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Pseudomonas aeruginosa Virulence Analyzed in a *Dictyostelium discoideum* Host System

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***Pseudomonas aeruginosa* is an important opportunistic pathogen that produces a variety of cell-associated and secreted virulence factors. *P. aeruginosa* infections are difficult to treat effectively because of the rapid emergence of antibiotic-resistant strains. In this study, we analyzed whether the amoeba *Dictyostelium discoideum* can be used as a simple model system to analyze the virulence of *P. aeruginosa* strains. The virulent wild-type strain PAO1 was shown to inhibit growth of *D. discoideum*. Isogenic mutants deficient in the *las* quorum-sensing system were almost as inhibitory as the wild type, while *rhl* quorum-sensing mutants permitted growth of *Dictyostelium* cells. Therefore, in this model system, factors controlled by the *rhl* quorum-sensing system were found to play a central role. Among these, rhamnolipids secreted by the wild-type strain PAO1 could induce fast lysis of *D. discoideum* cells. By using this simple model system, we predicted that certain antibiotic-resistant mutants of *P. aeruginosa* should show reduced virulence. This result was confirmed in a rat model of acute pneumonia. Thus, *D. discoideum* could be used as a simple nonmammalian host system to assess pathogenicity of *P. aeruginosa*.**

The bacterium *Pseudomonas aeruginosa* is an important causative agent of nosocomial infections, including severe pneumonia (10) and bacteremia. This opportunistic pathogen also colonizes the lungs of cystic fibrosis patients and leads to progressive lung damage, respiratory failure, and eventually death (3, 12). The seriousness of *P. aeruginosa* infections is further exacerbated by the rapid selection of antibiotic-resistant strains following antibiotic treatment (14).

Studies in mammalian hosts have shown that quorum sensing is important for the virulence of *P. aeruginosa* (28, 37, 42). Secreted components essential for *Pseudomonas* virulence, such as proteases, rhamnolipids, pyocyanin, and exotoxin A, are under the control of two quorum-sensing systems, *las* and *rhl* (Fig. 1) (31, 43). When the bacterial cell density reaches a certain threshold, the accumulation in the medium of signaling autoinducer molecules (3-oxo-C12-homoserine lactone [HSL] and C4-HSL) induces the *las* and *rhl* pathways, respectively, leading to transcription of virulence genes. Both systems involve a transcriptional regulator (LasR and RhlR, respectively) and an autoinducer synthase (LasI and RhlI, respectively). The *las* quorum-sensing system can also induce the transcription of *rhlR* and consequently activate, to some degree, the *rhl* quorum-sensing system (21).

Strategies to develop innovative treatments against *Pseudomonas* infections rely on the elucidation of virulence and antibiotic resistance mechanisms. These studies involve the characterization of mutant strains and analysis of their virulence. However, the assessment of bacterial pathogenicity in mammalian hosts is time-consuming and expensive. Therefore, alternative yet equally relevant host systems would be extremely useful. *Pseudomonas* is remarkable in its ability to infect a number of alternative host systems. Plants (34), insects (5, 15), and nematodes (41) are susceptible to *Pseudomonas* infections, revealing the ubiquitous nature of a number of its virulence factors (33). In this study, a single-celled organism, the amoeba *Dictyostelium discoideum*, was used to examine *P. aeruginosa* virulence factors. A good correlation between results obtained in the *Dictyostelium* model and in a mammalian host system was observed, demonstrating the usefulness of this system as a novel tool for the analysis of virulence determinants of *P. aeruginosa*.

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MATERIALS AND METHODS

Strains and culture conditions. The *D. discoideum* wild-type strain DH1-10 used in this study is a subclone of DH1 (8). Cells were grown at 21°C in HL5 medium (14.3 g of peptone per liter [Oxoid], 7.15 g of yeast extract per liter, 18 g of maltose per liter, 0.64 g of $Na_2HPO_4 \cdot 2H_2O$ per liter, 0.49 g of KH_2PO_4 per liter [pH 6.7]) (7) and subcultured twice a week. When indicated, *Klebsiella pneumoniae* was used as a growth substrate for *D. discoideum* (35).

P. aeruginosa strains used in this study are described in Table 1. PT5 is our laboratory wild-type PAO1 strain. PT502 was constructed by transducing the *lasI*::Tet mutation from strain PAO-JP1 (30) into the *rhlI* mutant strain PT454 by using the lipopolysaccharide-specific phage E79 *tv2* (26). Tc^r and Hg^r transductants were checked by Southern hybridization as described previously (17).

