Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an *in vivo* intraperitoneal foreign-body infection model

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Pseudomonas aeruginosa is an opportunistic human pathogen that causes chronic biofilm-based infections in host organisms. *P. aeruginosa* employs quorum sensing (QS) to control expression of its virulence, and to establish and maintain chronic infections. Under such conditions, the biofilm mode of growth contributes significantly to *P. aeruginosa* tolerance to the action of the innate and adaptive defence system and numerous antibiotics. In the present study, an *in vivo* foreign-body infection model was established in the peritoneal cavity of mice. Experimental data showed that QS-deficient *P. aeruginosa* are cleared more rapidly from silicone implants as compared to their wild-type counterparts. Concurrently, treatment with the QS inhibitor furanone C-30 of mice harbouring implants colonized with the wild-type *P. aeruginosa* resulted in a significantly faster clearing of the implants as compared to the placebo-treated group. These results were obtained with both an inbred (BALB/c) and an outbred (NMRI) mouse strain. The present results support a model by which functional QS systems play a pivotal role in the ability of bacteria to resist clearing by the innate immune system and strongly suggest that the efficiency of the mouse innate defence against biofilm-forming *P. aeruginosa* is improved when the bacteria are treated with QS drugs that induce QS deficiency.

Received 17 January 2007

Revised 5 March 2007

Accepted 13 March 2007

INTRODUCTION

In the USA, microbial infections on medical implants occur in more than 2 million surgical cases each year and infection is the main cause of biomedical implant failure (Emerson & Camesano, 2004; Gristina, 1987; Verkerke *et al.*, 1997). One explanation gaining momentum is that the biofilm mode of growth protects the colonizing bacteria and enables them to withstand host immune responses and exhibit high tolerance to treatment with the highest deliverable doses of antibiotics (Bjarnsholt *et al.*,

2005a; Costerton *et al.*, 1999; Hentzer *et al.*, 2003; Høiby *et al.*, 2001; Middleton *et al.*, 2002; Singh *et al.*, 2002). In general, infections on foreign bodies will not be cleared until the implants have been removed from the body (Neut *et al.*, 2005), and unfortunately reimplantation has a high failure rate and is prone to recolonization by similar problem-causing bacteria (Cherney & Amstutz, 1983).

One particularly notorious biofilm former that is known to cause infections on medically implanted foreign bodies is *Pseudomonas aeruginosa* (Braxton *et al.*, 2005; Neut *et al.*, 2005). *P. aeruginosa* uses cell-to-cell communication or quorum sensing (QS) to control the production and secretion of virulence factors, which, deployed at the right time and place, enables evasion of the host defences (Fuqua *et al.*, 1994). By producing and receiving small diffusible *N*-acyl-L-homoserine lactone (AHL) signal molecules, the bacteria are able to monitor their own population size and

Abbreviations: AHL, *N*-acyl-L-homoserine lactone; BAL, bronchoalveolar lavage; C4-HSL, *N*-butanoyl-L-homoserine lactone; CLSM, confocal laser scanning microscopy; GFP, green fluorescent protein; 3-oxo-C12-HSL, *N*-(3-oxododecanoyl)-L-homoserine lactone; PMN, polymorphonuclear leukocyte; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing.

