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Pseudomonas aeruginosa Cell-to-Cell Signaling Is Required for Virulence in a Model of Acute Pulmonary Infection

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Cell-to-cell signaling controls many virulence genes in *Pseudomonas aeruginosa*. We tested the virulence of *las* and *rhl* quorum-sensing mutants in neonatal mice. A *lasI rhlI* double mutant was nearly avirulent, and the respective single mutant strains were reduced in virulence compared with the wild-type strain. Quorum sensing plays a role in *P. aeruginosa* pneumonia in neonatal mice.

Pseudomonas aeruginosa frequently causes pneumonia (3, 6), septicemia (7), and other acute infections (25) in immunocompromised patients (for review see reference 2). This gram-negative bacterium also causes chronic lung infections in cystic fibrosis patients (11). Virulence of *P. aeruginosa* depends on both cellular and extracellular factors. Cell-associated pili, flagella, and lipopolysaccharide are important surface components of *P. aeruginosa* which facilitate attachment of the organism to host cell surfaces and activate immune responses. These cellular bacterial constituents are required for virulence in a number of models of *P. aeruginosa* infection (5, 8, 13, 24, 30, 31). Extracellular or secreted virulence factors, such as proteases (elastase and alkaline protease) and toxins (exotoxin A and the exoenzymes S, T, and U), have also been shown to be necessary for virulence in animal models of *P. aeruginosa* infection (1, 12, 13, 15, 28).

P. aeruginosa controls the expression of many of its extracellular virulence factors by quorum-sensing systems (reviewed in reference 9). Most quorum-sensing signals are acyl homoserine lactones (AHL), which diffuse in and out of gram-negative bacterial cells (14, 22). When a threshold AHL concentration is reached, the AHL binds a LuxR-type transcriptional activator that induces expression of certain genes. For *P. aeruginosa* two such systems have been described. The *las* system consists of *lasI* and *lasR* (encoding an AHL synthase and a transcriptional activator, respectively) (10, 18) and the AHL signal *N*-3-oxo-dodecanoyl homoserine lactone (19). A second *P. aeruginosa* quorum-sensing system (*rhl*) consists of *rhlI* and *rhlR* (encoding an AHL synthase and a transcriptional activator, respectively) (16, 17) and the AHL signal *N*-butyryl homoserine lactone (20, 33). These two *P. aeruginosa* quorum-sensing systems regulate expression of extracellular virulence factors (reviewed in reference 23).

The role of quorum sensing in *P. aeruginosa* virulence has only begun to be studied, although there is already great interest in using this system as a target for novel forms of antibacterial chemotherapy. A *P. aeruginosa lasR* mutant was avirulent in a neonatal mouse model of acute pulmonary infection (31). In models of systemic infection of both *Caenorhabditis elegans* and mice, a *lasR* mutant has been found to be significantly attenuated in its virulence (4, 29). Here we further examined quorum sensing in *P. aeruginosa* mutants which lack the AHL synthase genes *lasI* and *rhlI* (Table 1). Quantitative assays of elastase and rhamnolipid production demonstrated that these *P. aeruginosa* mutant strains were fully complemented when the respective *lasI* or *rhlI* gene (or both) was added back on a plasmid (21). We used a previously described neonatal mouse model of pulmonary infection to assay for *P. aeruginosa* virulence (30). Briefly, entire litters of 7- to 10-day-old strain BALB/cByJ mice (Jackson Labs) were intranasally

TABLE 1. *P. aeruginosa* strains used in this study

Strain	Relevant characteristic(s)	Source or reference
PAO1	Wild type	B. H. Iglewski laboratory
PDO100	Δ*rhlI*::Tn*501*	4
PAO-JP1	Δ*lasI*	21
PAO-JP2	Δ*rhlI*::Tn*501* Δ*lasI*	21

