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Inactivation of the *rhlA* gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes

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Abstract

Many of the virulence factors produced by the opportunistic human pathogen *Pseudomonas aeruginosa* are quorum-sensing (QS) regulated. Among these are rhamnolipids, which have been shown to cause lysis of several cellular components of the human immune system, e.g. monocyte-derived macrophages and polymorphonuclear leukocytes (PMNs). We have previously shown that rhamnolipids produced by *P. aeruginosa* cause necrotic death of PMNs *in vitro*. This raises the possibility that rhamnolipids may function as a 'biofilm shield' *in vivo*, which contributes significantly to the increased tolerance of *P. aeruginosa* biofilms to PMNs. In the present study, we demonstrate the importance of the production of rhamnolipids in the establishment and persistence of *P. aeruginosa* infections, using an *in vitro* biofilm system, an intraperitoneal foreign-body model and a pulmonary model of *P. aeruginosa* infections in mice. Our experimental data showed that a *P. aeruginosa* strain unable to produce any detectable rhamnolipids, due to an inactivating mutation in the single QS-controlled *rhlA* gene, did not induce necrosis of PMNs *in vitro* and exhibited increased clearance compared with its wild-type counterpart *in vivo*. Conclusively, the results support our model that rhamnolipids are key protective agents of *P. aeruginosa* against PMNs.

Keywords

Rhamnolipid; *Pseudomonas aeruginosa*; mouse models; biofilm; PMN

Pseudomonas aeruginosa is an opportunistic human pathogen causing serious infections in immuno-compromised individuals, and is the most frequent Gram-negative, bacterial

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etiologic agent associated with infections of indwelling catheters and foreign-body implants (1). *P. aeruginosa* also chronically infects the lungs of cystic fibrosis (CF) patients (2). Common for these infections are that the bacteria are rarely eradicated by administration of high doses of conventional antibiotics, leaving only one option to eradicate *P. aeruginosa*, which is removal of the infected lungs or implants (3,4). Re-colonization, after transplantation, is, however, almost impossible to avoid in the CF patient's lungs (3), and replaced foreign bodies are also prone to re-colonization (5). One explanation for the complexity of eradicating *P. aeruginosa* in these clinical settings is that this microbe forms biofilms, e.g. microcolonies, which have been observed both on medically inserted foreign bodies and in the lungs of CF patients (4,6–8). The biofilm mode of growth is known to protect *P. aeruginosa* against the host immune defense and enable tolerance against conventional antibiotics (7,9,10). Because of the high density of cells in the biofilm mode of growth, *P. aeruginosa* is able to make use of its cell-to-cell communication [quorum sensing (QS)] systems. *P. aeruginosa* uses two *N*-acyl-L-homoserine lactone (AHL) signal molecule-based QS systems: the *las* and *rhl* systems. Both systems are organized with a transcriptional regulator, LasR and RhlR, and a synthetase, LasI and RhlI, that synthesize the signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively (11,12). In addition to the AHL-based QS systems, *P. aeruginosa* also uses the *Pseudomonas* quinolone signal (PQS) system. The QS systems of *P. aeruginosa* have been shown to be hierarchically arranged, with the *las* system controlling the *rhl* system (13,14) and the PQS system positioned between the *las* and *rhl* systems (15). However, it has been postulated that the *rhl* system can be activated independent of the *las* system, and it has been suggested that the PQS system controls this activation (16).

