Pseudomonas aeruginosa tolerance to tobramycin, hydrogen peroxide and polymorphonuclear leukocytes is quorum-sensing dependent

Thomas Bjarnsholt,1 Peter Østrup Jensen,2 Mette Burmølle,3 Morten Hentzer,4 Janus A. J. Haagensen,1 Hans Petter Hougen,5 Henrik Calum,2 Kit G. Madsen,1 Claus Moser,2 Søren Molin,1 Niels Høiby2 and Michael Givskov1

Correspondence
Michael Givskov
immg@pop.dtu.dk

1Centre for Biomedical Microbiology, BioCentrum, Technical University of Denmark, DK-2800 Lyngby, Denmark
2Department of Clinical Microbiology, Rigshospitalet, DK-2100 Copenhagen Ø, Denmark
3Department of Microbiology, University of Copenhagen, DK-1307 Copenhagen K, Denmark
4Carlsberg Research Center, DK-2500 Valby, Denmark
5Institute of Forensic Medicine, University of Copenhagen, DK-2100 Copenhagen Ø, Denmark

The opportunistic human pathogen Pseudomonas aeruginosa is the predominant micro-organism of chronic lung infections in cystic fibrosis (CF) patients. P. aeruginosa colonizes the CF lungs by forming biofilm structures in the alveoli. In the biofilm mode of growth the bacteria are highly tolerant to otherwise lethal doses of antibiotics and are protected from bactericidal activity of polymorphonuclear leukocytes (PMNs). P. aeruginosa controls the expression of many of its virulence factors by means of a cell–cell communication system termed quorum sensing (QS). In the present report it is demonstrated that biofilm bacteria in which QS is blocked either by mutation or by administration of QS inhibitory drugs are sensitive to treatment with tobramycin and H2O2, and are readily phagocytosed by PMNs, in contrast to bacteria with functional QS systems. In contrast to the wild-type, QS-deficient biofilms led to an immediate respiratory-burst activation of the PMNs in vitro. In vivo QS-deficient mutants provoked a higher degree of inflammation. It is suggested that quorum signals and QS-inhibitory drugs play direct and opposite roles in this process. Consequently, the faster and highly efficient clearance of QS-deficient bacteria in vivo is probably a two-sided phenomenon: down regulation of virulence and activation of the innate immune system. These data also suggest that a combination of the action of PMNs and QS inhibitors along with conventional antibiotics would eliminate the biofilm-forming bacteria before a chronic infection is established.

INTRODUCTION

The progressive deterioration of the lungs in patients suffering from cystic fibrosis (CF) is mainly caused by an inflammatory response to chronic endobronchial biofilm-forming bacterial infection (Koch & Høiby, 1993; Konstan & Berger, 1997). The progressive development of the lung disease is the main cause of morbidity and mortality in CF patients (Davis et al., 1996), and up to 80% of young adults suffering from CF are chronically infected with P. aeruginosa (Koch & Høiby, 2000; Gilligan, 1991; Bauernfeind et al., 1987). The bacteria form biofilms in the host, which makes the bacteria tolerant to antibiotic treatment and the action of the host defence system. Contributing to this is their ability by mutation to develop conventional resistance to antibiotics such as β-lactam antibiotics (Ciofu, 2003; Bagge et al., 2004a, b).

Although several mechanisms have been postulated to explain the reduced susceptibility of biofilms to antimicrobials, it is becoming evident that biofilm tolerance to antimicrobial treatments is multifactorial with one factor being the low physiological activity of the bacterial cells deep below the surface of the biofilm where anaerobic conditions exists. Different barriers, such as ionic trapping of antibiotics, do not seem to play a critical role (Stewart & Costerton, 2001; Gilbert et al., 2002). However, free
chromosomal β-lactamase in and around the biofilm may contribute significantly (Giwerzman et al., 1992; Ciofu 2003).

As an active means to evade the host defences, *P. aeruginosa* controls the production and secretion of virulence factors by cell–cell communication signals in a process termed quorum sensing (QS; Fuqua et al., 1994). The concentration of signal molecules reflects the number of bacterial cells and at a certain threshold value the population becomes 'quorate', i.e. a sufficient number of cells have amassed to make behavioural decisions.