thereby pool their activities accordingly. When the bacterial density, and thus the concentration of secreted signal molecules, reaches a threshold, the signal molecules bind to a transcriptional regulator, forming a complex that induces the transcription of target genes (Fuqua *et al.*, 1994). *P. aeruginosa* employs two AHL-mediated QS circuits, the *las* and *rhl* QS systems. Both systems are organized with a transcriptional regulator, LasR or RhlR, and a synthetase, LasI or RhlI. LasI and RhlI synthesize the two AHL signal molecules *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) and *N*-butanoyl-L-homoserine lactone (C4-HSL), respectively (Ochsner & Reiser, 1995; Passador *et al.*, 1993; Pearson *et al.*, 1994, 1995). The QS systems of *P. aeruginosa* are hierarchically arranged, with the *las* QS system controlling the *rhl* QS system (Latifi *et al.*, 1996; Pesci *et al.*, 1997). In addition to the AHL signal molecules produced by *P. aeruginosa*, a third signalling compound, denoted the *Pseudomonas* quinolone signal (PQS), has been identified. LasR QS has been shown to regulate PQS production and since PQS enhances the expression of *rhlI* and *rhlR*, it is suggested that PQS activity forms a regulatory link between the *las* and *rhl* QS systems (Diggle *et al.*, 2003; Pesci *et al.*, 1997). The signal molecule 3-oxo-C12-HSL is known to pose immunomodulatory effects. Thus it was shown to cause neutrophil apoptosis and thereby to facilitate infection (Tateda *et al.*, 2003). Furthermore, 3-oxo-C12-HSL has been shown to inhibit T-cell proliferation and to suppress the production of the cytokine IL-12 and tumour necrosis factor alpha (TNF- α). These are all involved in the bactericidal T_H1 response, which is important in the response to bacterial infections (Telford *et al.*, 1998). The PQS signal is also known to exhibit immunomodulatory properties in that it was shown that PQS inhibits T-cell proliferation as 3-oxo-C12-HSL. In contrast to 3-oxo-C12-HSL, PQS induces the production of TNF- α (Hooi *et al.*, 2004). These findings indicate that the signal molecules both promote bacterial communication and production of virulence factors, and suppress the response of the host immune system. This leads to an increased survival potential of the bacteria (Hooi *et al.*, 2004).

P. aeruginosa in which QS is blocked either by mutation or by administration of QS-inhibitory drugs has been shown to be attenuated with respect to virulence (Bjarnsholt *et al.*, 2005a, b; Hentzer *et al.*, 2003; Rasmussen *et al.*, 2005a, b). Davies *et al.* (1998) showed that biofilms of a *P. aeruginosa* QS mutant are more susceptible to SDS than the wild-type. This apparent synergistic action with antimicrobial treatment was corroborated by Hentzer *et al.* (2003), who demonstrated that *P. aeruginosa* PAO1 biofilms became more susceptible to tobramycin and SDS treatments when pre-treated with QS inhibitors such as the halofuranones C-30 and C-56. Both compounds have been shown to exhibit QS inhibitory and attenuating effects *in vivo*; mice with PAO1 lung infections cleared bacteria significantly faster when treated with furanone C-30 or C-56 as compared to the untreated control group (Hentzer *et al.*, 2003; Wu *et al.*,

2004). A wide range of both natural and chemically synthesized compounds have been tested for QS-inhibitory effects both *in vitro* and *in vivo*. In addition to C-30 and C-56, garlic extract, 4-nitropyridine-*N*-oxide (4-NPO) and patulin are some of the compounds that have been shown to inhibit QS and to significantly reduce *P. aeruginosa* biofilm tolerance to tobramycin *in vitro* (Rasmussen *et al.*, 2005a, b). Garlic extract and patulin were also demonstrated to promote rapid clearance of *P. aeruginosa* in a pulmonary infectious mouse model (Bjarnsholt *et al.*, 2005b; Rasmussen *et al.*, 2005b). Recently, Bjarnsholt *et al.* (2005a) also showed that blocking QS, either by mutation or by inhibition with a QS-inhibitory drug, resulted in *P. aeruginosa* biofilms sensitive to treatment with H₂O₂ *in vitro*. Macrolides such as azithromycin have been proposed to inhibit QS (Tateda *et al.*, 2001). However, the concentrations used to determine the effect on QS-controlled gene expression were growth inhibitory. Azithromycin reduces the production of several of the virulence factors of *P. aeruginosa*, including elastase and rhamnolipids. It remains to be demonstrated whether these effects are specifically mediated through the quorum sensors and consequently independent of growth inhibition. Whatever the underlying mechanism is, azithromycin does improve the respiratory function in cystic fibrosis patients (Saiman, 2004; Southern & Barker, 2004).