The QS systems of *P. aeruginosa* have been shown to regulate the expression of approximately 160 genes (17,18), where a number of these control the production and secretion of several virulence factors, e.g. exotoxin A, proteases and rhamnolipids. Even though the interplay of these genes is not exactly known, they most likely contribute to the establishment and persistence of the bacteria in the host [reviewed by van Delden and Iglewski (19)]. Rhamnolipids act as heat-stable extracellular hemolysins (20), and are known to lyse polymorphonuclear leukocytes (PMNs) (21,22) and monocyte-derived macrophages (23), resulting in necrotic cell death. Rhamnolipids have also been detected in sputum from CF patients chronically infected with *P. aeruginosa* (24). *P. aeruginosa* produces several different rhamnolipids (25), and Jensen et al. (22) have been able to isolate an active fraction from *P. aeruginosa* PAO1 supernatant that induced necrosis of PMNs. The major component of this fraction was shown to be the di-rhamnolipid, rhamnolipid B (2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid). The synthesis of rhamnolipids proceeds by two sequential glycosyl transfer reactions, each catalyzed by a specific rhamnosyltransferase (26). The first rhamnosyltransferase is encoded by the *rhlAB* operon, and is responsible for the formation of mono-rhamnolipids. Recently, Zhu and Rock (27) showed that RhlA is the only enzyme needed to generate the lipid component, β-hydroxydecanoyl-β-hydroxydecanoate (HAA), of rhamnolipids by utilizing β-hydroxydecanoyl-acyl carrier protein intermediates from the fatty acid synthesis. A mutation in the *rhlA* gene prevents transcription of the *rhlB* gene, which is hypothesized to encode the catalytic subunit of the rhamnosyltransferase (28), and abolishes the production of the precursor of mono-rhamnolipid, HAA (29). However, Zhu and Rock (27) suggest that RhlB catalyzes the conversion of HAA to mono-rhamnolipid by itself. The second rhamnosyltransferase encoded by the *rhlC* gene converts mono-rhamnolipids to di-rhamnolipids. The expression of the *rhlAB* operon and the *rhlC* gene is coordinately regulated by the *rhl* QS and PQS systems (30).

We have previously provided evidence that PMNs are unable to eradicate *P. aeruginosa* cells organized as *in vitro* biofilms (10), and that this is associated with QS-regulated phenotypes, most notably with the production of rhamnolipids (22). In the present report, we demonstrate that a single mutation in the QS-controlled *rhlA* gene makes biofilm cells incapable of causing PMN necrosis. Additionally, this single mutation caused an increased eradication of bacteria in two different infectious animal models.

MATERIAL AND METHODS

Bacterial strains

All experiments were performed with the wild-type *P. aeruginosa* strain (PAO1) obtained from Professor Barbara Iglewski (University of Rochester Medical Center, NY, USA) and its isogenic derivatives. The strain is QS-proficient, except for reduced production of C4-HSL previously noted for this *P. aeruginosa* variant (31). Construction of the isogenic *rhlA::gentamicin* mutant was carried out as described by Pamp and Tolker-Nielsen (32), and likewise, construction of the *rhlA*+ complemented *rhlA* strain carrying the pEX1.8-*rhlA*+*B*+ plasmid including the *rhlA* promoter. The strains were tagged with a plasmid-based mini-Tn7 transposon system (pBK-miniTn7-*gfp*3) constitutively expressing a stable green fluorescence protein (GFP) according to Koch et al. (33).

Growth of bacteria and determination of rhamnolipid content

Bacteria from freezer stocks were plated onto blue agar plates (State Serum Institute, Denmark) and incubated at 37°C overnight. Blue agar plates are selective for Gram-negative bacilli (34). One colony was used to inoculate overnight cultures grown in Luria–Bertani (LB) medium at 37°C with shaking. For measurement of rhamnolipids in the supernatants of bacteria grown in the planktonic state and stationary biofilms, AB trace minimal medium containing 3mM glucose was used (32). Stationary biofilms were grown in test plates with six wells (92006, TPP) at 37°C. The biofilms were mechanically removed with a pipette, mixed with 1ml of ethyl acetate and the content of rhamnolipids was determined.

LC-ESI-MS data were used to calculate a standard curve for rhamnolipid B (concentration vs total ionization current). The rhamnolipid standards used for calculating the concentration curve were analyzed immediately before as well as after analysis of the samples, in order to minimize potential differences in ionization levels of rhamnolipid between the samples. Concentration values have been normalized from a standard of rhamnolipid B. In the analysis, the total rhamnolipid concentration was derived from the six major rhamnolipids, with the following masses $[M+NH_4]^+$: 668.4, 694.4, 696.4, 522.4, 548.4; and 550.4. These equate to C10-C10-rha-rha, an unidentified C10-C12Δ-rha-rha, C10-C12-rha-rha, and the respective mono-rhamnose derivatives.

BHPLC-MS analysis was performed using an agilent 1100 series HPLC connected to a micromass LCT TOF MS.

Animals

Female BALB/c mice were purchased from Taconic M&B A/S (Ry, Denmark) at 9–11 weeks of age and were maintained on standard mouse chow and water *ad libitum* for a minimum period of 1 week before the challenge. All experiments were authorized by the National Animal Ethics Committee, Denmark.

