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Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*

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Quorum sensing (QS) denotes a density-dependent mode of inter-bacterial communication based on signal transmitter molecules. Active QS is present during chronic infections with the opportunistic pathogen *Pseudomonas aeruginosa* in immunocompromised patients. The authors have previously demonstrated a QS-regulated tolerance of biofilm bacteria to the antimicrobial properties of polymorphonuclear leukocytes (PMNs). The precise QS-regulated effect on the PMNs is, however, unknown. Incubation of human PMNs with supernatants from dense *P. aeruginosa* cultures showed that the QS-competent *P. aeruginosa* induced rapid necrosis of the PMNs. This mechanism was also observed in mouse lungs infected with *P. aeruginosa*, and in sputum obtained from *P.-aeruginosa*-infected patients with cystic fibrosis. Evidence is presented that the necrotic effect was caused by rhamnolipids, production of which is QS controlled. The results demonstrate the potential of the QS system to facilitate infections with *P. aeruginosa* by disabling the PMNs, which are a major first line of defence of the host. Furthermore, the study emphasizes the inhibition of QS as a target for the treatment of infections with *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an opportunistic human pathogen, uncommon as a natural flora, but infecting hospitalized and immunocompromised patients, such as burn victims and AIDS patients. In particular, in patients with the autosomal recessive disease cystic fibrosis (CF), chronic *P. aeruginosa* lung infections represent the major morbid complication (Koch & Høiby, 1993). The lung infection experienced by CF patients can be divided into two stages: the colonizing non-mucoid state, and the chronic mucoid state. The chronic infection is preceded by intermittent colonizations and infections by non-mucoid *P. aeruginosa* for a mean period of 12 months (Høiby, 1974; Johansen & Høiby, 1992). During this stage, *P. aeruginosa* is apparently cleared. The gradual transition from the colonizing state to the chronic infection is characterized by a phenotypical switch from the non-mucoid to the mucoid state. This may be caused primarily by H_2O_2 liberated by the polymorphonuclear leukocyte (PMNs) (Mathee *et al.*, 1999), and, as recently demonstrated, by anaerobiosis of *P. aeruginosa* in the mucopurulent masses of the bronchioles (Worlitzsch *et al.*, 2002). The presence of *P. aeruginosa* growing in biofilms, i.e. microcolonies surrounded by a self-made polysaccharide matrix, is a hallmark of the chronic lung infection (Høiby, 1974). Within the biofilm, the bacteria are protected against the numerous surrounding PMNs, and they exhibit a remarkable tolerance to antibiotic treatments (Donlan & Costerton, 2002; Drenkard, 2003). The ability of bacteria to invade CF lungs in the first place is enabled by the high content of mucus and the concomitant cilia

Abbreviations: AHL, *N*-acylhomoserine lactone; BAL, broncheoalveolar lavage; CF, cystic fibrosis; C4-HSL, *N*-butanoyl-L-homoserine lactone; fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; 3-oxo-C12-HSL, *N*-3-oxododecanoyl-L-homoserine lactone; PI, propidium iodide; PMN, polymorphonuclear leukocyte; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing; 4Q, 4-quinolone.

A time-lapse movie showing the rapid death and disintegration of PMNs is available as supplementary data with the online version of this paper.

dysfunctionality, which result in a poor self-cleaning capacity of the CF lung (Knowles & Boucher, 2002; Gibson *et al.*, 2003). How the bacteria survive the encounter with the summoned PMNs prior to the formation of the protective biofilm is only partly understood, as no primary defects of PMNs from CF patients have been reported. The outcome of *P. aeruginosa* lung infections has recently been demonstrated to be at least partly dependent on quorum-sensing (QS)-regulated mechanisms (Bjarnsholt *et al.*, 2005a; Wu *et al.*, 2001), and the presence of QS activity during chronic *P. aeruginosa* lung infections in CF patients has been demonstrated (Storey *et al.*, 1998; Middleton *et al.*, 2002). QS, or cell-to-cell communication, is a regulatory mechanism by which bacteria respond to the population density (Fuqua *et al.*, 1996). The QS systems of *P. aeruginosa* have recently been intensively investigated, and they are responsive to chemically different signal molecules: one based on *N*-acylhomoserine lactone (AHL) signal molecules, and one based on 4-quinolones (4Qs). The AHL-based circuits are encoded by the Las and Rhl systems, each of which is based on LuxR and LuxI homologues. The two systems operate with specific signal molecules: *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12-HSL) for the *lasR*-encoded receptor, and *N*-butanoyl-L-homoserine lactone (C4-HSL) for the *rhlR*-encoded receptor. The 4Q-based system, also known as the *Pseudomonas* quinolone signal (PQS) system, is somewhat interspaced between the Las system and the Rhl system (McKnight *et al.*, 2000; Pesci *et al.*, 1999; Diggle *et al.*, 2006). The organization and interaction of the QS system in *P. aeruginosa* has been thoroughly investigated; however, it is not yet completely understood. The Las and Rhl systems have been identified as being hierarchically ordered, with the Las system in control of the Rhl system (Pesci *et al.*, 1997). It has been suggested, however, that the Rhl system can be switched on independently of the Las system. Diggle *et al.* (2003) proposed that this induction is governed by the PQS system. Intriguingly, LasR is required for the optimal production of the 4Q signal, whereas exogenously added PQS restores the expression of *lasB* in a *lasR* mutant background.

METHODS

Bacterial strains. The wild-type *P. aeruginosa* PAO1 used for the planktonic and biofilm *in vitro* experiments was obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu; strain PAO0001). This isolate has served as the DNA source for the *Pseudomonas* Genome Project (www.pseudomonas.com), and, subsequently, as a template for the design of the *P. aeruginosa* GeneChip (Affymetrix). The Δ*lasR rhlR* and Δ*lasI rhlI* mutants were constructed using the knockout systems described by Beatson *et al.* (2002). The knockout mutants were verified by Southern blot analysis, and by screening for AHL production (quorum signals). Strains for verification of genotypes were obtained from the University of Washington, Seattle, WA, USA (see Table 1).