Phagocytic cells, such as macrophages and polymorphonuclear leukocytes (PMNs), play an important role in innate immunity as the first line of host defences (Goldsby *et al.*, 2003). When PMNs are exposed to bacteria, they react by phagocytosis and secretion of antimicrobial agents, including oxygen radicals. However, wild-type *P. aeruginosa* biofilms have been shown to impair the activity of PMNs *in vitro*. Bjarnsholt *et al.* (2005a) observed that wild-type biofilms survived exposure to freshly isolated PMNs, whereas a Δ *lasR* *rhlR* QS mutant biofilm became penetrated and partly eliminated. There are at least two reasons for this: the wild-type biofilm failed to activate the PMNs to produce H₂O₂ and the PMNs appeared paralysed on top of the biofilm bacteria (Bjarnsholt *et al.*, 2005a). In addition, *P. aeruginosa* wild-type biofilms were able to activate PMNs when they had been grown and developed in the presence of furanone C-30 (Bjarnsholt *et al.*, 2005a). Recently, Jensen *et al.* (2007) showed that *P. aeruginosa* lyses the PMNs by QS-regulated excretion of rhamnolipid, strongly suggesting that this is a mechanism by which *P. aeruginosa* can evade the host innate immune system. Since rhamnolipid production and hence killing of PMNs was impaired in a QS mutant, we envision that QS-inhibitory drug treatment of *P. aeruginosa*-based biofilm infections would be expected to induce susceptibility to the action of PMNs and thereby promote clearing. In the present investigation we set out to probe this on an *in vivo* silicone implant model of *P. aeruginosa* infection.

METHODS

Bacterial strains. The *P. aeruginosa* wild-type strain was obtained from Professor Barbara Iglewski (University of Rochester Medical

Center, NY, USA). Construction of the corresponding *ΔlasR rhlR* mutant was described by Bjarnsholt *et al.* (2005a). Both strains, wild-type and the *ΔlasR rhlR* mutant, were tagged with a plasmid-based mini-Tn7 transposon system (pBK-miniTn7-*gfp3*) constitutively expressing a stable green fluorescent protein (GFP) according to Koch *et al.* (2001).

Preparation of implants. Silicone implants were cut out from a sheet of pyrogen-free silicone with a height of 0.8 mm, in sizes of 5 × 5 mm. Implants were sterilized in 0.9% sodium hypochlorite overnight, and washed in sterile distilled water before use. A maximum of 10 implants was placed in 50 ml shake flasks with 20 ml of cultures of the *P. aeruginosa* wild-type or its isogenic *ΔlasR rhlR* mutant. The cultures were prepared by centrifuging (3000 g) overnight cultures grown in Luria-Bertani (LB) followed by resuspension in 0.9% NaCl to an OD₆₀₀ of 0.05 (for pre-colonizing the implants destined for BALB/c mice) or 0.5 (for NMRI mice). The implants were incubated in the flasks for 20 h at 37 °C and shaking at 110 r.p.m. to get bacterial adhesion.

Animals. Female BALB/c and NMRI mice were purchased from M & B Laboratory Animals at 9–11 weeks of age. The mice were maintained on standard mouse chow and water *ad libitum* for a minimum of 1 week before challenge. All animal experiments were authorized by the National Animal Ethics Committee, Denmark.

Challenge protocol. The mice were anaesthetized by subcutaneous injections in the groin area with 0.15 ml (BALB/c) or 0.25 ml (NMRI) Hypnorm/midazolam (Roche) (2.5 mg Hypnorm ml⁻¹ and 1.25 mg midazolam ml⁻¹ in sterile water). An incision of approximately 1 cm was made in the groin area, allowing access to the peritoneal cavity, in which the implant was inserted. The incision was sutured with silk and healed without any complications. After insertion of the implants the mice were injected with 1 ml 0.9% NaCl in the neck-skin area to avoid dehydration. Pentobarbital (DAK), 2.0 ml (kg body weight)⁻¹, was used to euthanize the mice at termination of the animal experiments (Pedersen *et al.*, 1990).

Bacteriology. After removal from the mice, silicone implants were placed in 5 ml 0.9% NaCl and whirlly-mixed for 30 s. The bacteria were then scraped off with a sterile scalpel and whirlly-mixed for another 60 s, after which 100 µl was serially diluted and plated on blue agar plates (State Serum Institute, Denmark), for bacterial visualization. Blue agar plates are selective for Gram-negative bacilli (Høiby, 1974). The plates were incubated at 37 °C overnight.