The corresponding regulator genes of the three efflux pump-overexpressing mutants PT149 (MexEF-OprN overexpressor), PT625 (MexAB-OprM overexpressor), and PT648 (MexCD-OprJ overexpressor) were sequenced. The strains PT149 (NfxC) and PT637 (NfxC, *mexE*) were described recently in detail (16). Briefly, the *mexT* transcriptional activator gene (24) is interrupted by an 8-bp insertion in our *P. aeruginosa* wild-type strain, PT5. In the NfxC mutant PT149, the 8-bp insert is not present, yielding a functional *mexT* gene able to cause overexpression of the MexEF-OprN efflux system. To obtain PT637, the *mexE* gene was inactivated in PT149, restoring wild-type antibiotic susceptibility. PT625 did not contain any mutation in the *mexR* regulator gene of the *mexAB-*

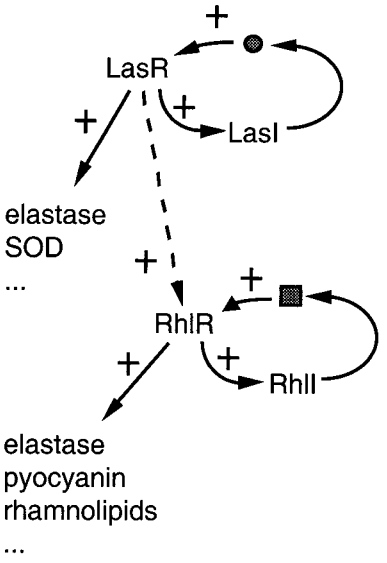

FIG. 1. Quorum sensing in *P. aeruginosa*. In *P. aeruginosa*, secreted components such as proteases (LasB elastase), rhamnolipids, and pyocyanin are under the transcriptional control of two quorum-sensing systems, *las* and *rhl*. Both systems involve a transcriptional regulator (LasR and RhlR, respectively) and an autoinducer synthase (LasI and RhlI, respectively). When the bacterial density reaches a threshold, the accumulation in the medium of signaling autoinducer molecules (● and ■) induces the *las* and *rhl* pathways, leading to transcription of virulence genes. The *las* quorum-sensing system can also induce, to some extent, the *rhl* quorum-sensing system. SOD, superoxide dismutase.

oprM efflux operon. However, since the strain was shown by Western blot analysis to overexpress the MexAB-OprM efflux pump (Köhler et al., unpublished observation), the strain was considered to be a NalC mutant (40). Strain PT648, which overexpresses the MexCD-OprJ system, was found to contain a 2-bp (AC) deletion at codon 19 of its cognate repressor gene, *nfxB*, resulting in overexpression of MexCD-OprJ.

Bacteria were grown overnight at 37°C on Luria-Bertani (LB) agar. Single colonies were inoculated into 5 ml of PB (2% [wt/vol] peptone, 0.3% [wt/vol] $MgCl_2 \cdot 6H_2O$, 1% [wt/vol] K_2SO_4) (11) in a 50-ml flask and grown at 37°C for 8 h prior to use. Under these conditions, similar optical densities at 600 nm (OD_{600}s) were obtained for each strain, and the induction of quorum sensing was maximal. The growth of various strains in rich medium (PB) and in defined M9 salts medium (23), supplemented with 0.2% glucose, 2 mM $MgSO_4$, and trace elements, was tested by measuring the OD_{600}.

Effect of *P. aeruginosa* on *D. discoideum* growth. The inhibition of *D. discoideum* clonal growth by *P. aeruginosa* was assayed by mixing 200 *D. discoideum* cells with 300 μl (6×10^8 CFU) of *K. pneumoniae* culture and 10 μl (10^7 CFU) of *P. aeruginosa* culture and plating immediately on SM agar (7). The plates were then incubated for 5 days at 25°C to allow the growth of *Dictyostelium* clones.

Quantitative measurements of *D. discoideum* growth on a lawn of *P. aeruginosa* cells alone were obtained by first plating 200 μl (2×10^8 CFU) of *P. aeruginosa* culture on SM agar. The bacterial lawn was then spotted with eight 5-μl droplets containing serial dilutions of *D. discoideum* cells (50,000 cells in drop 1, 10,000 cells in drop 2, 2,000 cells in drop 3, etc., to 1 cell in drop 8). The plates were incubated for 5 days at 25°C, and the highest dilution at which *D. discoideum* growth was visible was recorded.

Effect of *Pseudomonas* supernatants on *Dictyostelium* cells. *Pseudomonas* bacteria (PT5, PT531, or PT712) were grown in HL5 medium at 37°C overnight (16 h). The bacteria were then pelleted by centrifugation (10 min at 7,000 × *g*), and the supernatant was collected and filtered (0.22-μm pore size). Bacterial supernatants were applied to *Dictyostelium* cells observed in phase contrast with a Zeiss Axiovert 100 microscope, and pictures were recorded every 30 s with a Hamamatsu Orca camera and analyzed with OpenLab 3 software. Cells were counted, and their number was plotted as a function of time after addition of bacterial supernatant.