Foreign-body infection model

Silicone implants were prepared as described previously by Christensen et al. (35), with modifications. A bacterial pellet from a centrifuged overnight culture was resuspended in

0.9% NaCl to an OD_{600nm} of 0.1. Animals were challenged according to the method of Christensen et al. (35). Bacterial colonization of implants was determined according to the method of Christensen et al. (35), except that the implants were placed in 2ml 0.9% NaCl after removal from the mice. Mice were anesthetized by s.c. injections in the groin area with hypnorm/midazolam (Roche) [one part hypnorm (0.315 mg fentanyl citrate/ml and 10 mg fluanisone/ml), one part midazolam (5 mg/ml) and two parts sterile water). Pentobarbital (DAK), 10.0 ml/kg body weight, was injected i.p. to euthanize the mice at the termination of the experiments.

Pulmonary infection model

Immobilization of *P. aeruginosa* in seaweed alginate beads was performed as described by Pedersen et al. (36), with modifications. Bacterial overnight cultures were centrifuged for 10 min and the supernatants were discarded. The bacterial pellet was resuspended in 4.5 ml LB medium, and 0.5 ml was mixed with seaweed alginate for production of beads. Alginate beads were washed twice in 0.1M $CaCl_2$ dissolved in 0.9% NaCl. Just before the challenge, the suspension was adjusted to 6×10^6 CFU/ml in 0.1M $CaCl_2$ dissolved in 0.9% NaCl. Animals were challenged according to the methods described by Moser et al. (37), with modifications. The mice received a local anesthetic, bupivacaine (SAD), on the incision site for post-operative pain. After the infection procedures, the mice were injected s.c. with 0.75 ml of 0.9% NaCl in the neck-skin area to avoid dehydration. At termination of the experiments, isolated lungs were placed in 5ml of 0.9% NaCl and kept on ice until homogenization for 15–20 s (SilentCrusher M, Heidolph, Germany). Serial dilutions were plated onto blue agar plates for colony counting. The plates were incubated at 37°C overnight and CFU/lung was determined. The mice were anesthetized and euthanized as described for the implant model.

Biofilms in flow cells

The flow system was assembled and prepared as described by Christensen et al. (36). Biofilms were grown at 37°C in continuous-culture, once-through, threechannel, flow cells with individual channel dimensions of 1×4×40mm perfused with sterile AB trace minimal medium containing 0.3mM glucose as described by Pamp and Tolker-Nielsen (32). Overnight cultures were diluted to 0.1 at OD_{600nm} in 0.9% NaCl, and 250 μl was used for inoculation per channel. All microscopic observations and image acquisitions were performed using a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Germany). Images were obtained with a ×40/dry objective. To visualize dead bacterial cells and necrotic PMNs, propidium iodide (PI) (P-4170; Sigma) was used, whereas expression of GFP was used as a measure for live bacterial cells. Image scanning was carried out at 488nm (green) and 543nm (red) laser line from an Ar/Kr laser. Imaris software package (Bitplane AG) was used to generate pictures of the biofilm.

Preparation of PMNs

Isolation of PMNs was performed as described by Bjarnsholt et al. (10), with modifications. Human blood was collected from normal healthy volunteers in BD Vacutainers containing 0.129Msodium citrate. PMNs were resuspended in RPMI 1640 with $NaHCO_3$ to obtain a concentration of 1.5×10^7 PMNs/ml.

PMN treatment of biofilms

The experiment was performed as described by Bjarnsholt et al. (10). We evaluated the biofilm and PMN interactions after 15, 30, 60, 90, 120, 180 and 240min. Necrotic PMNs were demonstrated as increased red fluorescence from the supplemented DNA stain PI.

Staining of necrotic PMNs

Necrotic and apoptotic PMNs were stained according to Jensen et al. (22) and analyzed by flow cytometry. The experiment was repeated thee times using three different blood donors, obtaining similar results each time.

The dose–response assays were performed in mikrotiter dishes (Black Isoplate, Perkin Elmer). A sterile-filtered cell-free supernatant containing different rhamnolipid concentrations was mixed with PMNs with added PI. Necrotic cell death was measured as red fluorescence (excitation and emission wavelength 510 and 600 nm, respectively) every 15 min during the following 6 h on a plate reader at 37°C (Wallac 1420 VICTOR2 I; Perkin Elmer).