Production of *P. aeruginosa* supernatants. Planktonic cultures were grown in shake flasks (180 r.p.m.) with Luria–Bertani (LB) medium at 37 °C for 24 h. For complementation, C4-HSL and 3-oxo-C12-HSL were added to Δ*lasI rhlI* mutants. Inhibition of QS was achieved by adding 12.5 mg furanone C-30 ml^{-1} (Hentzer *et al.*, 2003) to the medium upon inoculation. Biofilm cultures were grown for 2 days at 37 °C in six-well Nunc multidishes (Nunclon), each well containing 20 ml LB medium. Supernatants from planktonic and biofilm cultures were sterile-filtered through Minisart filters (16543; Sartorius), pore size 0.20 μm, and they were stored at −20 °C until use.

Biofilms for direct interaction with PMNs. Biofilms were cultivated in continuous-culture once-through flow chambers, and these were perfused with sterile AB trace minimal medium containing 0.3 mM glucose, as described previously (Christensen *et al.*, 1999; Bjarnsholt *et al.*, 2005a).

Preparation of PMNs. Human blood samples were obtained by venous puncture from normal healthy volunteers, and collected in BD Vacutainers containing 0.129 M sodium citrate (367704; BD Diagnostics). The PMNs were isolated by erythrocyte sedimentation and density-gradient centrifugation, as previously described (Bjarnsholt *et al.*, 2005a).

PMN migration assay. Estimation of PMN migration against *P. aeruginosa* supernatants was carried out using Transwell trays (3415; Costar). Samples (350 μl) tested were: LB medium with 10 nM *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) (F3506; Sigma), LB medium, and sterile filtered supernatants from PAO1, Δ*lasR rhlR*, Δ*lasI rhlI*, and Δ*lasI rhlI* complemented with C4-HSL and 3-oxo-C12-HSL. A Transwell filter (pore size, 3 μm) was inserted in the well, and 100 μl isolated PMNs (2.5×10^6 cells ml^{-1} in RPMI 1640 with 5 % normal human AB+ serum) was added on top of the filter. Following incubation for 30 min at 37 °C, the Transwell filter was removed. A 100 μl volume was aspirated from the well, and added to a TruCount tube (340334; BD Biosciences) with 300 μl Facslysis (349202; BD Biosciences) containing 100 μg propidium iodide (PI) ml^{-1} (P-4170; Sigma). After incubation in the dark for a minimum of 10 min, the samples were analysed by flow cytometry, and the number of migrated PMNs was calculated according to: migrated PMNs = (cells counted/beads counted)(beads added/volume of cells added) $\times 10^3 \times 0.35$. Data from each set-up were normalized by setting migration against fMLP to 100 %.

PMN killing by supernatants. The isolated PMNs (2.5×10^6 cells ml^{-1}), and all tested sterile filtered supernatants, were equilibrated with 2.5 μg PI ml^{-1}, and incubated at 37 °C for 15 min, before mixing 50 μl isolated PMNs with 350 μl sterile filtered supernatant, followed by immediate analysis of PI staining with flow cytometry.

PMN killing by biofilms. In order to inoculate PMNs into the biofilm chambers, the flow was stopped, and the flow cells were clamped off. Isolated PMNs (100 μl, 2.5×10^6 cells ml^{-1}, stained with 2.5 μg PI ml^{-1}) were inoculated into each flow channel. The flow cells were incubated top down in a 37 °C water bath, with shaking, until microscopic inspection.

Haemolysis. Normal human venous blood collected in BD Vacutainers (100 μl) was mixed with 3.5 ml sterile filtered supernatant from batch cultures of *P. aeruginosa*. After 10 min, lysis was evaluated by visual inspection.

Experimental animals. Female BALB/cj mice were purchased from M&B Laboratory Animals at 10–11 weeks of age. The mice were of equal size, and were maintained on standard mouse chow and water *ad libitum* for 1 week prior to challenge. All animal experiments were authorized by the National Animal Ethics Committee, Denmark. The mouse experiments were performed as described by Pedersen *et al.* (1990).

Isolation and staining for endobronchial PMNs

Broncheoalveolar lavage (BAL). Exposed trachea of anaesthetized mice were canulated with a size 22 gauge catheter (OPTIVA* 2;

Table 1. Single-knockout mutants used in the study

Strains were obtained from the University of Washington, Seattle, WA, USA.

PA no.	Gene	Description	PA no.	Gene	Description
PA0026	*plcB*	Phospholipase C	PA1218		Hypothetical protein
PA0616		Hypothetical protein	PA1221		Hypothetical protein
PA0617		Probable bacteriophage protein	PA1367		Hypothetical protein
PA0620		Probable bacteriophage protein	PA1668		Hypothetical protein
PA0621		Hypothetical protein	PA1669		Hypothetical protein
PA0626		Hypothetical protein	PA1716	*pscC*	Type III secretion outer-membrane protein PscC precursor
PA0627		Hypothetical protein	PA1868	*xqhA*	Secretion protein XqhA
PA0668	*tyrZ*	Tyrosyl-tRNA synthetase 2	PA1898	*qscR*	QS control repressor
PA0669		Probable DNA polymerase	PA1899	*phzA2*	Probable phenazine biosynthesis protein
PA0685		Probable type II secretion protein	PA2194	*hcnB*	Hydrogen cyanide synthase
PA0843	*plcR*	Phospholipase accessory protein PlcR precursor	PA3105	*xcpQ*	General secretion pathway protein D
PA0844	*plcH*	Haemolytic phospholipase C precursor	PA3319	*plcN*	Non-haemolytic phospholipase C precursor
PA0962		Probable DNA-binding stress protein	PA3478	*rhlB*	Rhamnosyltransferase chain B
PA0985		Pyocin S5	PA3479	*rhlA*	Rhamnosyltransferase chain A
PA0997	*pqsB*	Homologous to β-keto-acyl-acyl-carrier protein synthase	PA3724	*lasB*	Elastase LasB
PA0998	*pqsC*	Homologous to β-keto-acyl-acyl-carrier protein synthase	PA3866		Pyocin protein
PA0999	*pqsD*	3-oxoacyl-[acyl-carrier-protein] synthase III	PA4210	*phzA1*	Probable phenazine biosynthesis protein
PA1003	*pqsR*	Transcriptional regulator	PA4217	*phzS*	Flavin-containing monooxygenase
PA1150	*pys2*	Pyocin S2	PA4304		Probable type II secretion system protein
PA1216		Hypothetical protein	PA5040	*pilQ*	Type 4 fimbrial biogenesis outer-membrane protein PilQ precursor
PA1217		Probable 2-isopropylmalate synthase	PA5262	*algZ*	Alginate biosynthesis protein AlgZ/FimS