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

Strain	Phenotype or genotype	Source or reference
PT5	PAO1 wild type	35 Laboratory collection
PT149	PT5NfxC (previously called PAO-7H)	18
PT454	PT5Δ*rhlI*::Tn*501* Hg^r	17
PT462	PT5Δ*rhlR*::Tn*501* Hg^r	17
PT466	PT5Δ*lasI* Tc^r	17
PT498	PT5Δ*lasR* Tc^r	17
PT502	PT5Δ*rhlI*::Tn*501* Δ*lasI* Hg^r Tc^r	This study
PT531	PT5*rhlR*::Tn*501* Δ*lasR* Hg^r Tc^r	17
PT625	PT5NalC	19
PT637	PT149*mexE*::ΩHg	20
PT648	PT5*nfxB*	19
PT712	PT5*rhlA*::Gm	17

To prepare rhamnolipids, wild-type strain PT5 was grown in M9 medium supplemented with 0.2% glycerol, 2 mM $MgSO_4$, trace elements, and 0.05% glutamate as a nitrogen source instead of NH_4Cl. After growth for 48 h at 37°C, cultures were centrifuged at 10,000 × *g* for 10 min, and rhamnolipids were extracted from the supernatant with 2 volumes of diethyl ether. Pooled ether extracts were extracted once with 20 mM HCl, and the ether phase was evaporated. The residue was dissolved in water. Rhamnolipid concentration was determined by the orcinol assay (27) with rhamnose as a standard, considering that 1 mg of rhamnose corresponds to 2.5 mg of rhamnolipid (27).

Virulence test in rats. The model of acute *P. aeruginosa* pneumonia used in this study is based on the model by Cash et al. (4) and has been modified as described previously (16). Briefly, bacteria (10^6 CFU) were injected in agar-enmeshed beads into anesthetized male Sprague-Dawley rats (Charles River, Saint Aubin les Elbeufs, France) via the transtracheal route. Animals develop an acute bronchopneumonia characterized by an increase in lung weight. Control animals, which were inoculated with noninfective beads, all survived. Virulence of strains was determined by comparing mortality and time to death. For deceased animals, the bacterial load in the lungs was determined after tissue homogenization by plating dilutions of the homogenate on LB agar plates.

RESULTS

Inhibition of *Dictyostelium* growth by *P. aeruginosa*: role of quorum sensing. *Dictyostelium* amoebae are unicellular organisms that feed phagocytically upon bacteria, such as *K. pneumoniae*. When *Dictyostelium* cells are plated with *K. pneumoniae* bacteria, each amoebal cell creates a plaque in the bacterial lawn, where bacteria have been phagocytosed (Fig. 2A). We observed that when wild-type *P. aeruginosa* bacteria were added to the *K. pneumoniae* lawn, growth of *Dictyostelium* was completely inhibited (Fig. 2B). These results are in agreement with those of earlier studies that demonstrated that *P. aeruginosa* was a particularly inadequate bacterial growth substrate for *D. discoideum* (9, 36). We therefore investigated whether this growth inhibition was related to the production of virulence factors by *P. aeruginosa*, in particular those controlled by the *las* and *rhl* quorum-sensing systems. Isogenic derivatives of our wild-type strain, PT5, mutated in one of the quorum-sensing genes *lasI*, *lasR*, *rhlI*, or *rhlR*, were tested for growth inhibition of *Dictyostelium* cells. Interestingly, *P. aeruginosa* mutants affected in the *rhl* system, namely the *rhlR* (Fig. 2C) and *rhlI* (Table 2) mutants, were permissive—i.e., caused no inhibition of *D. discoideum* growth in this qualitative assay. The *las* system, however, did not seem to play an essential role, since both the *lasR* (Fig. 2D) and *lasI* mutants (Table 2) were still inhibitory for *Dictyostelium* growth. As would be expected,