In *P. aeruginosa* the QS communication apparatus is composed of the *Las* and the *Rhl* systems. Both systems consist of two essential pairs of genes, *lasR* and *lasI*, and *rhlR* and *rhlI*, respectively. The I genes encode two synthetases that synthesize either 3-oxo-dodecanoyl-homoserine lactone (3-oxo-C12-HSL) for the las system or butyryl-homoserine lactone (C4-HSL) for the rhl system. The R genes encode regulatory proteins which, in concert with the signal molecules, activate gene expression of numerous target genes (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). The two QS systems of *P. aeruginosa* are hierarchically arranged, with the Las system being on top of the signalling cascade (Latifi et al., 1996; Pesci et al., 1997). Intertwined in this QS hierarchy is the quinolone signal (PQS) system, which provides a link between the Las and Rhl QS systems (Pesci et al., 1999) by cooperatively regulating the expression of *rhl* and *lasB* (McKnight et al., 2000). Recently, however, Medina et al. (2003) showed that the rhl system is also capable of operating on its own.

The QS system has on several occasions been shown to affect in vivo infections. In a murine model of chronic lung infection, a *las* rhlI mutant of *P. aeruginosa* was cleared faster than the wild-type (Wu et al., 2001). Rumbaugh et al. (1999) used a burned mouse model to show that QS-deficient mutants were less virulent than the wild-type. In accordance with this, Hentzer et al. (2003) showed that mice with pulmonary *P. aeruginosa* infections treated with the specific QS1 drug furanone C-30 exhibited clearing that was 1000-fold more efficient than mice treated with a placebo. Davies et al. (1998) and Hentzer et al. (2003) demonstrated a role for QS in the development of structural differentiated biofilms that showed tolerance to SDS and tobramycin treatment.

QS signal molecules have been reported to interfere with the host immune system. The first evidence for 3-oxo-C12-HSL acting as an immune modulator was published by DiMango et al. (1995), in which the production of IL-8 by respiratory epithelial cells was stimulated by 3-oxo-C12-HSL. This was supported by in vitro experiments on human cell lines by Smith et al. (2001). Smith et al. (2002) demonstrated that 3-oxo-C12-HSL induces production of cyclooxygenase-2 (cox-2) and prostaglandin E2 in human lung fibroblasts *in vitro*. Telford et al. (1998) showed that 3-oxo-C12-HSL, in a murine model, down regulated IL-12.

In addition, Tateda et al. (2003) reported that 3-oxo-C12-HSL, but not C4-HSL, in concentrations greater than 12 μM accelerated apoptosis in macrophages and neutrophils. It was confirmed by Chhabra et al. (2003) that it is the L-homoserine lactone ring acylated with a 3-oxo- or 3-hydroxy-substituted 12–14-carbon side chain which is optimum for immune modulation.

Once established, the chronic *P. aeruginosa* lung infection tolerates the highest deliverable doses of antibiotics (Anwar et al., 1992) and cannot be eradicated (Koch & Hoiby, 1993). The inflammatory response to the chronic infection is mainly characterized by the constant influx of polymorphonuclear leukocytes (PMNs), which surround the bacteria (Baltimore et al., 1989). Moser et al. (2000) showed that the immune response to the chronic *P. aeruginosa* lung infection in CF patients is predominantly the Th2 type (dominated by a PMN and antibody response). This correlates with the finding that inappropriate stimulation of the abundant PMNs is the major reason for the deterioration of the lung tissue during the inflammation of the chronic *P. aeruginosa* lung infection in CF patients (Koch & Hoiby, 1993; Konstan et al., 1995; Goldstein & Doring, 1986).

PMNs react to invading foreign organisms either by phagocytosis or secretion; in both cases the PMNs launch a cocktail of antimicrobial agents, in particular free oxygen radicals. H2O2 has a bactericidal effect, but the biofilm matrix of *P. aeruginosa* protects the bacteria since alginate is an oxygen radical scavenger. This has been reported to be due to the presence of catalase activity (Elkins et al., 1999). Palma et al. (2004) demonstrated by means of transcriptomic analysis that the early response of *P. aeruginosa* to H2O2 consists of an up regulation of protective mechanisms and a down regulation of primary metabolism. They reported more than 500 genes to be up or down regulated more than fivefold. Mathie et al. (1999) demonstrated that the conversion of *P. aeruginosa* to a mucoid phenotype is strongly promoted by the presence of H2O2.

The PMNs are the first line of defence for the human body against intruders and are an important part of the immune system in normal and healthy human beings. The PMNs have been termed Janus cells due to their beneficial and destructive properties (Nussler et al., 1999; Jensen, 2003), which are particularly evident in CF. It has been shown by in vitro experiments that the wild-type biofilm is impermeable to PMNs. They settle on top without disrupting the biofilm (Jesaitis et al., 2003).

In the present communication we have investigated the role of a functional QS system in the development of biofilm tolerance to tobramycin treatment and H2O2 exposure. Next, we investigated the role of a functional QS system in the extent of PMN phagocytosis and biofilm elimination. Finally, we have investigated the role of a functional QS system in development and magnitude of the oxidative burst. Accordingly we demonstrate that a lasR rhlR quorum mutant is cleared 100-fold more efficiently from a
pulmonary infection mouse model compared to the wild-type \textit{P. aeruginosa}.