Statistical analysis

To compare the bacterial counts (CFU) between two groups of mice, the Mann–Whitney *U*-test was used (analysis of nonparametric data) for calculating p-values in the statistical program GraphPad Prism (GraphPad software Inc., San Diego, CA, USA, version 5.0). To compare differences in the number of cleared silicone implants, the χ^2 test was used for calculating p-values. To compare differences in the fraction of necrotic PMNs, a paired *t*-test was used. p-values ≤ 0.05 were considered significant.

RESULTS

Detection of rhamnolipids in the supernatants of planktonic grown bacteria and in stationary biofilms

In order to evaluate the production of rhamnolipids, the wild-type *P. aeruginosa* and the corresponding *rhlA* mutant were grown as planktonic cells in shaking cultures and in stationary biofilms. Rhamnolipid production is not detectable until the stationary phase of growth in *Pseudomonas* (38); therefore, the rhamnolipid content from shaking cultures was measured after 24 and 48 h. However, no difference was observed in rhamnolipid production between 24 and 48 h. The stationary biofilms were grown for 24 or 72 h, and a doubling in rhamnolipid production was observed from 24 to 72 h. The average concentration (three different experiments) of rhamnolipids after 24 h for the wild-type *P. aeruginosa* was 104 μg rhamnolipids/ml when grown as shaking cultures and 35 μg rhamnolipids/ml when grow as stationary biofilms (Fig. 1). As expected, the *rhlA* mutant produced no measurable rhamnolipids (Fig. 1). In order to verify that the *rhlA* mutant was a true single *rhlA* mutant, we introduced a plasmid with the *rhlAB* genes (pEX1.8-*rhlA*+*B*+) into the *rhlA* mutant for complementation, and were able to restore the production of rhamnolipids in the *rhlA*+ complemented *rhlA* strain (planktonic: 304 μg rhamnolipids/ml; stationary biofilm: 242 μg rhamnolipids/ml) (Fig. 1). However, the *rhlA*+ complemented *rhlA* strain produced a larger amount of rhamnolipids compared with the wild type, possibly due to the fact that the plasmid-borne *rhlA*+*B*+ genes were constitutively expressed.

To establish the necrotic effect of the rhamnolipid concentrations by wild-type *P. aeruginosa* shown above, PMNs were incubated with a cellfree supernatant containing different concentrations of rhamnolipids. A clear time-dependent dose–response relationship was observed.

An *in vitro* biofilm of a QS-proficient but rhamnolipid-deficient mutant does not promote necrosis of PMNs

Previous studies have shown that a biofilm produced by the *P. aeruginosa* Δ*lasR rhlR* mutant is unable to cause necrosis of freshly isolated PMNs in contrast to its wild-type counterpart (22). Because rhamnolipids were found to be the agents causing necrosis of the

PMNs (21,22), we hypothesized that a biofilm formed by a *P. aeruginosa rhlA* mutant most likely would have lost the ability to induce necrosis of PMNs. However, because QS regulates several virulence factors, we could not be certain that other factors besides rhamnolipids also had an influence on the necrotic death of the PMNs, which is why we wanted to validate our hypothesis in an *in vitro* continuous-culture flow cell system. Biofilms of wild-type *P. aeruginosa* and the corresponding *rhlA* mutant were grown for 5 days. On day 5, PMNs were introduced into the flow chambers and incubated with the biofilms. To monitor necrosis and thereby death of the PMNs, PI was added together with PMNs. Two hours later, it was observed that a high fraction of PMNs had transformed into red flouresence after exposure to biofilms formed by wild-type *P. aeruginosa* (Fig. 2A, B), indicative of necrosis. In contrast, only a few red PMNs were observed after exposure to biofilms formed by the *rhlA* mutant (Fig. 2C, D). The experiment was repeated twice, yielding similar results as judged from several images captured aiming at the same location on each flow channel at different time points.

The fraction of necrotic PMNs was also analyzed by flow cytometry. PMNs were mixed with a sterile-filtered biofilm supernatant from either the wild-type *P. aeruginosa* or the *rhlA* mutant. As seen in the biofilm experiment, a higher fraction of PMNs became necrotic when mixed with the cell-free biofilm supernatant from wild-type *P. aeruginosa* as compared with when mixed with the cell-free biofilm supernatant from the *rhlA* mutant (9%/90%, 8%/88%, and 15%/100%; $p<0.0004$).