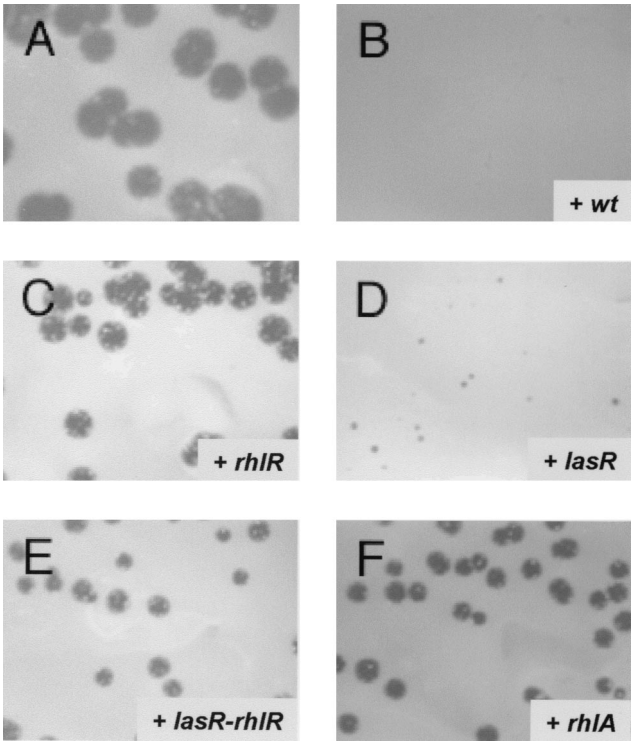

FIG. 2. Growth of *D. discoideum* clones in the presence of *Klebsiella* and *P. aeruginosa*. Approximately 200 *D. discoideum* cells were plated with a lawn of *K. pneumoniae* bacteria alone (A) or supplemented with *P. aeruginosa* strain PT5 (wild type [wt]) (B), PT462 (*rhlR* [C]), PT498 (*lasR* [D]), PT531 (*lasR-rhlR* [E]), or PT712 (*rhlA* [F]). Growth of *D. discoideum* created plaques in the bacterial lawn after 5 days of incubation at 25°C.

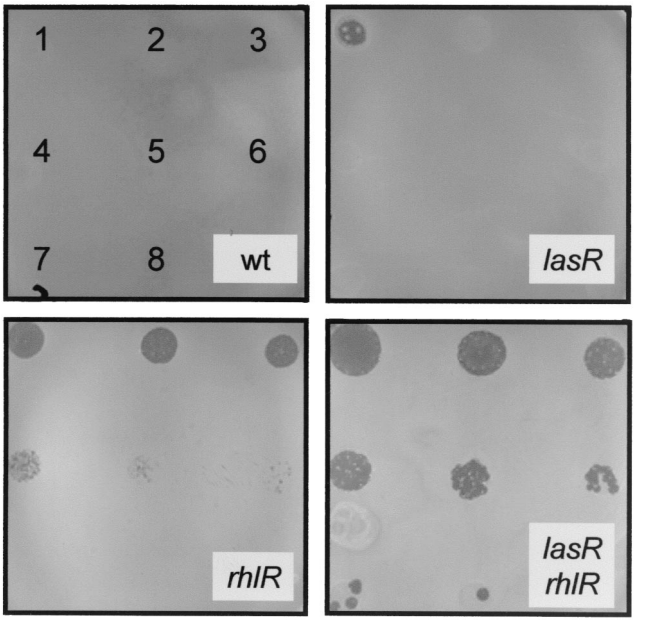

FIG. 3. Quantitative assessment of *D. discoideum* growth in the presence of *P. aeruginosa*. *Dictyostelium* cells were applied as droplets onto a lawn of pure *P. aeruginosa* bacteria, as represented in the upper left panel. The numbers of *Dictyostelium* cells applied were 50,000 in drop number 1, 10,000 in 2, 2000 in 3, etc., as described in Materials and Methods. After 5 days at 25°C, the ability of *Dictyostelium* cells to create plaques in the bacterial lawn was recorded. The bacteria used here were PT5 (wild type [wt]), PT498 (*lasR*), PT462 (*rhlR*), and PT531 (*lasR-rhlR*). In a scale measuring growth of *Dictyostelium* cells, the results obtained were scored 0 for wild-type PT5, 1 for PT498, 5 for PT462, and 8 for PT531.

the *rhlR-lasR* double mutant was also permissive for *Dictyostelium* growth (Fig. 2E; Table 2).

In these experiments, *Dictyostelium* was grown, however, in the presence of both *Klebsiella* and *Pseudomonas* bacteria.

TABLE 2. Growth of *D. discoideum* in the presence of various *P. aeruginosa* mutants

Strain	*D. discoideum* growth on substrate(s)[a]	
	K. pneumoniae + *P. aeruginosa*	*P. aeruginosa*[b]
PT5 (wild type)	−	0
PT462 (*rhlR*)	+	5
PT498 (*lasR*)	−	1
PT531 (*rhlR-lasR*)	+	8
PT454 (*rhlI*)	+	4
PT466 (*lasI*)	−	1
PT502 (*rhlI-lasI*)	+	8
PT712 (*rhlA*)	+	2
PT149 (NfxC)	+	4
PT637 (NfxC, *mexE*)	−	0
PT625 (NalC)	−	0
PT648 (*nfxB*)	−	1

[a] Clonal growth of *D. discoideum* was tested on a mixture of *K. pneumoniae* and *P. aeruginosa* cells, as described in Fig. 2. +, growth of *D. discoideum* on the given substrate; −, no growth of *D. discoideum*.

[b] The ability of *D. discoideum* cells to form plaques was tested on a lawn of pure *P. aeruginosa* cells as described in the legend to Fig. 3.