\section*{METHODS}

\subsection*{Bacterial strains.} The wild-type \textit{P. aeruginosa} PAO1 used for the planktonic and biofilm \textit{in vitro} experiments was obtained from the \textit{Pseudomonas} Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). This isolate has served as DNA source for the \textit{Pseudomonas} Genome Project (www.pseudomonas.com) and, subsequently, as template for the design of the \textit{P. aeruginosa} GeneChIP (Affymetrix). The \textit{lasR rhlR} mutant was constructed using previously described knock-out systems (Beatson et al., 2002). The knock-out mutants were verified by Southern blot analysis and by screening for acyl homoserine lactone production (quorum signals). A stable green fluorescent protein (GFP) constitutively expressed on images captured by SCLM were subjected to quantitative image analysis.

\subsection*{Methods} The \textit{P. aeruginosa} used for \textit{in vivo} experiments was obtained from Professor Barbara Iglewski (University of Rochester Medical Center, NY, USA). The \textit{lasR rhlR} mutant of the Iglewski PAO1 strain was generated using the same knock-out systems as for strain PAO0001 (Beatson et al., 2002). The knock-out mutants were verified by Southern blot analysis and by screening for quorum signal production.

\subsection*{Growth of bacteria.} Planktonic cultures were grown in shake flasks at 37 °C. Biofilms were cultivated in continuous-culture once-through flow chambers, perfused with sterile ABTrace minimal medium containing 0.3 mM glucose as described previously by Christensen et al. (1999). The tube biofilms were grown using the same media. The tube used was made of silicone 2-mm in diameter. The pump was set to 3 ml h\(^{-1}\). Furunan C-30 was added to medium when appropriate. Biofilm development was examined by SCLM using a Zeiss LSM 510 system (Carl Zeiss) equipped with an argon laser and a helium-neon laser for excitation of the fluorophores. Quantitative image analysis was performed using the \textsc{comstat} software (Heydorn et al., 2000). Stacks of horizontal-plane images captured by SCLM were subjected to quantitative image analysis using the \textsc{comstat} software. The program calculates the biomass of the biofilm. Simulated fluorescence projections and vertical cross-sections through the biofilms were generated by using the \textsc{imaaris} software package (Bitplane AG). Images were further processed for display by using the Photoshop software (Adobe).

\subsection*{Antimicrobial treatments.} Biofilm tolerance to tobramycin was assessed by supplementing the medium of 3-day-old \textit{P. aeruginosa} biofilms with tobramycin in two different concentrations, 10 μg ml\(^{-1}\) and 20 μg ml\(^{-1}\). The tobramycin treatments were continued for 48 h. Tolerance of biofilms to H\(_2\)O\(_2\) was assessed by adding 100 mM H\(_2\)O\(_2\) to the influent medium of 3-day-old biofilms. After 15 min the treatment was stopped by adding 0.2% sodium thiosulfate as a neutralizing agent to the inlet medium. Bacterial viability in biofilm cultures was assessed either by using the LIVE/DEAD BacLight \textit{bacillary} viability staining kit (Molecular Probes) or by using GFP-tagged bacteria together with propidium iodide, the ‘dead cell’ stain from the BacLight bacterial viability kit (Molecular Probes), as described elsewhere (Hentzer et al., 2001). Tolerance of planktonic cells to H\(_2\)O\(_2\) was assessed by introducing H\(_2\)O\(_2\), to a concentration of 100 mM, to each sample. After 15 min the reaction was inactivated by adding 0.2% sodium thiosulfate for 10 min as a neutralizing agent. This chemical has no effect on the cell viability. The viability of the treated cells was determined by BacLight \textit{bacillary} viability kit (Molecular Probes). The c.f.u. per ml was assessed by diluting and plating.

\subsection*{Preparation of PMNs.} Human blood samples were obtained from normal healthy volunteers, by venous puncture, and collected in BD vacutainers coated with heparin and lithium (Becton-Dickinson, 388330). The blood was mixed with dextran (T-500) 1:5 and the erythrocytes were sedimented for 40 min. The supernatant was applied to Lymphoprep (Axis-Shield PoC) and centrifuged at 2200 r.p.m. for 15 min at 5 °C. The supernatant was discarded and the neutrophils were treated with 2 ml 0.2% NaCl in order to lyse remaining erythrocytes. Lysis was terminated by adding 2 ml 1:6% NaCl and 6 ml Eagle-MEM (Bie & Berntsen). The cells were centrifuged at 350 r.p.m. for 10 min at 5 °C, the supernatant was discarded and the PMNs were resuspended in Eagle-MEM.