Johnson & Johnson Medical). BAL was performed by flushing six times with 1.5 ml ice-cold PBS without Ca^{2+} and Mg^{2+}. The BAL fluid was stored on ice until staining for necrotic PMNs. The mean recovery of BAL fluid was 1.1 ml (CV 13 %).

Staining for necrotic PMNs in the BAL fluid. Necrotic and apoptotic PMNs were stained with Annexin V-FITC Apoptosis Detection Kit I (556747; BD Biosciences), according to a modification of the preparation supplied by the manufacturer. BAL fluid (200 µl) was equilibrated by centrifugation with 2.5 ml cold 1× binding buffer (BD Biosciences) at 350 ***g*** for 7 min at 5 °C. To discriminate between necrotic and apoptotic PMNs, 100 µl 1× binding buffer containing 2.5 µg PI ml^{-1}, annexin V-FITC component (1 : 40), and the PMN phenotypic surface marker monoclonal allophycocyanin-conjugated rat anti-Ly 6G antibody (clone RB6–8C5; BD Biosciences) (1 : 50), was added to the pellet, and incubated for 15 min at room temperature in the dark. The incubation was terminated by addition of 400 µl 1× binding buffer, and the samples were analysed by flow cytometry.

Staining for the concentration of PMNs in the BAL fluid. A 200 µl volume of BAL fluid was added to a TrueCount tube. PMNs and total leukocytes were stained by adding 20 µl cold PBS containing phycoerythrin-conjugated monoclonal rat anti-mouse Ly 6G antibody (clone RB6–8C5; BD Biosciences; 1 : 20) and peridinin chlorophyll A protein-conjugated monoclonal rat anti-mouse CD45 antibody (clone RB6–8C5; BD Biosciences; 1 : 10). After incubation for 30 min on ice in the dark, 300 µl Facslysis solution was added, and the samples were incubated for at least 10 min prior to flow cytometry. PMN concentration was calculated according to: cells ml^{-1} = (cells counted/beads counted)(beads added/BAL fluid added) $\times 10^3$.

Flow cytometry. The samples were analysed using a FACSort (Becton Dickinson) equipped with a 15 mW argon-ion laser tuned at 488 nm, and a red diode laser emitting at 635 nm for excitation. Light scatter, time, and exponentially amplified fluorescence parameters from at least 10 000 events, were recorded in list mode. Necrotic PMNs were identified according to their increased PI fluorescence intensity, and their morphology was determined by light scatter. The instrument was calibrated using Calibrite beads (Becton Dickinson).

Quantitative lung bacteriology. For colony counting, the exposed lungs were isolated in 5 ml PBS, and homogenized on ice. A serial dilution of the lung homogenate was performed, and dilutions were plated on blue agar plates (States Serum Institute), which are selective for Gram-negative bacilli.

Proteinase K assay. Protein degradation by proteinase K was performed as described by the manufacturer (Promega).

Pyocyanin assay. The pyocyanin concentration was measured as described by Essar *et al.* (1990).

Statistics. Data are presented as means ± SEM; P values are from Student's two-tailed unpaired t tests, except for comparison of frequencies, which was done using a χ^2 test.

RESULTS AND DISCUSSION

PMNs are unable to migrate towards the supernatant of wild-type strain *P. aeruginosa* PAO1

We have previously described the presence of a QS-regulated phenotype that operates to paralyse PMNs *in vitro* (Bjarnsholt *et al.*, 2005a). This inspired us to further investigate the encounter between *P. aeruginosa* and the PMNs. The PMNs are the first major phagocytes to arrive during *P. aeruginosa* lung infection, and their activity is related to the early outcome of the infection (Jensen *et al.*, 2004). Based on our previous results, we speculated that QS-induced paralysis of the PMNs also affected migration. When freshly isolated PMNs were allowed to migrate towards sterile-filtered *P. aeruginosa* supernatants [obtained from the wild-type and the QS mutants (Δ*lasR rhlR* and Δ*lasI rhlI*), and from Δ*lasI rhlI* complemented with C4-HSL and 3-oxo-C12-HSL], no migration to the wild-type and complemented supernatant was observed (Fig. 1). This effect, however, was not caused by the presence of AHL molecules, as additional experiments showed that PMNs migrated freely towards concentrations of pure AHL signal molecules 3-oxo-C12-HSL and C4-HSL increasing from 0.1 to 50 μM (data not shown).

PMNs are lysed by a *P. aeruginosa* wild-type supernatant

Elaboration of this phenomenon found that sterile-filtered supernatant from wild-type and Δ*lasI rhlI* mutant strains, grown in the presence of both C4-HSL and 3-oxo-C12-HSL (10 μM), caused rapid damage to PMN plasma membranes, as demonstrated by increased fluorescence from supplemented PI during real-time flow cytometry (Fig. 2a).