Preparation of the QS inhibitor for injection. The furanone C-30 solution was prepared by dissolving the furanone C-30 powder in 96% ethanol to a concentration of 5 mg ml⁻¹. This solution was then diluted 1:40 in 0.9% NaCl. The mice were given 1.2 µg furanone C-30 (g body weight)⁻¹. The BALB/c mice weigh on average 20 g and the NMRI mice 30 g, thus 0.2 ml was injected into BALB/c mice and 0.3 ml into NMRI mice. The mice were treated with the inhibitor every 8 h (intraperitoneal injections) for 4 days starting just after insertion of the implant. Treatment was continued until 24 h before the mice were euthanized. The placebo group were injected with 0.2 ml (BALB/c) or 0.3 ml (NMRI) of a 2.4% ethanol solution (96% ethanol in 0.9% NaCl), corresponding to the amount of ethanol that the furanone C-30-treated group received.

Biofilm development on implants. Biofilm development on the implants was examined using confocal laser scanning microscopy (CLSM) (Zeiss LSM 510 system, Carl Zeiss) equipped with an argon laser for excitation of the fluorophores. Images were obtained with a 40 × 0.75 oil objective. Bacteria were visualized using GFP-tagging, and the IMARIS software package (Bitplane) was used to generate pictures of the biofilms.

Statistical analysis. To compare the data between two groups the Mann-Whitney *U*-test was used (analysis of non-parametric data). *P*-values ≤ 0.05 were considered significant. For calculating *P*-values, the statistical program StatView 5.0.1 (SAS Institute) was used.

RESULTS

Clearance of *P. aeruginosa* from silicone implants is QS dependent

The previous findings that QS-deficient *P. aeruginosa* *in vitro* biofilms are less tolerant to components of the host defence (Bjarnsholt *et al.*, 2005a; Hentzer *et al.*, 2003) led to the hypothesis that mice would be able to clear silicone implants colonized with a QS mutant faster than the wild-type. In order to test this hypothesis, we inserted silicone implants, pre-colonized with either the wild-type or the *ΔlasR rhlR* mutant, in the peritoneal cavity of two groups of mice respectively. Thirty-two BALB/c mice were assigned to each of the two groups from which six implants were removed each day at days 4, 7, 14 and 21 post-infection for determination of bacterial counts (c.f.u.) and a further two implants per day were examined by means of CLSM. At the time of implantation, the median c.f.u. for the wild-type and the *ΔlasR rhlR* mutant was adjusted to 7.1×10^5 . As shown in Fig. 1(a), the implants colonized with the QS mutant showed a rapid decline with respect to bacterial counts and on day 4 only 1.5×10^2 c.f.u. per implant were found. From day 7 post-infection, no bacteria were found on the implants. In contrast, the bacterial counts on the wild-type-colonized implants decreased more slowly and were, after 4, 7 and 14 days, 2.1×10^4 , 3.4×10^3 and 8.1×10^2 , respectively. However, on day 21 no bacteria were found on four out of the six removed implants (Fig. 1a). A significantly lower number of bacteria were present on the implants initially colonized with the *ΔlasR rhlR* mutant as compared to the wild-type at days 4, 7 and 14 post-infection ($P=0.01$; $P=0.007$ and $P=0.006$, respectively) (Fig. 1a).

Similar experiments were performed with NMRI mice except that the two groups consisted of 28 mice each, and the implants were removed at days 4, 7, 11 and 15 post-infection. Five implants were removed from each group at each time point for determination of bacterial counts and two implants per day were examined by CLSM. The c.f.u. per implant for each mouse was determined (Fig. 1b). At the time of implantation the median c.f.u. for the wild-type and the *ΔlasR rhlR* mutant were 1.0×10^7 and 8.8×10^6 , respectively. Similar to the BALB/c mice, we observed that the c.f.u. for implants colonized with the *ΔlasR rhlR* mutant decreased faster than the wild-type. From day 7 post-infection, no bacteria could be found on the QS mutant pre-colonized implants. The c.f.u. for the wild-type-colonized implants were found to decrease from day 0 to day 4 (5.6×10^3), thereafter reaching a steady state until day 11 (Fig. 1b). However, on day 15 post-infection, a large increase to 2.2×10^6 c.f.u. per implant was observed