\subsection*{PMN treatment of biofilms.} In order to inoculate PMNs into the biofilm chambers, the flow was stopped and the flow cells clamped off. PMNs in the order of 1.5 × 10\(^6\) were inoculated in each flow channel. The flow cells were incubated top down in a 37 °C water bath with shaking, until microscopic inspection.

\subsection*{Monitoring the oxidative burst of PMNs}

\subsection*{Biofilms.} Isolated PMNs were incubated for approximately 30 min in Eagle-MEM (3 × 10\(^5\) cells ml\(^{-1}\)) with 10% normal human AB serum and 5 μM SYTO 62 (Molecular Probes) to stain the nuclei (dsDNA). For detecting the oxidative burst of the PMNs, 0.1 mg 123-dihydrodorhodamine (123-DHR) ml\(^{-1}\) (D-1054, Sigma) was added to the PMNs in order to stain the H\(_2\)O\(_2\) in the phagosomes (Bassoe et al., 2003). 100 μl quantity of the PMN mixture was added to each biofilm.

\subsection*{Planktonic.} To 50 μl human peripheral blood was added 20 μl 0.01 mg 123-DHR ml\(^{-1}\) and this was incubated with 30 μl of either the wild-type \textit{P. aeruginosa} or the QS mutant for 15 min at 37 °C. The two cultures were grown in parallel, samples were collected every 30 min and the optical density was measured. Incubation was terminated by fixation in 1 ml fluorescence-activated cell sorter (FACS) Lysing Solution (349202, Becton Dickinson) diluted 10-fold in MilliQ water for 20 min. The samples were centrifuged (7 min, 350 g, 4 °C) and the supernatant was discarded. Three millilitres PBS was added, the samples were centrifuged (7 min, 350 g, 4 °C) and the supernatant was discarded. The DNA was stained by adding 200 μl cold PBS containing 100 μg propidium iodide (P-4170, Sigma) and the samples were incubated on ice for at least 10 min prior to flow cytometry analysis. The samples were analysed using a FACSort (Becton Dickinson) equipped with a 15 mW argon-ion laser tuned at 488 nm for excitation. Light scatter and exponentially amplified fluorescence parameters from at least 10,000 events were recorded in list mode after gating on light scatter to avoid debris, cell aggregates and bacteria. The instrument was calibrated using Calibrite (Becton Dickinson). PMNs were identified according to their morphology and their content of DNA.

\subsection*{Experimental animals.} Female BALB/c mice were purchased from M & B Laboratory Animals at 10 to 11 weeks of age. The mice were of equal size and were maintained on standard mouse chow and water \textit{ad libitum} for 1 week before challenge. All animal experiments were authorized by the National Animal Ethics Committee.

\subsection*{Immobilization of \textit{P. aeruginosa} in seaweed algal bead.} Immobilization of \textit{P. aeruginosa} in seaweed algal beads was performed as described by Pedersen et al. (1990). The suspension was adjusted to 2.5 × 10\(^6\) c.f.u. ml\(^{-1}\) and confirmed by colony counts; 0.04 ml of the suspension was instilled in the left lung of each mouse.

\subsection*{Challenge procedure.} The mice were anaesthetized by subcutaneous injection of 0.2 ml of Hyp/Mid [2.5 mg Hypnorm ml\(^{-1}\) (Janssen) and 1.25 mg midazolam ml\(^{-1}\) (Roche) in sterile water]. Each sedated mouse was fixed and its trachea was exposed and penetrated with an 18 G needle. The inoculum was instilled in the left
The mice were sacrificed at day 5 after infection. Histopathological evaluation was done blindly. Necrosis or with severe inflammation throughout the lung (moderate to severe inflammation) or 3 (severe inflammation with necrosis or with severe inflammation throughout the lung). The histopathological evaluation was scored as 0 (no inflammation), 1 (mild inflammation), 2 (moderate to severe inflammation) or 3 (severe inflammation with necrosis or with severe inflammation throughout the lung). The histopathological evaluation was done blindly.

**RESULTS AND DISCUSSION**

**Increased sensitivity towards tobramycin is QS dependent**

The effect of the QS inhibitors furanone C-30 and C-56 on biofilm sloughing and sensitivity to tobramycin treatment (Hentzer et al., 2003) led us to hypothesize that a functional QS system in general might play a role in the development of the characteristic biofilm tolerance to a variety of antimicrobial treatments. To investigate this hypothesis two sets of biofilms were inoculated, one with the wild-type *P. aeruginosa* and one with the Δ*lasR rhlR* QS-receptor mutant. The latter strain does not produce detectable amounts of the signal molecules and is considered non-responsive to externally added quorum signals.