Clearance of a *P. aeruginosa rhlA* mutant is increased in a foreign-body infection model

Our previous results raised the question of whether the production of rhamnolipids by wild-type *P. aeruginosa* significantly contributes to the bacterial persistence in the host. To address this, two different mouse models were chosen: a foreign-body infection model and a pulmonary infection model.

To investigate the role of rhamnolipids for a biofilm developing on a foreign body, silicone implants colonized with either the wild-type *P. aeruginosa* or the corresponding *rhlA* mutant were inserted into the peritoneal cavity of BALB/c mice. The mice were euthanized 3 days post-insertion and the implants were removed to determine the CFU/implant recovered from each mouse. The median CFU/implant measured on control implants not inserted into the mice was adjusted to an OD_{600nm} of 0.1, which correlate with 6.4×10^5–6.9×10^5 CFU/implant, and arbitrarily assigned the value of 100% in order to normalize the respective CFUs obtained 3 days after infection. Two experiments were pooled and the normalized CFUs for each mouse in each group are plotted in Fig. 3A. Of the 24 implants colonized with the *rhlA* mutant, 21 had no detectable CFU when removed from the mice. In contrast, only three out of 22 of the implants colonized with wild-type *P. aeruginosa* were free of bacteria ($p<0.0001$). There was also a highly significant difference ($p<0.0001$) in the total CFU recovered from the implants of mice infected with wild-type *P. aeruginosa* compared with the colonization levels associated with the *rhlA* mutant (Fig. 3A).

A *P. aeruginosa rhlA* mutant is less persistent in a pulmonary infection model

The ability of mice to eradicate the *rhlA* mutant was also studied in a pulmonary infection model. Two groups of mice were challenged intratracheally with alginate beads containing 6×10^6 CFU/ml of either wild-type *P. aeruginosa* or the corresponding *rhlA* mutant. Four mice in each group were euthanized after challenge to estimate the content of bacteria in the lungs (Fig. 3B). The median CFU/lung obtained from pooled data for two experiments from mice infected with the wild-type *P. aeruginosa* was 5.6×10^6 CFU/lung, while the mice infected with the *rhlA* mutant had a median of 1.8×10^6 CFU/lung. Data from two experiments were pooled and the respective medians were arbitrarily assigned the value of

100% and used to normalize the CFUs obtained 3 days after infection (Fig. 3B). A significant decrease in the bacterial levels was found on comparing mice infected with the *rhlA* mutant with mice infected with the wild-type *P. aeruginosa* ($p<0.005$) (Fig. 3B).

DISCUSSION

Previously, Bjarnsholt et al. (10) have shown that a *P. aeruginosa* QS-deficient strain growing in an *in vitro* biofilm was more susceptible to phagocytosis by PMNs compared with a biofilm formed by a QS proficient *P. aeruginosa* strain. Additionally, Jensen et al. (22) showed that PMNs became necrotic when exposed to a *P. aeruginosa* QS-proficient biofilm, in contrast to exposure to a QS-deficient biofilm. Rhamnolipids were identified as the necrotic factors (22) and have also been reported to play an important role in the QS-dependent development and differentiation of *in vitro P. aeruginosa* biofilms with mushroom-shaped multicellular structures separated by water-filled channels (39). In the light of previous and recent data, QS-controlled differentiation appears to account for both the increased tolerance to antimicrobials and the action of cellular components of the host's immune defense (10,22). With respect to the previous results, we set out to test our hypothesis that an inactivating mutation in a single QS-controlled gene, the *rhlA* gene, would considerably impair the ability of *P. aeruginosa* to induce necrosis of PMNs, and subsequently reduce bacterial persistence in *in vivo* settings.