Fig. 1. Implant bacteriology. Healthy BALB/c (a) and NMRI (b) mice had implants pre-colonized with wild-type PAO1 or the $\Delta lasR rhIR$ mutant inserted in the peritoneal cavity. The number of bacteria adhering to the implants was counted before insertion ($n=2 \times 6$ implants in both experiments). BALB/c mice: 6 implants from each group were removed on days 4, 7, 14 and 21 post-infection ($n=48$). NMRI mice: 5 implants were removed from each group on days 4, 7, 11 and 15 post-infection ($n=40$). Triangles represent wild-type bacteria, and squares the $\Delta lasR rhIR$ mutant. Each open triangle and square represents one mouse. Solid black symbols represent the medians for each day. The statistical significance of difference in clearance between the wild-type and $\Delta lasR rhIR$ mutant was tested by a Mann-Whitney *U*-test for non-parametric data. *P*-values are shown in the figure.

(Fig. 1b). Based on the *P*-values, $P=0.04$, $P=0.03$, $P=0.05$ and $P=0.02$, respectively, the experiment showed a significant difference in clearance between the wild-type- and QS mutant-colonized implants at all time points (Fig. 1b).

Macroscopic evaluation of the removed implants indicated that the host response against *P. aeruginosa* differed between the two mouse strains. The BALB/c mice that were able to clear the biofilms present on the implants showed no macroscopic signs of inflammation. The NMRI mice that were unable to clear the infection showed abscess formation in the tissue surrounding all the wild-type implants at day 15 post-infection. In neither of the mouse strains was pus or abscess formation observed around the $\Delta lasR rhIR$ implants.

CLSM examination of the implants

After removal from the mice, the implants were inspected by CLSM. The immediate observations were that a significant part of the cellular material adhering to the implants originated from the host and was auto-fluorescent (data not shown). Although this somewhat complicated identification of *P. aeruginosa* biofilms, areas free of mouse tissue were localized, making it possible to obtain images of microcolonies present on the implants removed from BALB/c mice. Fig. 2 shows images obtained from implants removed from BALB/c mice on day 4 after insertion. This revealed the presence of microcolony-based biofilms on the implants, though distinctly different from the highly structured and mushroom-shaped biofilms *P. aeruginosa* is known to develop under certain growth conditions in the *in vitro* flow-cell system (Klausen *et al.*, 2003). Microcolonies were also observed on wild-type-colonized implants removed on days 7 and 14 from BALB/c mice, and on days 7, 11 and 15 from NMRI mice (images not shown). We found a substantially higher number of microcolonies on the implants that were pre-colonized with the wild-type compared to the implants pre-colonized with the $\Delta lasR rhIR$ mutant (Fig. 2). Furthermore, the microcolonies found on the QS mutant-colonized implants were smaller in size. These observations are in agreement with the previous determinations of bacterial content by means of c.f.u. It is also noteworthy that both the $\Delta lasR rhIR$ and wild-type bacteria adhering to the implants were

Fig. 2. CLSM images of the wild-type (a, b) and $\Delta lasR rhIR$ mutant (c, d) colonizing silicone implants after removal from BALB/c mice at day 4 after insertion. Green fluorescent areas represent GFP-tagged microcolonies. (a, c) 3D images of the microcolonies adhering to the implants; (b, d) top views. Scale bars (50 μ m) are shown in (b) and (d). All images were obtained with a 40 $\times$ /0.75 oil objective.

not rod-shaped, as at the time of implantation, but rather sphere-shaped and smaller than exponentially growing, batch-cultured cells (Fig. 3).