The biofilms were allowed to grow and develop for 3 days. At day 3, the biofilms were each treated with either 10 μg tobramycin ml⁻¹ or 20 μg tobramycin ml⁻¹. Propidium iodide was added, together with the tobramycin, to the influent medium for continuously monitoring viability of the biofilm cells. A clear difference in the number of red (dead) cells was recorded within 48 h of treatment (Fig. 1). The QS-deficient biofilms were almost completely killed by both concentrations of tobramycin (Fig. 1e, f), whereas only cells at the surface layer of the wild-type biofilm were affected by the treatments (Fig. 1b, c). The experiment was repeated three times showing identical results.

To verify the bacterial viability stain by means of enumeration, we decided to grow biofilms in tubes, from which isolation of bacteria is more reproducible compared to the flow cells. Three-day-old tube biofilms of both the wild-type *P. aeruginosa* and the Δ*lasR rhlR* mutant were treated with tobramycin for 48 h. The bacteria were harvested and the cells were washed twice before plating. The result of the live-dead stain for tobramycin treatment was verified by this method (Fig. 2). No difference was observed between the untreated wild-type and QS mutant, whereas 10 μg tobramycin ml⁻¹ led to a 15-fold reduction in c.f.u. for the QS mutant compared to the wild-type, and 20 μg tobramycin ml⁻¹ led to 1000-fold reduced c.f.u. for the QS mutant compared to the wild-type. The experiment was repeated twice with identical results.

**Increased sensitivity towards H₂O₂ is QS dependent**

When exposed to bacteria, activated PMNs secrete toxic oxygen radicals and other reactive oxygen products. The tolerance of planktonic *P. aeruginosa* towards reactive...
oxygen was previously suggested by Hassett et al. (1999) to be under QS control using the lasI rhlI mutant, JP2. To investigate the response of our QS mutant and to compare the tolerance of wild-type P. aeruginosa and the ΔlasR rhlR mutant to H2O2, the two strains were grown in shake flasks in parallel and sampled. No difference in the c.f.u. ml−1 was observed between untreated cultures of the wild-type and the QS mutant, but a 1000-fold difference in c.f.u. ml−1 was observed between the wild-type and the QS mutant when exposed to 100 mM H2O2 for 15 min (data not shown).

Since a functional QS system seems to be involved in the protection of planktonic P. aeruginosa to oxidative stress, we investigated whether this was also true for biofilms. Two sets of biofilms were allowed to grow and develop for 3 days. At day 3, 100 mM H2O2 (final concentration) was added to the influent medium. After 15 min the H2O2 was inactivated and the effect of the H2O2 treatment on viability was visualized by means of propidium iodide staining. As shown in Fig. 3(a, c) the QS-mutant biofilm appears to be slightly more densely populated than the wild-type biofilm before the treatment (although this does not affect the outcome of the experiment). Fig. 3(b) shows the wild-type after the treatment and hardly any dead cells are detected. In contrast, very few QS mutant cells are alive in Fig. 3(d); the cells are either red or yellow, which arises from imposing red on green. The experiment was repeated twice showing identical killing of the QS mutant. It should be noted that cells can still appear green fluorescent even though they are dead since we used a stable version of GFP to tag the bacteria. In contrast, the cells that appear red are dead according to our control experiments and the product information from Molecular Probes. Green and red cells were sorted by FACS and the red fraction did not produce any c.f.u., whereas the green fraction produced a number of c.f.u. corresponding to the FACS count (data not shown).
Increased sensitivity of QS mutants to PMN activity

*P. aeruginosa* is thought to evade the host immune system by means of QS-regulated gene expression and establish itself by means of the biofilm mode of growth, which in turn offers protection from the action of the immune system. We speculated that a wild-type biofilm would be less vulnerable to the action of the PMNs compared to a QS-deficient biofilm. To test this hypothesis, we compared the effect of PMNs on biofilms formed by the wild-type and the ΔlasR rhlR mutant. The biofilms were allowed to grow and develop for 3 days. As judged from microscopic inspection, both types of biofilms developed similarly; however, the QS-mutant biofilm created more biomass than the wild-type, 277 000 \( \mu m^3 \mu m^{-2} \) compared to 190 000 \( \mu m^3 \mu m^{-2} \) according to calculations by COMSTAT (Heydorn et al., 2000; \( P < 0.03 \); Fig. 4(a, d)). On day 3 freshly isolated PMNs were inoculated into the biofilm flow chambers. The mixed PMN-biofilms were monitored every 30 min. After 2-5 h both the penetration of PMNs and the remaining biomass of the two biofilms were significantly different according to calculations by COMSTAT (Heydorn et al., 2000; \( P \leq 0.04 \)). The medians of the calculated biomasses were 170 000 \( \mu m^3 \mu m^{-2} \) for the QS-mutant biofilm and 213 000 \( \mu m^3 \mu m^{-2} \) for the wild-type biofilm. This is especially interesting since we detected more biomass for the QS mutant prior to treatment.