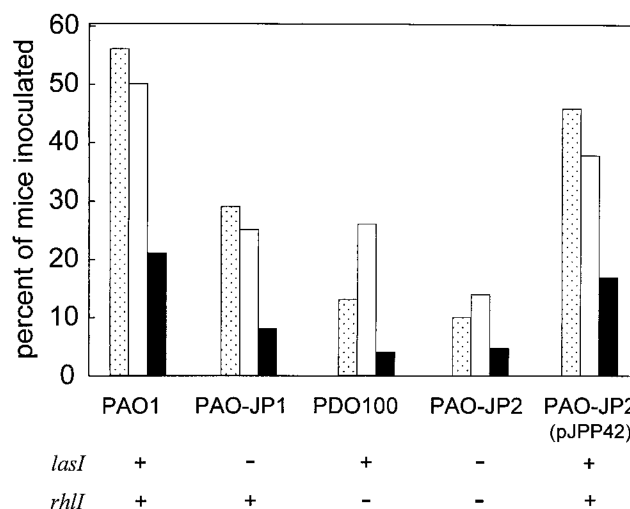

FIG. 1. Contribution of *P. aeruginosa* AHL synthase genes *lasI* and *rhl* in the pathogenesis of pneumonia, bacteremia, and mortality in neonatal mice. The percentage of the total number of mice that developed pneumonia (dotted bars), bacteremia (white bars), or died (black bars) by 18 h following intranasal inoculation with the *P. aeruginosa* strains indicated is shown: PAO1, $n = 34$; PAO-JP1 $n = 24$; PDO100, $n = 23$; PAO-JP2, $n = 21$; and PAO-JP2(pJPP42), $n = 21$. *P* values are indicated in the text.