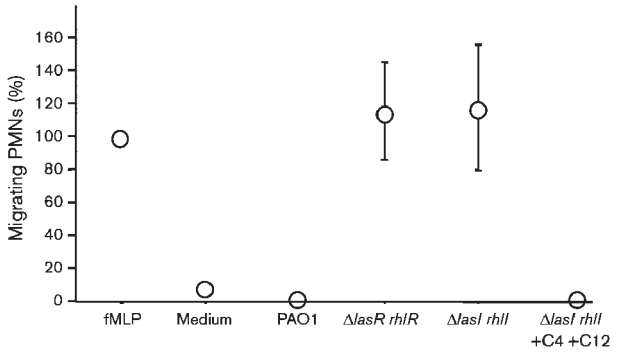

Fig. 1. Effects of QS on PMN migration. The mean PMN migration towards supernatants from *P. aeruginosa* with competent QS (PAO1) and deficient QS (Δ*lasR rhlR* and Δ*lasI rhlI*), and a QS-deficient mutant (Δ*lasI rhlI*) complemented with C4-HSL (C4) and 3-oxo-C12-HSL (C12) (10 μM), was calculated as the percentage of human PMNs migrated towards supernatants from *P. aeruginosa*, as compared with migration towards fMLP (10 nM). Standard error bars are indicated ($n=6$).

Within the first minute after the increase in PI fluorescence was recorded, the light scatter of the PMNs decreased below the threshold of detection, suggesting a rapid disintegration of the PMNs (data not shown). The rapid death and disintegration was confirmed by time-lapse recording using a combination of fluorescence and light-transmission microscopy (Fig. 2b, and supplementary movie available with the online version of this paper). Furthermore, this analysis revealed that the PMN DNA was released during disintegration. In contrast, the PMN plasma membrane remained intact when mixed with supernatants derived from Δ*lasI rhlI* and Δ*lasR rhlR* mutants. This necrotic effect contrasts with the previously reported acceleration of PMN apoptosis caused by *P. aeruginosa* QS signal transmitter molecules (Tateda *et al.*, 2003). Pure C4-HSL and 3-oxo-C12-HSL at concentrations up to 50 μM failed to induce rapid necrosis (data not shown).

PMNs are lysed when in contact with *in vitro* biofilms of wild-type *P. aeruginosa*

To determine if the cytotoxic effect accounted for paralysis of PMNs (Bjarnsholt *et al.*, 2005a), PMNs were incubated on an *in vitro* biofilm. Necrosis was observed when PMNs were incubated with QS-proficient *P. aeruginosa* biofilms grown for 4 days in flow cells (Fig. 3), but it was not observed when the PMNs were incubated on a QS-deficient biofilm grown under identical conditions. The cytotoxic effect was not specific for the PMNs, as demonstrated by the haemolytic activity of sterile-filtered supernatant from QS-competent *P. aeruginosa* cultures (Fig. 4).

PMNs disappear in mice infected with wild-type *P. aeruginosa*

To determine if the necrotic effect was restricted to *in vitro* settings only, a pulmonary infectious mouse model was used for verification. Previously, we established a correlation between QS deficiency and faster clearing of infecting bacteria in the mouse model (Bjarnsholt *et al.*, 2005a). BALB/cj mice were infected with either wild-type or QS-deficient mutant, both of which were alginate embedded. At the time points 3, 6, 18 and 24 h post-infection, BAL fluid was obtained, after which the lungs were homogenized. Analysis of the BAL fluid by means of flow cytometry from mice infected with the wild-type *P. aeruginosa* showed a high proportion of dead PMNs in the endobronchial space, as detected by their strong PI fluorescence. In accordance with our *in vitro* data, significantly fewer intact PMNs (estimated by weak PI fluorescence and low annexin V staining) were found in the BAL fluids from mice infected with the wild-type *P. aeruginosa*. After 18 and 24 h, an increased number of bacteria were observed in the lungs of mice infected with the wild-type *P. aeruginosa* (Fig. 5). This increase in the number of bacteria, however, is transient, as previous experiments have shown that the number of wild-type bacteria decreases on days 3 and 5 (Bjarnsholt *et al.*, 2005a). Previously, we reported an elevated concentration of the PMN-recruiting interleukin MIP-2 (a murine IL-8 analogue)