The wild-type biofilms seemed to withstand the activity of the PMNs (the PMNs settled and remained paralysed on top of the biofilm, see Fig. 4(b, c)). In contrast, in the case of the ΔlasR rhlR mutant (Fig. 4(e, f)) the PMNs penetrated the biofilms down to the substratum, resulting in a significantly reduced biomass of the remaining bacteria. As seen from Fig. 4(g, h) bacteria were both attached to and phagocytosed by the PMNs. This grazing phenomenon was not observed when the PMNs were exposed to a wild-type biofilm.

During the course of the experiments, it was discovered that a ΔlasR rhlR biofilm appeared more loose and wobbly than the wild-type biofilms. We speculate that this could be due to a lack of exopolymeric substances, e.g. exopolysaccarides, as described by Shih & Huang (2002). If true, this suggests that a ΔlasR rhlR biofilm exhibits less physical hindrance to PMN action than a wild-type biofilm. This, taken together with the sensitivity to \( H_2O_2 \) treatment, might at least in part explain why PMNs more efficiently graze on the ΔlasR rhlR biofilm.

PMN activation is affected by a QS-controlled mechanism

Wu et al. (2001) found that pulmonary infections caused by a lasI, rhlI mutant induced a faster and stronger immune response against the bacterial infection in the early phase as judged from the severity of lung pathology, higher lung IFN-\( \gamma \) production, stronger oxidative burst of blood PMNs and faster antibody response compared to the wild-type counterpart. The authors speculated that the wild-type produced extracellular products which would interfere with activation and oxidative burst of the PMNs. We therefore investigated whether the difference in biofilm tolerance to PMN activity was influenced by differences in activation of the PMNs.

Wild-type *P. aeruginosa* and the ΔlasR rhlR mutant biofilms were allowed to grow and develop for 3 days. The PMNs were injected into the biofilm flow chambers and the development of oxidative burst was followed over a period of 2 h. The results are shown in Fig. 5. A marked difference in green fluorescence signal intensity was observed for the PMNs incubated on the wild-type biofilms compared to the ΔlasR rhlR biofilms. In a similar experiment performed on a biofilm consisting of a 1:1 mixture of wild-type *P. aeruginosa* and the ΔlasR rhlR mutant, only a slight activation of the PMNs was observed (data not shown).

From these experiments we conclude that a wild-type biofilm, in contrast to a QS-deficient biofilm, fails to fully activate the PMNs to produce \( H_2O_2 \). The experiments were repeated multiple times with similar outcomes. In addition, the thickness of the biofilm is an important factor; we observed that a (presumably pre-quorate) thin wild-type biofilm (less than approximately 40 \( \mu m \) thick) was capable of activating the PMNs to about the same extent as the QS mutant (data not shown). This suggests that components of the QS system block PMN activation.

A flow-cytometer-based analysis was used to quantify the oxidative burst of the PMNs. In this case the PMNs were activated by planktonic *P. aeruginosa* cells. Cultures of wild-type *P. aeruginosa* or ΔlasR rhlR mutants were followed in parallel, and samples were extracted at different OD\(_{600}\) densities. Peripheral blood was obtained from eight different individuals. Bacteria, blood and 123-DHR were mixed for 15 min at 37 \( ^{\circ}C \). Fig. 6 shows that the fraction of activated PMNs mixed with the wild-type is less than the fraction of activated PMNs mixed with the QS-mutant bacteria. The activation, and especially the difference in activation between the wild-type bacteria and the QS mutant, increased with increasing bacterial density. This further supports the view that components of the QS system block PMN activation.

**Activation of oxidative burst is controlled by QS signal molecules**

The above results suggest that secreted virulence factors or the quorum signal molecules produced by the wild-type biofilms (or both) block activation of the PMNs. It has recently been shown that treatment with 12–50 \( \mu M \) 3-oxo-C12-HSL causes PMN viability loss in a concentration-dependent manner (Tateda et al., 2003).