While the presence of *Klebsiella* ensures that an adequate food supply for *Dictyostelium* is present, it also makes the results more complex to interpret. For example, *Pseudomonas* bacteria might directly inhibit *Dictyostelium* growth, or they might inhibit the growth of the nutrient lawn of *Klebsiella* and thus indirectly inhibit *Dictyostelium* growth. To rule out the latter possibility, the ability of *D. discoideum* cells to grow on a lawn of pure *P. aeruginosa* bacteria was tested. To obtain quantitative results, a test was developed that allowed us to determine how many amoebal cells are necessary to create a plaque in a lawn of pure *P. aeruginosa* bacteria. In this test, 5-μl droplets were applied to a lawn of *P. aeruginosa*, with each droplet containing a defined number of *Dictyostelium* cells (droplet 1, 50,000 cells; droplet 2, 10,000 cells; droplet 3, 2,000 cells; etc., to droplet 8, 1 cell). Under these conditions, even 50,000 *Dictyostelium* cells failed to create a plaque in a lawn of wild-type *P. aeruginosa* PT5 cells (Fig. 3). In a scale measuring the growth of *Dictyostelium*, the wild-type strain was therefore scored as 0 (Table 2). When the *lasR* mutant was tested, 50,000 *Dictyostelium* cells created a plaque, while the next dilution (10,000 cells) failed to do so (Fig. 3). Consequently, the *lasR* mutant was scored as 1 (Table 2). The *rhlR* mutant permitted amoebal growth in the first five dilutions (Fig. 3) and was hence scored as 5 (Table 2). The *rhlR-lasR* double mutant was even more permissive for *Dictyostelium* growth, even at the highest dilution (Fig. 3), and thus obtained a score of 8 (Table 2). This new assay therefore defines a scale from 0 to 8, where the least

permissive (fully inhibitory) strains are scored as 0 and the most permissive (least inhibitory) strains are scored as 8 (Table 2). With this test, it was apparent that the *rhl* quorum-sensing system was essential for efficient inhibition of *Dictyostelium* growth, since both *rhlR* and *rhlI* mutants were significantly more permissive (scores of 5 and 4, respectively) than the wild-type strain (score of 0). Surprisingly, *lasR* and *lasI* mutants (score of 1) were only marginally more permissive than the wild type, although when combined with an *rhl* mutation, *lasR* and *lasI* mutants resulted in fully permissive strains, obtaining a score of 8 (Table 2).

Effect of secreted virulence factors on *D. discoideum* cells. To test whether secreted factors are at least in part responsible for the growth inhibition of *Dictyostelium*, filtered culture supernatants of the wild-type strain and of the *lasR-rhlR* double mutant PT531 were incubated with *Dictyostelium* cells. Examination by phase-contrast microscopy showed a rapid lysis of *Dictyostelium* cells completed after a 10-min exposure to wild-type supernatants (Fig. 4A, upper panels). Under the same conditions, the supernatant of the *lasR-rhlR* double mutant PT531 did not induce significant lysis of *Dictyostelium* cells (Fig. 4A, lower panels). These results indicate that wild-type bacteria secrete, under the control of the quorum-sensing systems, one or several factors that disrupt the *Dictyostelium* cells and lead to fast lysis.

Since mutants in the *rhl* system were particularly permissive for *Dictyostelium* growth, we tested whether rhamnolipids, the synthesis of which depends mainly on the *rhl* system, were involved in the fast lysis of *Dictyostelium* cells. We therefore tested the effect of supernatants from the *rhlA* mutant PT712, which is specifically defective in rhamnolipid synthesis, but is not affected in the quorum-sensing circuit (17). Supernatant from this *rhlA* mutant did not cause lysis of *Dictyostelium* cells (Fig. 4B), suggesting that rhamnolipids are essential for this effect. We then purified rhamnolipids from the supernatants of the wild-type strain PT5 and showed that these rhamnolipids at a final concentration of 10 μg/ml were able to cause lysis of the *Dictyostelium* cells (data not shown).

Since rhamnolipids are only one of the numerous virulence factors controlled by the quorum-sensing circuit, we tested growth inhibition by the *rhlA* mutant PT712, which is specifically affected in rhamnolipid production. We found that this strain was indeed permissive for *Dictyostelium* growth in a qualitative test in the presence of *Klebsiella* bacteria (Fig. 2F). In the quantitative test with PT712 as the only food source, however, the *rhlA* mutant obtained a score of only 2 (Table 2). Since the *rhlR* mutant (score of 5) was much more permissive, these results suggest that rhamnolipid-induced lysis of *Dictyostelium* cells is not the only factor responsible for growth inhibition by the wild-type strain and that other products under the control of the *rhl* quorum-sensing system are essential for the inhibition of *Dictyostelium* growth.