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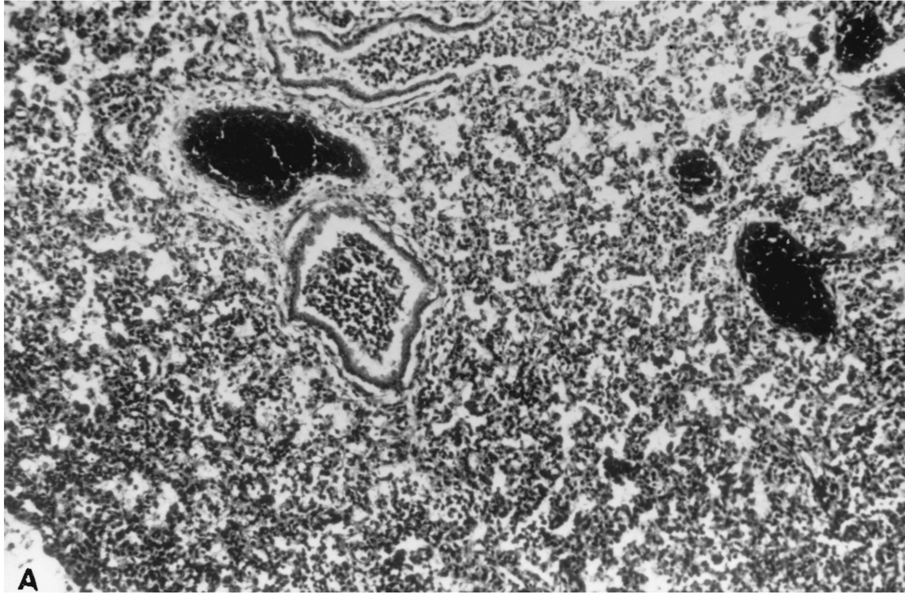
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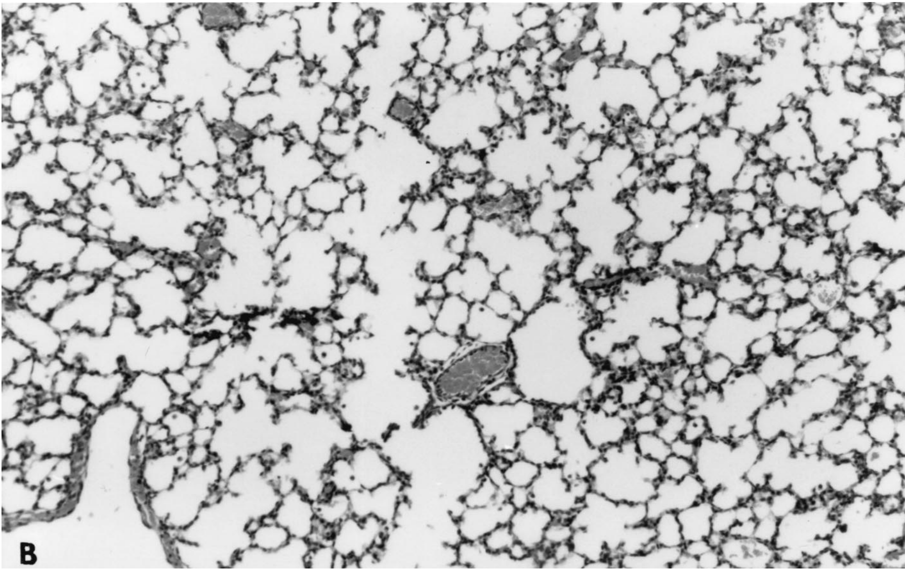
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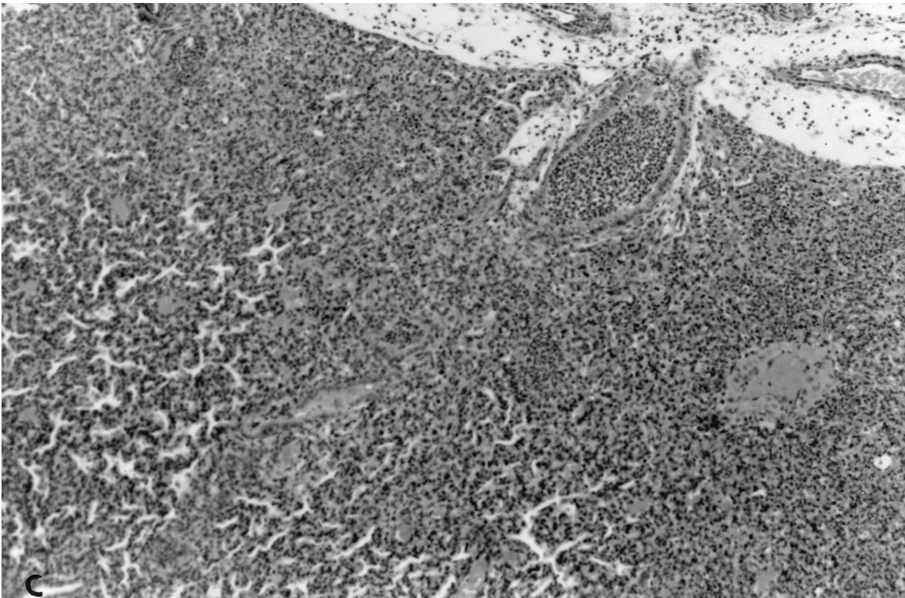
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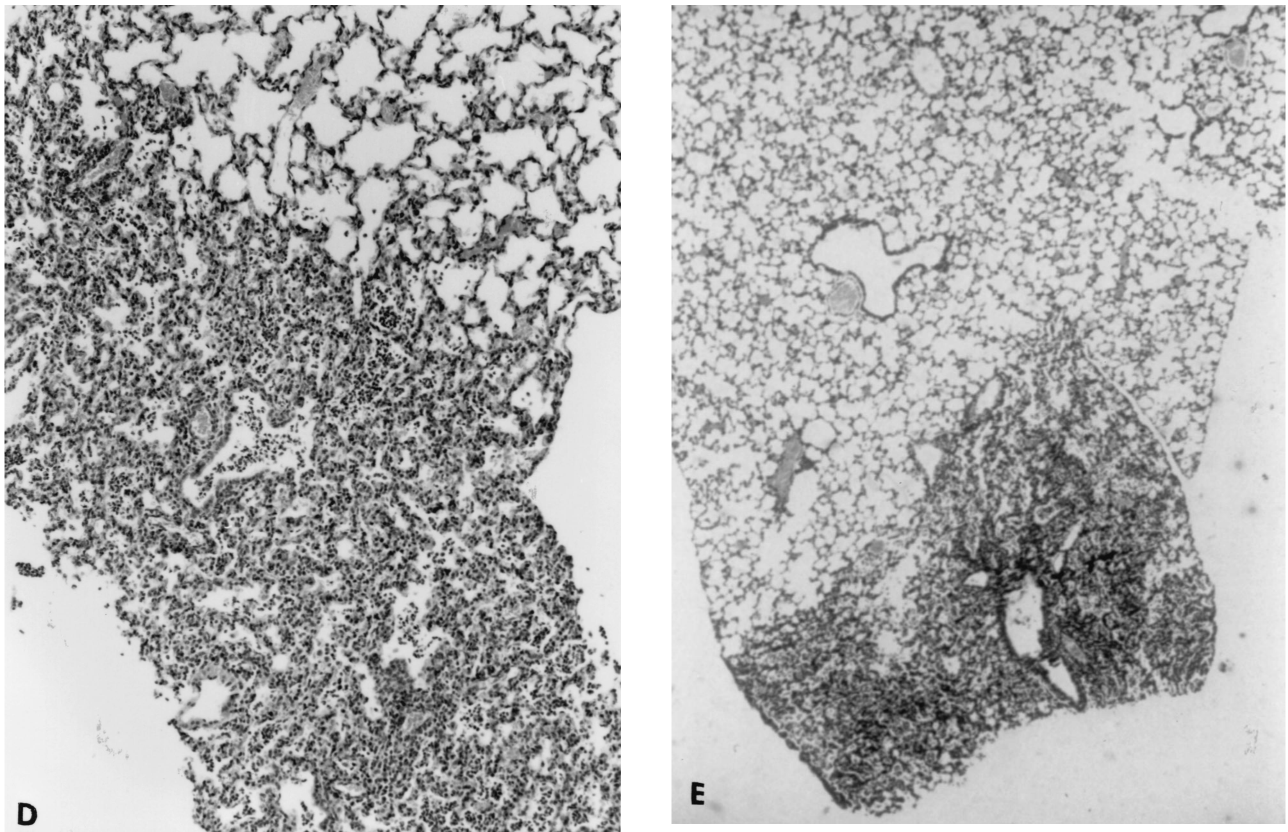