We found that *in vitro* biofilms of the *P. aeruginosa rhlA* mutant grown in a continuous-culture flow cell system only induced necrosis of a few PMNs in contrast to biofilms formed by the wild-type counterpart (see Fig. 2). These data were supported by flow cytometry measurements, where a significantly higher fraction of necrotic PMNs were observed when the PMNs were exposed to the biofilm supernatant of wild-type *P. aeruginosa* compared with exposure to the corresponding *rhlA* mutant. The inability to cause necrotic death of PMNs was attributed to the fact that the *rhlA* mutant did not produce any detectable rhamnolipids. The wild-type *P. aeruginosa* strain, however, produced an average of 35 μg rhamnolipid/ml when grown as stationary biofilms. When wild-type *P. aeruginosa* was grown planktonically in shaking cultures, the rhamnolipid content contented was three times higher than the ones detected for stationary biofilms (Fig. 1). This is in accordance with the recent findings of Morici et al. (40), who showed that AlgR downregulates Rhl QS in biofilms, but not in planktonic cultures, and as a consequence, downregulates the expression of a number of RhlR-controlled genes, including those involved in rhamnolipid synthesis. Consequently, a mature *in vitro* biofilm of *P. aeruginosa* would be expected to produce reduced levels of rhamnolipids compared with planktonic cultures.

As a result of the *in vitro* findings presented in the present study, we speculated that rhamnolipids were the major QS-regulated virulence factor in the foreign body and pulmonary infectious mouse models. Previously, we have shown that a functional QS system plays a key role in the ability of *P. aeruginosa* to persist in a foreign-body infection model and a pulmonary infection model (10,35). In the present study, both the wild-type *P. aeruginosa* and the *rhlA* mutant were QS-proficient, except that the *rhlA* mutant was not able to produce any detectable rhamnolipids. Nonetheless, we still found that the *rhlA* mutant was eradicated more rapidly from both the silicone implants and the lungs, as compared with the wild-type counterpart. To prevent an infection, the human body relies on the epithelial barrier and mechanical clearance, but if these mechanisms fail, the cells of the innate immune system will seek to eradicate the invading pathogenic microbes. Cells of the innate immune system include phagocytic cells, such as PMNs, that internalize and kill whole microorganisms. PMNs are the first immune cells at the site of infection, and it is well accepted that they play a very important role in acute infections. However, the defective clearance of bacteria, which also occur in the CF lung, results in a large fraction of

the PMNs undergoing necrosis (41), possibly when they encounter biofilms of *P. aeruginosa* and virulence factors, such as rhamnolipids. Recent investigations by us support this, as we observed a significantly larger fraction of dead PMNs in the broncheoalveolar lavage fluid from mice infected with a QS-proficient *P. aeruginosa*, compared with mice infected with a QS-deficient strain (22). From *in vitro* experiments, we have observed that rhamnolipids are able to induce necrosis of PMNs and hemolysis of red blood cells (22), and others have shown that rhamnolipids are capable of inducing necrosis of macrophages (23). For these reasons we believe that rhamnolipids are able to cause necrotic death of the host's innate immune cells *in vivo*, resulting in a reduced clearing of wild-type *P. aeruginosa*. Moreover, we think that the importance of rhamnolipids as virulence factors to a certain degree depends on the genetic background of the bacterial strain used for virulence evaluations, the site of infection in the host and perhaps additional factors likely to contribute to the establishment and persistence of *P. aeruginosa* in the foreign-body and pulmonary infection models.

In conclusion, we found that the persistence of *P. aeruginosa* carrying an inactivating mutation in a single QS-controlled gene, the *rhlA* gene, was significantly reduced in two different animal models of infection compared with the parent wild type. Furthermore, a functional *rhlA* gene was found to be essential for biofilms formed by *P. aeruginosa* to induce necrosis of PMNs. The presented data support a model where rhamnolipids are major contributors to a biofilm 'shield' that provides protection against the most abundant type of phagocytes that arrive at the infection site – the PMNs.