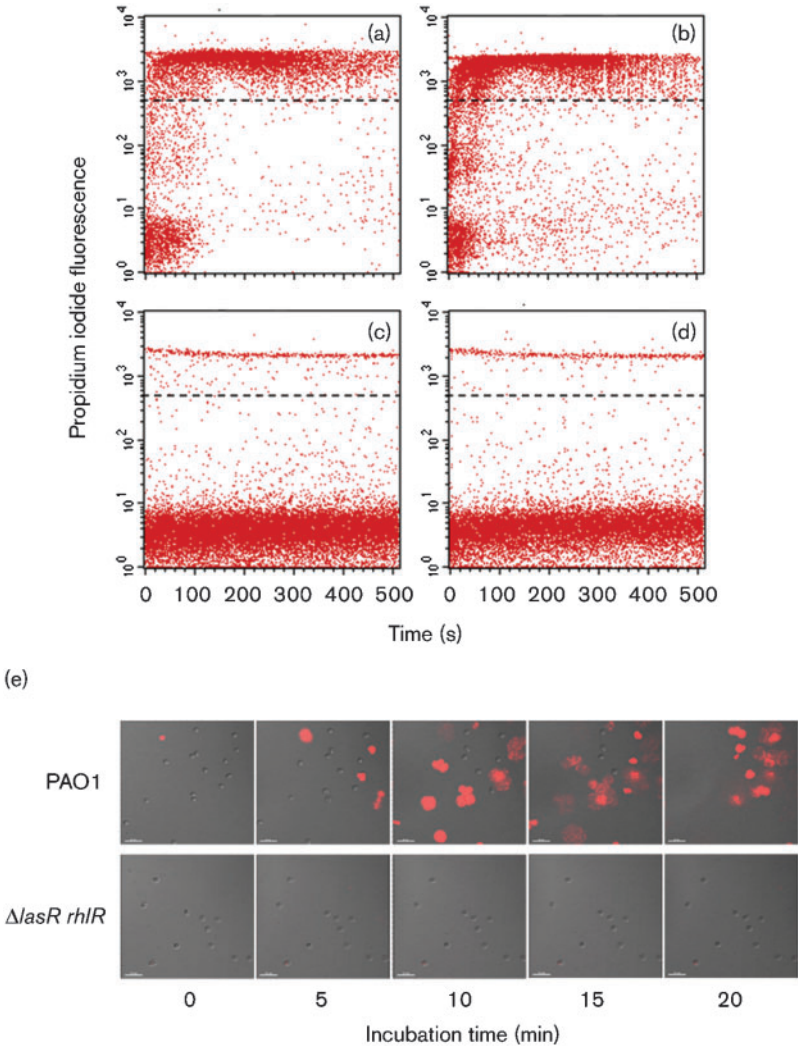

Fig. 2. QS-regulated killing of PMNs. (a–d) Real-time flow cytometry of PI-stained human PMNs mixed with sterile filtered supernatants from *P. aeruginosa*. Dashed lines represent the lower PI fluorescence intensity used for discriminating necrosis. (a) Supernatant from *P. aeruginosa* with competent QS (PAO1). (b) Supernatant from *P. aeruginosa* with deficient QS (Δ*lasI rhlI*) complemented with C4-HSL and 3-oxo-C12-HSL. (c) Supernatant from *P. aeruginosa* with deficient QS (Δ*lasR rhlR*). (d) Supernatant from *P. aeruginosa* with deficient QS (Δ*lasI rhlI*). (e) Micrographs of PMN disintegration and DNA release induced by QS, and recorded by combined fluorescence and light microscopy. Human PMNs were mixed with sterile filtered supernatants from QS-competent (PAO1) and QS-deficient (Δ*lasR rhlR*) *P. aeruginosa*, and stained with PI. Bars, 50 μm.

when mice were infected with the wild-type (Bjarnsholt *et al.*, 2005b). We therefore suggest that the results obtained at each time point represent snapshots of a continuous process, i.e. the PMNs are constantly being recruited to the sites of infections, but, in the wild-type situation, a substantial fraction of the incoming PMNs disintegrate due to the cytotoxic bacterial activity, which, in turn, allows the bacterial population to expand. The recruited PMNs in mice infected with the QS-deficient mutant manage to prevent an increase in the number of bacteria (Fig. 5). We suggest that this difference is caused by a QS-regulated reduction of the PMN functionality. To estimate the clinical relevance of

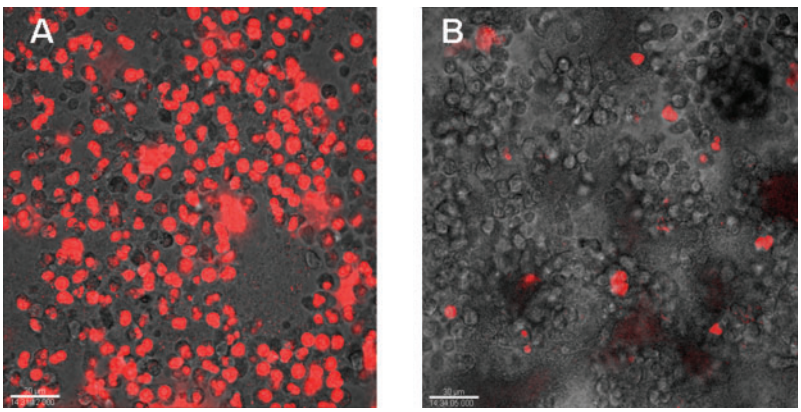

Fig. 3. QS-regulated killing of PMNs by *P. aeruginosa* biofilms visualized by combined fluorescence and light microscopy. Four-day-old biofilms, grown in continuous-culture once-through flow chambers, were inoculated with human PMNs, and stained with the DNA stain PI. A greater number of PMNs became necrotic on the QS-competent PAO1 biofilm (A) compared with the QS-mutant (Δ*lasR rhlR*) biofilm (B). Bars, 30 μm.

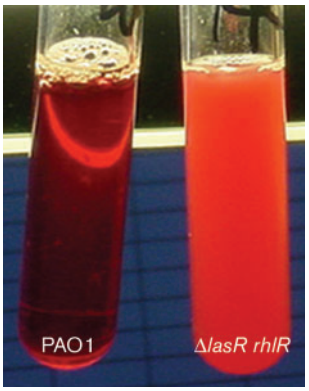

Fig. 4. QS-regulated haemolysis of whole blood. Human peripheral blood was mixed with sterile filtered supernatants from *P. aeruginosa*. Haemolysis was only induced with supernatant from QS-competent *P. aeruginosa* (PAO1).

cytotoxic metabolites from *P. aeruginosa*, sputum from CF patients chronically infected with and without *P. aeruginosa* was compared. Of the sputum samples from patients with *P. aeruginosa* infections, 9 of 13 induced rapid PMN necrosis in our *in vitro* assay, whereas 0 of 3 samples from patients without *P. aeruginosa* induced PMN necrosis ($P \leqslant 0.03$).