Analysis of multidrug-resistant mutants of *P. aeruginosa* in the *Dictyostelium* model. *P. aeruginosa* possesses several drug efflux pumps of broad specificity, which contribute to its intrinsic and acquired resistance to a wide range of antimicrobial agents. We recently observed that *P. aeruginosa* strains that overexpress one of the four multidrug efflux pumps, namely the MexEF-OprN efflux system, show reduced production of secreted virulence factors, including elastase, pyocyanin, and rhamnolipids (20). This was found to result from reduced expression of the *rhlI* gene responsible for the synthesis of the C4-HSL autoinducer in *P. aeruginosa* (20). Since *rhl* mutants were more permissive in our *Dictyostelium* host system than the wild type, we tested the behavior of multidrug efflux mutants in this model. We used isogenic derivatives of our wild-type strain PT5 overexpressing either the MexAB-OprM (NalC), MexCD-OprJ (*nfxB*), or MexEF-OprN (NfxC) efflux system. Interestingly, the multidrug-resistant MexEF-OprN overproducer (NfxC) was more permissive for *Dictyostelium* growth (score of 4) than the antibiotic-susceptible parental strain (score of 0) (Table 2). On the contrary, the MexAB-OprM (NalC) and MexCD-OprJ (*nfxB*) overproducers were as inhibitory as the wild-type strain (Table 2).

Overexpression of efflux pumps results in most cases from mutations occurring in the cognate regulator genes, which may encode a transcriptional repressor protein (MexR for MexAB-OprM and NfxB for MexCD-OprJ) or an activator protein (MexT for MexEF-OprN). In the wild-type strain PT5, MexEF-OprN is not expressed due to the insertion of 8 bp in the coding sequence of the *mexT* activator gene (20, 24). In the MexEF-OprN overproducer PT149, this insertion is removed, resulting in a functional activator protein, MexT. To test whether the permissivity of strain PT149 was due to the overexpression of the MexEF-OprN efflux pump per se or to possible pleiotropic effects caused by MexT, we tested strain PT637, which expresses a functional MexT activator, but is mutated in the *mexE* gene and is hence as susceptible to antibiotics as the wild-type strain (20). As shown previously (20), this strain expresses wild-type levels of pyocyanin, elastase, and rhamnolipids. Indeed in the *Dictyostelium* host system, PT637 (NfxC *mexE*) was as inhibitory as the isogenic wild-type strain (Table 2). This demonstrates that overexpression of the MexEF-OprN efflux pump per se in strain PT149 (NfxC) accounts for the reduced ability to inhibit *Dictyostelium* growth.

The *Dictyostelium* model is predictive for *P. aeruginosa* virulence in a rat model. To establish the correlation between *Dictyostelium* and a mammalian host system, the three efflux pump mutants and the wild-type strain PT5 were tested in the well-established model of acute pneumonia in rats (4, 16). Bacteria were injected into rat trachea, and virulence of strains was determined by assessing mortality and time to death. In this model, the wild-type strain PT5 caused mortality in 72% of the infected rats. MexAB-OprM and MexCD-OprJ overproducers exhibited a slight decrease in virulence, which was not statistically significant compared to that of the wild type (Table 3). Interestingly, the MexEF-OprN overproducer PT149 (NfxC) did not cause any mortality (100% survival). When the *mexE* gene was inactivated in the latter strain, virulence was restored to almost wild-type levels (PT637) (Table 3), suggesting that MexEF-OprN overexpression is responsible for the reduced virulence of the NfxC mutant PT149. Analysis of the lungs of deceased animals confirmed that for all strains studied, death was accompanied by high bacterial loads (Table 3). All of the mutants used in this study showed growth curves that were similar to those of the wild type, in both rich and defined minimal media (data not shown), suggesting that the decreased virulence of the MexEF-OprN overproducer was not due to reduced growth. The results from the rat model correlate well with those obtained in the *Dictyostelium* model, which could