FIG. 2. Histopathology associated with infection due to *P. aeruginosa* strains. Shown are hemotoxylin-and-eosin-stained sections of murine lung 18 h following inoculation with *P. aeruginosa*. (A) PAO1-infected lung showing widespread pneumonia, with consolidation and polymorphonuclear leukocytes visible in small bronchi; (B) normal uninfected control lung; (C) PAO1-JP2(pJPP42)-infected lung showing severe pneumonia; (D) PAO-JP1-infected lung showing focal pneumonia, with several regions of normal airspaces visible; (E) PAO-JP2-infected lung showing focal pneumonia present peripherally and large areas of normal tissue. Magnifications, ×100 (A to D) and ×50 (E).

inoculated with 1.5×10^9 CFU of *P. aeruginosa* per mouse (30). Inocula were prepared from 14- to 17-h cultures grown at 37°C with shaking in M9 medium (27) containing 0.2% glucose and 1 mM $MgSO_4$. Mice were returned to the mother for 24 h and then sacrificed. Lung and spleen tissue was prepared for bacteriology (enumeration of CFU per tissue homogenate by plate counts on MacConkey-lactose agar [Difco Corp., Detroit, Mich.]) and histopathology as described previously (30). Pneumonia was defined by two criteria: (i) the presence of $>10^3$ CFU of *P. aeruginosa* per lung homogenate and (ii) histopathological evidence of destruction of the lung parenchyma, edema, and leukocyte infiltration. Bacteremia was defined as the presence of at least one CFU of *P. aeruginosa* per spleen (30).