The QS inhibitor furanone C-30 blocks cytotoxicity *in vitro*

Since the effect of QS blockers, such as furanone C-30, results in accelerated clearance of *P. aeruginosa* in the pulmonary mouse model (Hentzer *et al.*, 2003), it was of interest to determine if the induction of PMN necrosis by treating a growing batch culture with furanone C-30 could be blocked. We observed that furanone-C-30-treated cultures of the wild-type, and a Δ*lasI rhlI* mutant grown in the presence of exogenously added AHL signal molecules, completely blocked development of both the PMN necrotic (Fig. 6) and the haemolytic effect (not shown).

Identification of the QS-regulated cytotoxin

Based upon these results, we conclude that the QS-regulated necrotic PMN factor is an extracellular product that causes PMN malfunction both *in vivo* and *in vitro*. As for *P. aeruginosa*, a minimum of 172 genes are QS regulated, and many of these are only annotated as hypothetical proteins (Hentzer *et al.*, 2003). Several of the QS-regulated genes encode known virulence factors, including proteins, lipids and secondary metabolites (Hentzer *et al.*, 2003; Wagner *et al.*, 2003; Schuster *et al.*, 2003). In addition, several virulence factors with potentially necrotic capacity are not QS regulated, and these include exotoxin A and pepA (exoU is not present in PAO1). Furthermore, the type III secretion apparatus is dependent on cell–cell contact for delivery of toxins; in contrast, the effect we describe in the present report is not dependent on this process. AHLs have been described to induce apoptosis in macrophages and PMNs (Tateda *et al.*, 2003); however, we were unable to replicate the fast killing of the PMNs by treatment with pure AHL signal molecules (data not shown). To identify the compound responsible for the rapid necrotic killing, the supernatant of the wild-type was examined as described below. The active component was identified as rhamnolipid B, which belongs to a class of well-known biosurfactants from *P. aeruginosa*. To verify this, single-knockout mutants were investigated, including rhamnolipid, hydrogen cyanide, elastase and phospholipase C; for a complete list of strains tested refer to Table 1. Supernatants of these single-knockout mutants suggested that rhamnolipids were responsible for the necrotic activity identified by flow cytometry of PI-stained PMNs. Induction of PMN necrosis was also found to be independent of the pyocyanin content (data not shown). *P. aeruginosa* produces quinolones (PQS) as another part of its QS apparatus, and these molecules have been shown to induce apoptosis and reduce T-cell proliferation (Calfee *et al.*, 2005; Pritchard, 2006). No necrotic effect was observed in the PMNs upon direct addition of up to 100 μM PQS (data not shown). We did, however, find that Δ*pqsB*, Δ*pqsC*, Δ*pqsD* and Δ*pqsR* mutants did not produce the necrotic effect, indicating that the PQS system is involved in the regulation of the production of the compound(s) causing PMN necrosis.

The principal necrotic metabolite was identified from supernatants as 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid, which is also known as rhamnolipid B. Supernatants of outgrown *P. aeruginosa* PAO1 batch cultures were found to contain 100–200 μg rhamnolipid B ml^{-1}, whereas no rhamnolipids

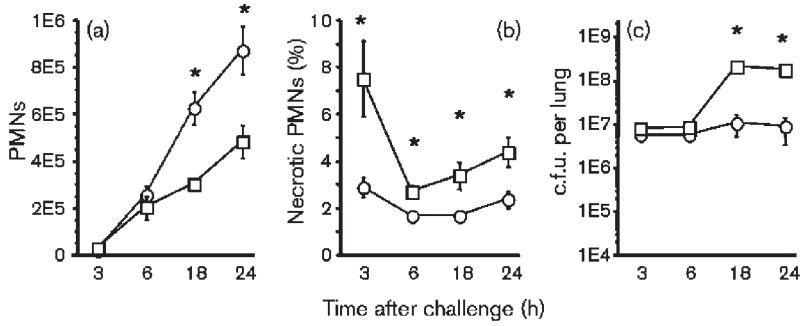

Fig. 5. Significance of QS during *P. aeruginosa* lung infection. BALB/cj mice were inoculated with alginate-embedded QS-competent (PAO1, □) or QS-deficient (Δ*lasR rhlR*, ○) *P. aeruginosa* in the left lung, and sampled at 3, 6, 18 and 24 h. (a) Mean number of intact PMNs in the BAL fluid. (b) Mean percentage of necrotic PMNs in the BAL fluid. (c) Mean number of c.f.u. in the lungs. Standard error bars are indicated. *$P < 0.01$, $n = 10$; unpaired *t* test.

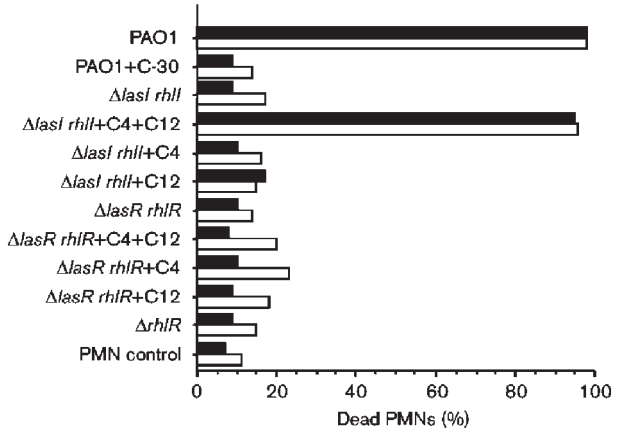

Fig. 6. Inhibition and complementation of the QS-regulated fast killing of PMNs. The contents of dead PMNs were analysed by flow cytometry of human PMNs stained with PI, and mixed with sterile filtered supernatants from cultures of *P. aeruginosa* for 5 (black bars) and 10 min (white bars). Inhibition of QS was done by adding synthetic furanone C-30 to PAO1 during culture, and with double-knockout (Δ*lasR rhlR* and Δ*lasI rhlI*) and single-knockout (Δ*lasR*) mutations. Complementation was performed by growing Δ*lasI rhlI* in the presence of C4-HSL (C4) and/or 3-oxo-C12-HSL (C12). The bars show representative values from several experiments.

were detected in Δ*rhlA* mutants and a Δ*lasR rhlR* mutant. In addition, PQS mutants were found to produce far less rhamnolipid B than the wild-type. Incubating PMNs with wild-type supernatant containing approximately 100 μg rhamnolipid B ml^{-1} induced necrosis as fast as incubation with 100 μg purified rhamnolipid B ml^{-1}. Earlier investigations support our finding, as rhamnolipids are known to lyse PMNs (Shryock *et al.*, 1984), erythrocytes (Johnson & Boese-Marrazzo, 1980), and monocyte-derived macrophages (McClure & Schiller, 1992).