Indeed the 3-oxo-C12-HSL signal has previously been
demonstrated to act as an immune modulator (DiMango et al., 1995; Smith et al., 2001, 2002; Telford et al., 1998; Chhabra et al., 2003; Tateda et al., 2003). To investigate whether the lack of PMN activation was caused by the presence of the quorum signal molecules, we grew biofilms of the \( \Delta lasR \ rhlR \) mutant for 3 days. Three hours prior to challenge with PMNs, both signal molecules (1 \( \mu \)M 3-oxo-C12-HSL and 2 \( \mu \)M C4-HSL) were added to the influent media of the flow chambers. As seen in Fig. 5(d) the PMNs remained inactivated after incubation with the quorum signal-treated \( \Delta lasR \ rhlR \) biofilm. These results were confirmed by several experiments. Since the \( \Delta lasR \ rhlR \) mutant is unable to produce QS-regulated virulence factors as it is unable to respond to externally added signal molecules, this experiment suggests that QS signals directly affect the magnitude of the oxidative PMN burst.

**Fig. 4.** QS-dependent tolerance of \( P. \ aeruginosa \) biofilms towards PMNs. Three-day-old biofilms of wild-type \( P. \ aeruginosa \) (a) and a \( \Delta lasR \ rhlR \) mutant (d), both expressing GFP as a tag, were exposed to PMNs for 2-5 h. The PMNs appear red fluorescent by SYTO-62 staining. (b) 3-D projection and (c) cross-section showing the wild-type biofilm with PMNs on top. (e) 3-D projection and (f) cross-section showing the \( \Delta lasR \ rhlR \) mutant fully penetrated by PMNs and the disappearance of much of the biomass. As seen from the enlargement of PMNs exposed to a QS-deficient biofilm frame (g) and isolated PMNs (h), the green fluorescent bacteria are attached to and phagocytosed by the PMNs.
observed between the several repetitions. No difference in oxidative burst was C-30-treated wild-type biofilm and were confirmed by PMNs respond with oxidative burst when exposed to a chambers. The results presented in Fig. 5(b) show that the freshly isolated PMNs were inoculated into the flow biofilm. The biofilm was grown for 3 days in the presence of 10 μM C-30. The PMNs fluoresce green (indicative of oxidative burst) compared with the PMNs in (a). (d) Quorum-signal-mediated inactivation of PMNs present on a wild-type biofilm. 3-Oxo-C12-HSL (1 μM) and C4-HSL (2 μM) were added to the 3-day-old ΔlasR rhlR mutant biofilm 3 h prior to inoculation with the PMNs.

 Activation of PAO1-exposed PMNs by the furanone C-30 Biofilms of wild-type P. aeruginosa or the ΔlasR rhlR mutant were allowed to grow and develop for 3 days in the absence or presence of 10 μM furanone C-30. At day 3, freshly isolated PMNs were inoculated into the flow chambers. The results presented in Fig. 5(b) show that the PMNs respond with oxidative burst when exposed to a C-30-treated wild-type biofilm and were confirmed by several repetitions. No difference in oxidative burst was observed between the ΔlasR rhlR mutant grown in the presence or absence of C-30; in both cases the PMNs became and remained activated (data not shown).

 A ΔlasR rhlR mutant is cleared rapidly in vivo BALB/c mice were used to establish a pulmonary model of chronic lung infection. The immune response of the BALB/c mice is Th2 dominated (Moser et al., 1999), resembling the immune response of the CF patients towards the chronic P. aeruginosa infection (Moser et al., 2000). Two groups of mice were challenged with alginate beads containing either wild-type P. aeruginosa or the ΔlasR rhlR mutant (Pedersen et al., 1990). Each of the two groups consisted of 72 mice. After the challenge, the mortality rate was noted for 5 days. Deaths within 24 h post-challenge were rejected since they might be caused by post-surgical trauma. In the wild-type-infected group 73 % (35/48) of the mice died, whereas only 46 % (27/59) died in the group infected with the ΔlasR rhlR mutant (P<0.005).

 Clearance of bacteria from the lungs was assessed on day 1 and day 5 post-challenge (Fig. 7). On day 1 post-challenge there was a 100-fold difference between the two groups, with the ΔlasR rhlR group being cleared fastest (P<0.004). On day 5, the lungs in nine out of ten mice in the group of mice infected with ΔlasR rhlR were sterile, whereas for the mice infected with the wild-type, five out of seven contained P. aeruginosa (P<0.02). This is in accordance with the data by Hentzer et al. (2003) using C-30 to block QS in the lungs.

