonia (53) and in a murine corneal infection model (37), although the role of alginate in those models was not tested. Production of the alginate capsule should not be affected by the absence of the galU gene product, given its lack of direct involvement in the alginate biosynthetic pathway. Indeed, the galU mutants of P. aeruginosa described in this study can be made phenotypically mucoid by transfection with a cloned algT gene (17), which counteracts the usual inhibition of alginate synthesis (J. B. Goldberg, unpublished observations). The P. aeruginosa alginate capsule plays a significant role in the chronic lung infection seen in patients with cystic fibrosis (34) and has recently been shown to be a virulence factor for oropharyngeal colonization of transgenic mice with cystic fibrosis, even when expressed at a low level by a typical nonmucoid strain (5). While alginate has not traditionally been thought to play a role in acute pneumonia, there are suggestions in recent literature that it might (50, 56).

A prior report evaluating the LPS-rough P. aeruginosa strain AK1012 in an acute murine pneumonia model found that the rough strain was cleared more efficiently than a smooth strain 4 h after administration of a relatively low inoculum (31). Interestingly, the rough strain elicited almost threefold higher numbers of polymorphonuclear leukocytes in the bronchoalveolar lavage fluid. While the reasons for the increased polymorphonuclear leukocyte influx were not evaluated, it is possible that the inability of epithelial cells to take up the rough strain via CFTR might lead to overexuberant inflammation initiated by the resident macrophages and other antigen-presenting and phagocytic cells. This speculation would also fit with our own observation that the mice dying after challenge with the galU mutants did not die from continued bacterial growth in the lungs or from dissemination of bacteria. It is likely that these mice died from severe lung injury, as suggested by the lung weights and the findings on histological analysis at the earlier time point. We are currently investigating the character and degree of inflammation in pneumonia due to the galU mutants. This model is highly relevant to the study of lung injury due to pneumonia in humans because in most human cases of pneumonia, bacterial levels can be controlled by antibiotic therapy, even when exuberant inflammation is seen.
had defects in other surface proteins, such as IscA for S. flexneri (43) and flagella for E. coli (25). Among all these studies, however, the common theme of impaired survival of galU mutants in the face of host factors such as complement does emerge, as we found in the P. aeruginosa galU mutants.

We did not uncover evidence of a defect in the TTSS of the galU mutants of P. aeruginosa in terms of their ability to produce cytotoxicity in vitro. This is important because one might expect the TTSS to be very sensitive to perturbations in the integrity of the outer membrane. A possible explanation for the resistance of the TTSS of P. aeruginosa to LPS defects is the high phosphate content of the P. aeruginosa LPS core, which is thought to play a role in maintaining the integrity of the outer membrane (55). These results also suggest that in the absence of the LPS outer core, other bacterial factors such as pili (6) and/or the type III secretion apparatus itself allow sufficient bacterial contact for delivery of ExoU via the TTSS. Overall, the current study confirms the importance of the LPS O antigen and outer core in the pathogenesis of P. aeruginosa infections of the eye and lung. We observed that, in corneal infections, galU mutants of P. aeruginosa were highly attenuated regardless of cytotoxic potential. This result was expected, given the known role of the outer core of LPS in binding to CFTPR on the corneal epithelial cells to initiate infection (59–61). Prior studies with LPS-rough algC mutants and AK1012, which have the same LPS defect as the galU mutants described here, found decreased bacterial adherence to and internalization by corneal epithelial cells (59) and, more recently, impaired intracellular viability (12) of LPS-rough strains. The increased serum sensitivity of the galU mutants may also contribute to their attenuated virulence in corneal infections, in light of the fact that complement has been shown to play a critical role in bacterial clearance in murine corneal infections (21). Furthermore, in the setting of high extracellular bacterial numbers in tear fluid predicted to result from decreased adherence and internalization of LPS-rough strains, susceptibility to complement and other serum factors present in the tear fluid would be expected to play an even more prominent role.

In lung infections, the galU mutants were also attenuated with respect to both lethality and survival of the bacteria in the lung and bloodstream. The attenuation in lethality and bacterial dissemination following pneumonia was likely due to the more rapid clearance of the galU mutants by the complement system in the bloodstream. It is possible that in the context of the severe pneumonia produced by the galU mutants, nonviable bacteria were released into the bloodstream. Such nonviable bacteria would not be detected in our model and could conceivably contribute to the inflammatory response. Systemic spread of bacteria might simply be a marker for severe pneumonia and damage of epithelial barriers. Indeed, experiments with a rabbit model of septic shock following instillation of a cytotoxic P. aeruginosa strain (PA103) into the lungs suggested that it was the leakage of inflammatory mediators such as tumor necrosis factor alpha into the systemic circulation from damaged lung epithelial barriers rather than bacteremia alone that caused septic shock (28). Nevertheless, it is clear from our results that the galU gene and, thus, an intact LPS is required for efficient systemic spread of viable bacteria during pneumonia but is not required for infection confined to the lung, for the elicitation of acute lung injury, or for pneumonia-induced mortality. Further investigations into the infections caused by galU mutants of P. aeruginosa will help elucidate the pathogenesis of lung injury and of bacterial dissemination during pneumonia.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI50036 (a Mentored Clinical Scientist Development Award [K08] granted to G.P.P.), AI22555 (G.B.P.), AI50230 (J.B.G.), and AI37652 (J.B.G.).

REFERENCES