Rhamnolipid isolation and identification

Sterile filtered culture supernatant from PAO1 (3 l) was extracted three times in ethyl acetate (3 × 2 l), and the ethyl acetate was removed under vacuum to yield 1.07 g yellow solid material. This was adsorbed to celite, and applied to a 10 g isolute DIOL column pre-equilibrated with 100 % heptane. Fractions were eluted as follows: two fractions of 50 % dichloromethane (DCM) in heptane; two fractions of 100 % DCM; 20, 30, 40, 50, 60 and 80 % ethyl acetate in DCM; 100 % ethyl acetate; 10 % methanol in ethyl acetate; and, finally, two 100 % methanol washes. All fractions were 12 ml, except for the last methanol wash, which was 50 ml. PMN necrotic activity was seen in the last three fractions; these fractions were combined, and then further fractionated on a 20 g StrataX C_{18} column. Elution was with a stepped acetonitrile/water gradient starting at 100 % water, increasing to 100 % acetonitrile in 10 % steps, skipping 10 and 90 % acetonitrile fractions, and collecting 50 ml per fraction. The primary PMN necrotic activity was detected in the seventh fraction (80 % acetonitrile). LC-MS showed a molecular mass of 651.3881 g mol^{-1} (Fig. 7), corresponding to a molecular formula of $C_{32}H_{58}O_{13}$ (4 dbe). Other ions were observed at 359.3, 505.3, 668.4 and 673.4 g mol^{-1},

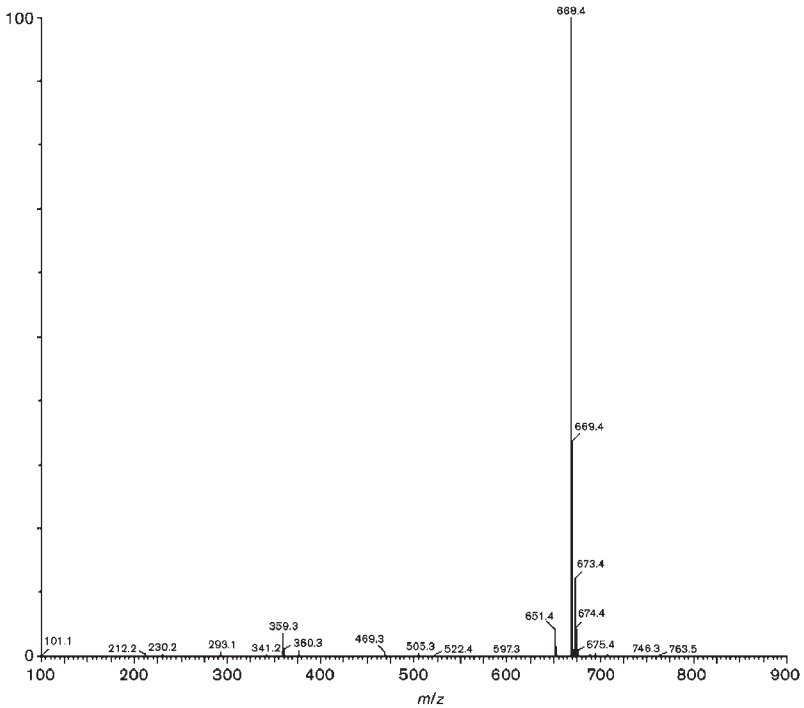

Fig. 7. Mass spectrum of rhamnolipid B purified from supernatant of *P. aeruginosa* PAO1.

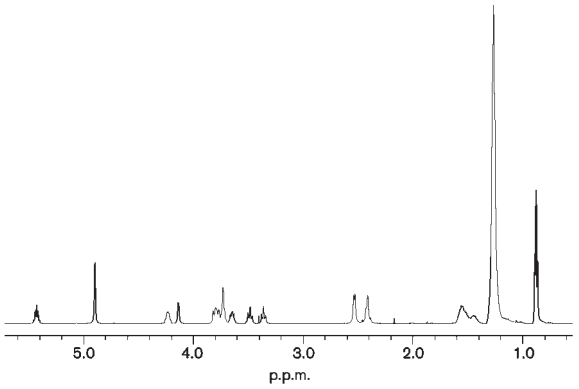

Fig. 8. ^{1}H NMR spectrum of rhamnolipid B purified from supernatant of *P. aeruginosa* PAO1. The spectrum was referenced to $CDCl_3$ at δ_H 7.26, and obtained on a Varian Inova operating at 500 MHz.

corresponding to $[M+H\text{-}2\times\text{rhamnose}]^+$, $[M+H\text{-rhamnose}]^+$, $[M+NH_3]^+$ and $[M+Na]^+$, respectively.

The ^{1}H NMR spectrum (Fig. 8) showed two anomeric signals, several glycosidic proton signals, and a large number of aliphatic signals. The ^{13}C NMR spectrum indicated the presence of two carbonyl ester carbons, two anomeric carbons, several oxygenated carbons, with the remaining signals corresponding to aliphatic and methyl carbons. Analysis of the 2D NMR spectra (Table 2) showed two rhamnose and two fatty acid units, suggesting that the molecule was a rhamnolipid, which is a class of compounds well known from *P. aeruginosa*. Extremely good agreement was seen between the experimental NMR data and that published by Sim *et al.* (1997) for the compound 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoic acid, or rhamnolipid B.