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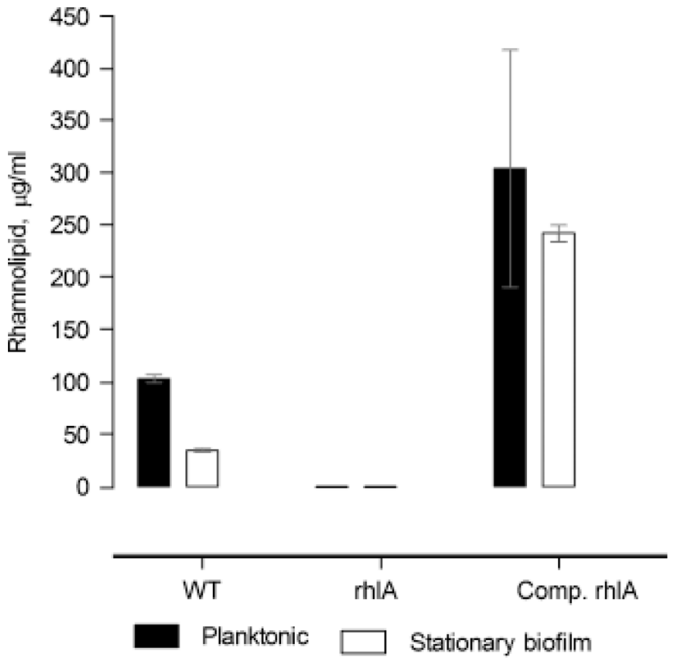

Fig. 1.

Rhamnolipid production by wild-type *Pseudomonas aeruginosa*, its corresponding *rhlA* mutant and the *rhlA*+ complemented *rhlA* strain. Production of rhamnolipids was measured in planktonic grown shaking cultures (black) and a stationary grown biofilm after 24 h (white). The average concentration (±SEM) of rhamnolipids from three experiments is shown.

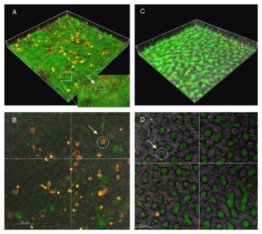

Fig. 2.

Pseudomonas aeruginosa biofilms at day 5 (green) exposed to polymorphonuclear leukocytes (PMNs) for 120 min at 37°C and stained with the DNA stain propidium iodide (red). Wild-type *P. aeruginosa* biofilms with extensive PMN necrosis [necrotic PMNs (red color) are indicated by a ring and an arrow in (B), and dead bacteria are indicated in magnification in (A) by a triangle and an arrow]. (C, D) *rhlA* mutant biofilms mainly with intact PMNs as indicated by an arrow a and ring in (D). (A, C) 3D images; (B, D) top views. The biofilms were visualized by combined fluorescence and light microscopy. Scale bars (50 μm) are shown in (B) and (D). All images were obtained with a ×40/dry objective.

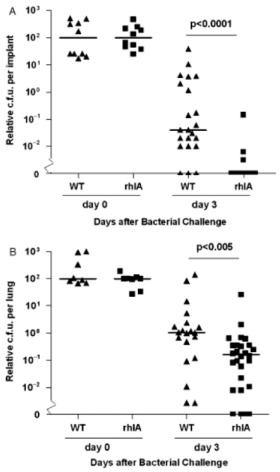

Fig. 3.

(A) Clearance of wild-type *Pseudomonas aeruginosa* and the *rhlA* mutant in the foreign-body infection model. Implants were removed 3 days post-insertion and the CFU/implant was determined. There was a significant difference in CFU recovered on comparing mice infected with wild-type *P. aeruginosa* with mice infected with the *rhlA* mutant ($p<0.0001$). The median CFU/implant measured on control implants, not inserted into the mice, was adjusted to an OD_{600nm} of 0.1, correlating to 6.4×10^5–6.9×10^5 CFU/implant. The median CFU/implant on control implants not inserted into the mice, from pooled data from two experiments, was arbitrarily assigned the value of 100% and used to normalize the respective CFUs obtained 3 days after infection. RhlA, *rhlA* mutant (n=24); WT, wild type (n=22). (B) Clearance of wild-type *P. aeruginosa* and the *rhlA* mutant in a pulmonary infection model 3 days post-infection. There was a significant difference in the recovery of bacteria on comparing mice infected with the wild-type *P. aeruginosa* with mice infected with the *rhlA* mutant ($p<0.005$). Four mice were euthanized immediately after the challenge to establish the inocula. The median CFU/lung, obtained from pooled data from two experiments, from mice infected with the wild-type *P. aeruginosa* was 5.6×10^6 CFU/lung, while mice infected with the *rhlA* mutant had a median of 1.8×10^6 CFU/lung. The respective medians were arbitrarily assigned the value of 100% and used to normalize the CFUs obtained 3 days after infection. rhlA, *rhlA* mutant (n=28); WT, wild type (n=20). Squares and triangles represent CFU/lung in individual mice; scale bars represent the median.