Fig. 5. PMN activation measured by oxidative burst. Three-day-old biofilms of wild-type P. aeruginosa and the ΔlasR rhlR mutant were exposed to PMNs for 2 h. The oxidative burst was visualized by the green fluorescence emitted when 123-DHR is oxidized to 123-rhodamine due to production of H2O2. (a) Wild-type; (c) ΔlasR rhlR mutant. (b) Furanone C-30-mediated activation of PMNs present on a wild-type biofilm. The biofilm was grown for 3 days in the presence of 10 μM C-30. The PMNs fluoresce green (indicative of oxidative burst) compared with the PMNs in (a). (d) Quorum-signal-mediated inactivation of PMNs present on a ΔlasR rhlR biofilm. 3-Oxo-C12-HSL (1 μM) and C4-HSL (2 μM) were added to the 3-day-old ΔlasR rhlR mutant biofilm 3 h prior to inoculation with the PMNs.

Fig. 6. Bacterial-cell-density-dependent activation of PMNs by wild-type and ΔlasR rhlR P. aeruginosa as measured by flow cytometry. The bacteria were grown in shake flasks and samples were retrieved every 30 min starting at OD600 0.1. Blood from eight different normal persons was used (n=8). The PMNs were incubated with either wild-type P. aeruginosa or the ΔlasR rhlR mutant at 37 °C for 15 min and the reaction was stopped by placing the sample on ice. Next, the sample was run through the flow cytometer and the fraction of PMNs emitting green florescence was counted. The fraction of activated PMNs was calculated by the count of PMNs emitting green signal divided by the total counts of PMNs. The circles show the mean of each time point. The y axis shows the increased percentage of PMNs activated by ΔlasR rhlR, calculated by subtracting the fraction of PMNs activated by exposure to the wild-type from the fraction of PMNs activated when exposed to the QS mutant. A statistically significant fraction of PMNs was activated when exposed to the ΔlasR rhlR mutant compared to the wild-type, tested by a paired t-test: OD600 0.11, NS; OD600 0.17, NS; OD600 0.26, NS; OD600 0.44, P<0.03; OD600 0.48, P<0.05; OD600 0.73, NS; OD600 0.90, NS; OD600 1.10, P<0.04; OD600 1.30, P<0.04; OD600 1.50, P<0.008. NS, Not statistically significant.
of the mice. The faster and highly efficient clearance of infecting bacteria could then be two sided: down regulation of virulence and activation of the PMNs as observed in the above in vitro experiments and in vivo by Wu et al. (2001).

The ΔlasR rhlR mutant gave rise to a more severe lung inflammation

Seven mice of each group from the experiment described above were evaluated for histopathological changes. On day 1 post-challenge there were no significant differences in the number of mice with lung inflammation as based on histopathology. On the other hand, the degree of inflammation detected was significantly higher in the group of mice infected with the ΔlasR rhlR mutant (P<0.009). These results are consistent with the previous in vitro and in vivo investigations and indicate that quorum signals from the infecting bacteria block the primary innate immune response. On day 5, no difference was detected between the two groups. The lack of difference at this point, we believe, lies in the fact that quorum mutants had been efficiently cleared from the host.

Conclusion

The present investigation supports a model in which P. aeruginosa employs quorum signals to manipulate the host immune response during the initial stage of infection, and we believe that this plays an important role in the protection against the antimicrobial action of the PMNs.

First, in vitro biofilms formed by QS mutants are more susceptible to H2O2 treatment. Second, human PMNs fail to produce H2O2 when exposed to biofilm bacteria and quorum signals but are activated when exposed to the QSI drug C-30. Experiments performed in vivo support our in vitro data and suggest that the immune system is activated to a higher level when mice are infected with the ΔlasR rhlR mutant compared to the wild-type. As a consequence the mutants are cleared faster than their wild-type counterparts.

The present results also indicate that the immune response to biofilm-growing P. aeruginosa can be modulated by means of QSI drugs. Furthermore, Hentzer et al. (2003) demonstrated synergistic effects of C-30 and the antibiotic tobramycin. Biofilms grown in the presence of C-30 were less tolerant to tobramycin treatment compared to their untreated counterparts. This raises the question as to whether conventional antimicrobial treatments of P. aeruginosa biofilms in general will be more efficient in the presence of QSI drugs. Prophylactic administration of QSI drugs would presumably prevent the formation of highly resistant biofilms on medical devices as well as in the lungs of CF patients. If true, a combination of the action of PMNs and administered conventional antibiotics would then eliminate the biofilm-forming bacteria before a chronic infection is established. The present study has been carried out with non-mucoid P. aeruginosa strains, which dominate in the early phase of pulmonary infections. Strains from chronically infected CF patients are mucoid. Experiments are in progress to study whether PMNs and mucoid strains will give results similar to those presented here.

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REFERENCES


Baltimore, R. S., Christie, C. D. & Smith, G. J. (1989). Immuno-histopathologic localization of Pseudomonas aeruginosa in lungs from...