When mice were inoculated with the *P. aeruginosa lasI rhlI* double mutant (strain PAO-JP2) ($n = 21$), only 1 mouse died, and 2 mice had $>10^3$ CFU per lung (Fig. 1). In contrast, 19 of the 34 animals inoculated with the wild-type strain, PAO1, developed confluent pneumonia throughout the lungs, and mortality occurred in 21% of the inoculated animals. Bacteremia was also reduced in mice infected with the quorum-sensing double mutant compared to that in mice infected with the wild type. Sections of lung tissue from an animal with $>10^3$ CFU per lung of the *lasI rhlI* strain, PAO-JP2, showed only a mild focal pneumonia (Fig. 2). The lung tissue of mice infected with the parental wild-type *P. aeruginosa* strain, PAO1, showed a much more severe confluent pneumonia. The quorum-sensing double mutant strain, PAO-JP2, caused significantly less pneumonia ($P < 0.001$), bacteremia ($P < 0.01$), and mortality ($P < 0.05$) than the wild-type strain as calculated using a Z test of proportions. To determine the relative contributions to virulence of the two quorum-sensing systems, the *lasI* and *rhlI* single mutant strains (PAO-JP1 and PDO100, respectively) were tested. These strains were both less virulent than the parental strain (Fig. 1). Strain PAO-JP1 (*lasI*) caused significantly less pneumonia and bacteremia ($P < 0.05$ for each) than strain PAO1. Significantly fewer mice developed pneumonia ($P < 0.001$) or bacteremia ($P < 0.05$) or died ($P < 0.05$) when inoculated with strain PDO100 (*rhlI*) than when inoculated with strain PAO1. While the *lasI* mutant caused pneumonia in approximately 30% of the animals, the *rhlI* mutant was associated with pneumonia in only 15%, although both single mutants caused bacteremia in 25% of the mice. These differences in the host response to strains PAO-JP1 and PDO100 may indicate that *rhlI* is required for the expression of certain gene products which specifically stimulate airway inflammation and result in pneumonia. The results suggest that both the *las* and *rhl* quorum-sensing systems are important for virulence of *P. aeruginosa* in the neonatal mouse model.

To confirm that virulence can be restored by complementation of the *P. aeruginosa lasI rhlI* double mutant with functional *lasI* and *rhlI* genes, mice were inoculated with strain PAO-JP2 transformed with pJPP42 expressing *rhlI* and *lasI* described previously (21). Fifty percent of the animals inoculated with strain PAO-JP2(pJPP42) developed pneumonia and 38% developed bacteremia. These findings were similar to results obtained with the parent strain, where pneumonia and bacteremia occurred in 56 and 50% of the animals, respectively. These results indicated that the low level of virulence associated with the mutant strain PAO-JP2 was due to the lack of functional

lasI and *rhlI* genes, as complementation of the wild-type genes restored virulence to nearly wild-type levels.

The effects of the *lasI* and *rhlI* mutations on *P. aeruginosa* virulence are consistent with the effects of these mutations on *P. aeruginosa* production of the virulence factors elastase and rhamnolipid (21). The focal nature of the pneumonia stimulated by strain PAO-JP2 suggests that elastase and possibly rhamnolipid expression are important in allowing the dissemination of infection and invasion of organisms into the bloodstream. Even early in the establishment of pulmonary infection, as demonstrated in this model of pulmonary disease, it appears that AHL-dependent coordination of bacterial gene expression is important in pathogenesis.

Rumbaugh et al. have recently tested the strain PAO1 *lasI* and *rhlI* mutants in a mouse burn model of *P. aeruginosa* infection and showed that these mutants were significantly less virulent than the wild-type strain (26). Thus, in two independent studies using different models of infection, mutations in quorum-sensing genes resulted in decreased virulence. A recent report has shown that at least 39 genes are controlled by quorum sensing in *P. aeruginosa* (32). Many of these genes have unknown functions, or the corresponding null mutant strains have not yet been tested in animal models for virulence. Our results indicate that functional quorum-sensing systems are important for the development of *P. aeruginosa* acute pneumonia. Further work will be needed to pinpoint which quorum-sensing-controlled virulence factors are essential for *P. aeruginosa* virulence in the neonatal mouse model of pneumonia.

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