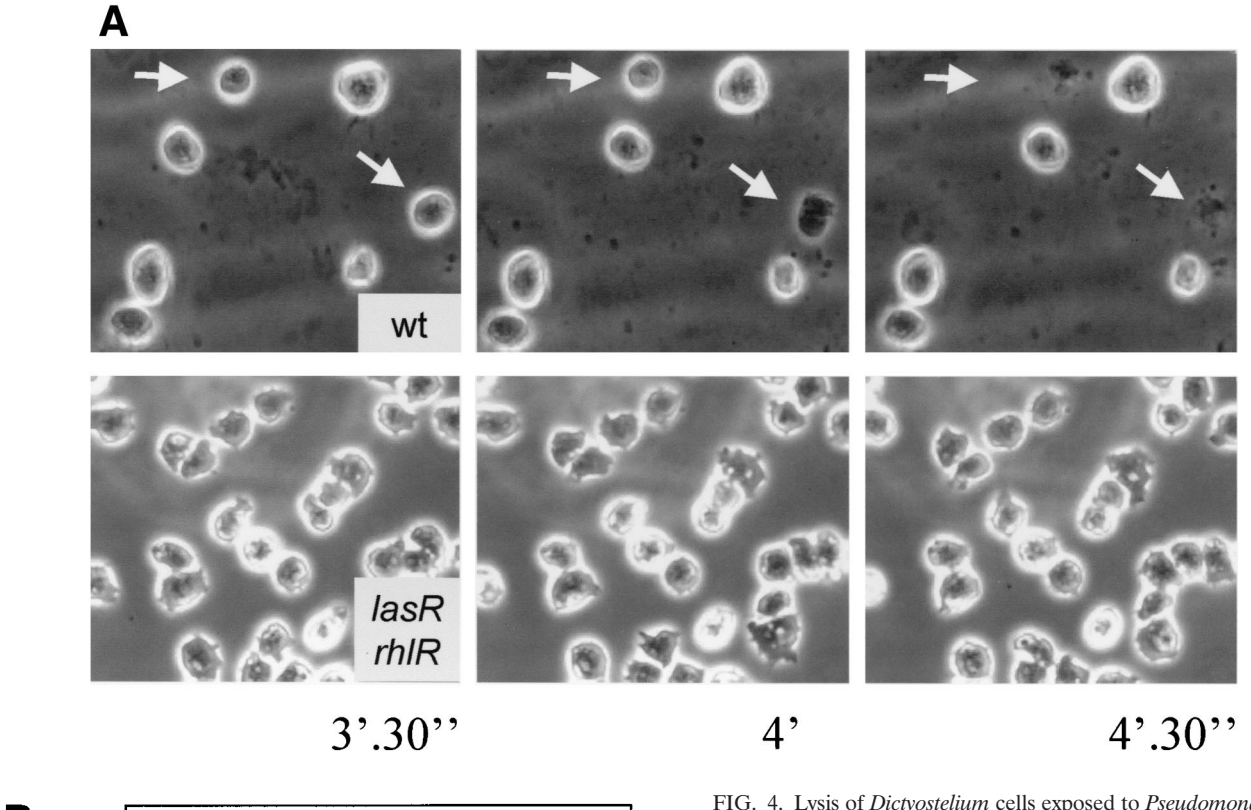

FIG. 4. Lysis of *Dictyostelium* cells exposed to *Pseudomonas* supernatants. *Dictyostelium* cells were exposed to culture supernatants of wild-type (wt) strain PT5 (A, upper panels) and the double mutant PT531 (A, lower panels) and observed with a phase-contrast microscope. Arrows indicate individual *Dictyostelium* cells being lysed by wild-type supernatant during the indicated time frame. The kinetics of cell lysis induced by supernatants of the wild type (PT5), the *lasR-rhlR* double mutant (PT531) and the *rhlA* mutant (PT712) was determined by counting *Dictoystelium* cells under the microscope in a field containing approximately 100 cells at time zero (B).

therefore be used as a novel and simple assay for testing the virulence properties of *P. aeruginosa* strains.

DISCUSSION

In the present study, *D. discoideum* was used to study the virulence of *P. aeruginosa*. With this unicellular system, the *rhl* quorum-sensing system of *P. aeruginosa* was found to be critical for inhibition of *D. discoideum* growth, while the *las* system appeared to be less important. In particular, rhamnolipids were shown to induce lysis of *Dictyostelium* cells. Furthermore, the model allowed us to predict the reduced virulence of a multidrug-resistant efflux mutant of *P. aeruginosa*, a result confirmed subsequently in a rat model of acute pneumonia.

Importance of quorum-sensing systems in mammalian and *Dictyostelium* host systems. It was reported previously that mutations affecting the quorum-sensing circuitry resulted in less virulent strains in mammalian hosts (28, 29, 37, 42). However, in these experiments, the *rhl* system did not seem to play a more predominant role in virulence than the *las* system. Indeed, in the burn wound infection model and in the acute pneumonia model, *las* and *rhl* quorum-sensing mutants exhibited similar decreases in virulence (28, 29, 37). In these studies, however, a particular PAO1 isolate, referred to here as PAO-BI, was used as the wild-type strain. PAO-BI was reported earlier to exhibit particularly low pathogenicity in a mouse corneal infection model (32). We also observed in the pneumonia model a higher mortality rate with PT5 (70%) than that observed previously with strain PAO-BI (21%) (28). These observations correlate well with our recent finding that

TABLE 3. Virulence of *P. aeruginosa* efflux mutants in a rat model of acute pneumonia

Strain	Inoculum (10^6 CFU)	*n*	% Mortality[a]	*P*	Time to death (days)	Lung wt (g)[b]	Bacterial counts in lungs (log CFU/g)
PT5 (wild type)	3.0	22	72		1.6 ± 0.63	3.98 ± 0.50	8.30 ± 0.54
PT625 (NalC)	1.7	18	50	0.19	2.1 ± 1.0	3.84 ± 0.9	8.53 ± 0.38
PT648 (*nfxB*)	2.1	11	45	0.15	1.6 ± 0.89	4.83 ± 1.35	8.85 ± 0.28
PT149 (NfxC)	2.1	18	0	<0.001	NR[c]	ND[d]	ND
PT637 (NfxC, *mexE*)	0.9	12	42	0.006	2.8 ± 1	4 ± 1.21	8.44 ± 0.48

[a] Statistical significance ($P < 0.05$) was evaluated with a Student's *t* test calculated versus PT5, except for PT637, which was calculated against PT149.
[b] Lung weight of uninfected rat, 1.0 ± 0.2 g.
[c] NR, not relevant.
[d] ND, not determined.