Conclusion

Functional QS systems are present in the vast majority of *P. aeruginosa* isolates from the urinary tract, the lower respiratory tract and wound infections (Schaber *et al.*, 2004), and the presence of functional QS systems significantly delays clearing of the bacteria in experimental studies of *P. aeruginosa* pulmonary infections and thermal wounds (Wu *et al.*, 2001; Bjarnsholt *et al.*, 2005a; Rumbaugh *et al.*, 1999). Interestingly, *P. aeruginosa* frequently loses the ability to produce the long-chain signal molecule over time (Heurlier *et al.*, 2006), suggesting a pivotal role of QS during the initial stages of infection. It is now widely accepted that *P. aeruginosa* adapts to the lung environment, to the point of evolving several subpopulations throughout the lung. One of the main adaptations involves production of alginate,

Table 2. NMR data of rhamnolipid B

Data were acquired on a Varian Inova machine at 500 MHz for ^{1}H, and 300 MHz for ^{13}C. Spectra were referenced to $CDCl_3$ at δ_H 7.26. The shifts for the atoms numbered from 2 to 7, and from 12 to 17, were not included in the table as they all overlapped in the region of δ_H 1.2–1.5 for proton, and δ_C 25–30 for carbon. COSY, correlated spectroscopy; HMBC, heteronuclear multiple-bond correlation.

Number	δ_H, mult., (J_{HH} Hz)	COSY	^{13}C	HMBC
1	0.87, d	1.29	13.9	–
8	4.23, sept	2.41, 1.45	70.9	–
9	2.41, m	4.23	39.4	29.1, 24.6
10	–	–	171.0	–
11	0.86, d	1.29	13.9	–
18	5.43, p, (6.2)	1.56, 2.53	70.2	24.9, 171.3, 173.6
19	2.53, d, (5.9)	5.43	39.1	171.3, 71.2
20	–	–	173.0	–
1′	4.89, br s	4.13	94.2	68.0, 70.1, 71.5, 79.7
2′	4.13, br s	4.89, 3.77	70.3	71.0, 72.3
3′	3.77, dd, (2.4, 10.3)	4.13, 3.48	71.1	–
4′	3.48, t, (9.1)	3.77, 3.73	72.2	70.5, 68.0, 17.7
5′	3.73, br q, (7.0)	3.48, 1.26	68.5	71.0
6′	1.27, d, (6)	3.73, 1.45	17.4	–
7′	4.89, br s	3.73	102.1	68.0, 70.1, 71.5, 79.7
8′	3.73, m	4.89, 3.80	79.4	102.5, 73.4
9′	3.80, dd, (1.8, 9.6)	3.36, 3.73	70.3	–
10′	3.36, t, (9.4)	3.80, 3.65	73.2	173.6, 70.5
11′	3.65, dt, (5.6, 14)	3.36, 1.27	67.5	71.0, 68.7, 17.7
12′	1.26, d, (6)	3.65, 1.56	24.9	–

which is believed to represent a long-term defence mechanism. In contrast, the killing of the PMNs may be one of the mechanisms that facilitates establishment of early *P. aeruginosa* lung infections. In support of this model, a higher number of necrotic PMNs has been detected in CF lungs infected with *P. aeruginosa*, as compared with other bacterial infections (Watt *et al.*, 2005). In addition, CF lungs chronically infected with *P. aeruginosa* contain high amounts of extracellular DNA, which is probably derived from necrotic PMNs (Lethem *et al.*, 1990; Shah *et al.*, 1996). The ability of *P. aeruginosa* to defend itself aggressively against PMNs by production of rhamnolipids may be a prerequisite for the development of the chronic infection with *P. aeruginosa*. As rhamnolipids have been detected in sputum from CF patients with chronic *P. aeruginosa* lung infections (Kownatzki *et al.*, 1987), and nonmucoid clinical isolates have been shown to produce more haemolytically active rhamnolipids than mucoid isolates (McClure & Schiller, 1992), we believe that our observations add significantly to understanding the basis of how QS regulates the phenotype of *P. aeruginosa* for successful establishment of chronic infections, as seen in the CF lung. Several characteristics of the infected CF lungs are currently considered as being able to promote the persistence of *P. aeruginosa*. In this context, our demonstration of QS-regulated secretion of cytotoxic amounts of rhamnolipids by *P. aeruginosa* may add significantly to understanding the establishment and persistence of the infection: impairment of neutrophil migration caused by the viscous mucus has been proposed as a mechanism for decreased bactericidal activity (Matsui *et al.*, 2005). Baltimore *et al.* (1989), however, have clearly shown that the neutrophils are able to accumulate very close to the microcolonies of *P. aeruginosa* in the CF lung. Despite this massive PMN accumulation, the microcolonies persist, suggesting another mechanism by which the bacteria impair the nearby neutrophils. Impairment of the neutrophils has also been proposed to be caused by QS-regulated proteases cleaving surface receptors on the neutrophils (Kharazmi *et al.*, 1984a, b); however, neutralizing antibodies against these proteases are present in the lungs of chronically infected CF patients (Doring *et al.*, 1985). We found that QS-induced PMN necrosis was not caused by proteins. In addition, the formation of biofilm has been recognized as a mechanism for protection against phagocytosis (Donlan & Costerton, 2002); however, we previously demonstrated that biofilm-induced protection against phagocytosis by neutrophils is dependent on QS (Bjarnsholt *et al.*, 2005a). Further work is necessary to elucidate the role and consequences of the presence of QS-regulated rhamnolipds during the colonization and infection of CF lungs.

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