PAO-BI produces smaller amounts of extracellular virulence factors controlled by the *rhl* quorum-sensing system than our PAO1 strain PT5 (20). Four other PAO1 strains from four different laboratories were also tested in the *Dictyostelium* assay. Only PAO-BI was permissive for *Dictyostelium* growth (score of 4), while the other PAO1 strains were not permissive (score of 0). Since it was observed that in the PAO-BI strain, the *rhl* quorum system is attenuated (20), it would seem logical that in this situation, the *las* quorum-sensing system becomes more important for virulence, as was observed in mammalian systems. Interestingly, a *lasR* mutant in the PAO-BI background resulted in a *Pseudomonas* mutant fully permissive for *Dictyostelium* growth (score 8). In contrast, in the PT5 background, the *lasR* mutant remained inhibitory (score 1). As detailed below, these results suggest that the genetic backgrounds of the *P. aeruginosa* strains used might account for differences regarding the relative roles played by the *las* and *rhl* quorum-sensing systems in *P. aeruginosa* virulence. The fact that PAO-BI exhibits low virulence in both mammalian and *Dictyostelium* host systems and that, in this strain, the *las* quorum-sensing system is essential in both systems is further evidence for a good correlation between these two model systems.

Antibiotic resistance and virulence. Analysis with the *Dictyostelium* model revealed that a multidrug-resistant MexEF-OprN-overproducing strain is less inhibitory than the isogenic wild-type strain. This strain was also less virulent in a rat model of acute pneumonia. This is in agreement with our recent report on reduced virulence factor production by the NfxC mutant PT149. Indeed, the MexEF-OprN overproducer showed drastic decreases in pyocyanin, rhamnolipid, and elastase production, which result from reduced levels of C4-HSL autoinducer produced by this strain (20). Interestingly, in this study, we also found that strain PAO-BI overproduced the MexEF-OprN efflux pump and showed decreased production of rhamnolipids and elastase compared to our wild-type PT5 strain (20). This might explain why both PT149 and PAO-BI show the same permissive phenotype (score of 4) in the *Dictyostelium* assay.

Several studies with other bacterial pathogens have shown that resistance to antibiotics can be associated with reduced virulence. For example, several antibiotic-resistant strains of *Salmonella enterica* serovar Typhimurium (1, 14) and *Staphylococcus aureus* (25) are less pathogenic. The lower infectivity of these antibiotic-resistant strains, however, could often be attributed to decreased growth rates and has thus been thought to result mostly from a decrease in bacterial fitness (2, 22, 38). In the case of the MexEF-OprN overproducer (NfxC), however, growth rates were identical to that of the wild-type strain (data not shown) and should therefore not account for its decrease in virulence. Instead, it appears that, in this particular case, acquisition of antibiotic resistance affects bacterial virulence by interfering with the *P. aeruginosa* quorum-sensing systems.

***Dictyostelium* as a host model for bacterial pathogens.** *Dictyostelium* amoebae feed upon bacteria in the soil, and it is not surprising to observe that certain bacteria have developed strategies to resist this predator. Our results suggest that in the case of *Pseudomonas*, similar mechanisms are crucial for virulence in mammalian systems and in amoebae. The role of quorum-sensing systems was particularly important in our experiments. Many other virulence mechanisms have also been described for *Pseudomonas*, and their role in a *Dictyostelium* model was not tested here. It will be interesting in the future to determine whether other virulence mechanisms, including cytotoxic mechanisms, can also be analyzed with the *Dictyostelium* host system.

Interestingly, *Legionella pneumophila* was shown recently to replicate intracellularly in amoebae by mechanisms similar to those used for growth inside macrophages (13, 39). In particular, *dot*/*icm* mutants of *L. pneumophila* have lost the ability to replicate intracellularly in both mammalian and *Dictyostelium* cells (39). It is therefore likely that many bacterial virulence mechanisms can be analyzed by using *Dictyostelium* amoebae as a host system.

The use of *Dictyostelium* as a host model has several advantages. First, the simplicity and reproducibility of the *Dictyostelium* system surpass those of other mammalian as well as nonmammalian systems. Second, *D. discoideum* represents a powerful genetic system to analyze host-pathogen relationships. Indeed, efficient genetic tools are available to allow the isolation of *Dictyostelium* mutants with increased or decreased sensitivity to pathogens and the identification of the corresponding genes. Mutants affected in the organization of the endocytic and phagocytic pathways have also been isolated and characterized (6, 8). It will be interesting to determine how alterations of the phagocytic machinery modify the relationship of *Dictyostelium* cells with various pathogens.

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