Furanone C-30 treatment of wild-type *P. aeruginosa*-colonized implants

Since the QS mutant was cleared faster than the wild-type bacteria, administration of a functional QS-inhibitory drug would be expected to have a similar impact. Forty NMRI mice had implants pre-colonized with wild-type *P. aeruginosa* PAO1 (OD₆₀₀ 0.5) inserted in the peritoneal cavity at day 0. Immediately after this, the mice were separated into two groups (20 in each), which received intraperitoneal injections of either furanone C-30 [$\sim 1 \mu\text{g}$ (g body weight)⁻¹] or placebo with 8 h intervals for the following 4 days. This concentration of drug had previously been shown to significantly improve clearance of *P. aeruginosa* from a pulmonary model of infection (Hentzer *et al.*, 2003). No macroscopic effect on the host was observed when treating the mice with furanone C-30. Twenty-four hours after the last injection, the implants were removed at day 5. This protocol was repeated in two individual experiments [denoted (a) and (b)] in which the c.f.u. were adjusted to 8.2×10^6 per implant. Determination of c.f.u. was used to assess the effect of furanone C-30 treatment on clearance, and the c.f.u. per implant for each mouse in each group was determined and the medians plotted in Fig. 4(a, b). The remaining implants were used for CLSM studies. In experiment (a), the c.f.u. determined for the treatment and placebo groups showed medians of 800 and 2.6×10^6 , respectively ($P=0.005$). In

Fig. 3. CLSM images of implants colonized with wild-type *P. aeruginosa*. The image to the left was taken after colonization but before insertion, while the image to the right is of an implant removed 15 days after insertion from a NMRI mouse. It is noteworthy that both the $\Delta lasR rhIR$ and wild-type bacteria adhering to the implants, after removal from the mice, were not rod-shaped, as at the time of implantation, but rather sphere-shaped and smaller than exponentially growing, batch-cultured cells. This phenomenon was observed when the implants were removed 7 and 15 days after insertion ($n=8$). Scale bars ($10 \mu\text{m}$) are shown on the images. The images were obtained with a $40\times/0.75$ oil objective and then magnified five times.

Fig. 4. Implant bacteriology. Healthy NMRI mice had implants pre-colonized with wild-type PAO1 inserted in the peritoneal cavity. (a) and (b) are two individual experiments carried out identically with NMRI mice, where 24 implants were used in each study for bacteriology. The mice were in each experiment separated into two groups and were injected with either furanone C-30 [$\sim 1 \mu\text{g}$ (g body weight)⁻¹] or placebo at 8 h intervals for 4 days. On day 5 post-infection the implants were removed and the number of bacteria adhering to the implant was counted. Each mouse is represented by one square, and solid squares represent the median of the bacterial counts for each group. Bacterial counts per implant were calculated before insertion [(a) $n=8$ and (b) $n=12$]. The statistical significance of difference in clearance between the furanone C-30-treated and placebo groups was tested by a Mann-Whitney *U*-test for non-parametric data. *P*-values for the experiments are shown in the figure.

experiment (b), the median c.f.u. were 25 (furanone C-30-treated) and 8.8×10^3 (placebo) ($P=0.0007$).

A similar experiment was performed with BALB/c mice, where the furanone C-30 treatment also was found to promote clearing of the bacteria from the implants compared to the placebo-treated group ($P=0.03$) (data not shown).

CLSM examination of the implants

CLSM was used to study the removed implants. Due to the low bacterial counts on the implants from the furanone C-30-treated NMRI mice in experiment (b), no images of bacteria could be obtained. However, bacteria organized in microcolonies were found on several occasions on the

implants from the placebo groups from experiments (a) and (b), and also some bacteria were observed on the furanone C-30-treated implants from experiment (a) (data not shown). With BALB/c mice, it was also possible to find microcolonies on the implants removed from both the furanone C-30-treated and untreated BALB/c mice. A higher number of microcolonies was observed on the implants from the untreated mice as compared to those treated with furanone C-30.

DISCUSSION

Many researchers share the view that biofilms are sanctuaries in a hostile environment, which release planktonic cells that may spark systemic infections. Numerous studies of *in vitro* biofilms have revealed a characteristic series of events, which include surface attachment followed by growth and proliferation into a characteristic self-made, exopolymeric embedded state where the bacteria show an extreme tolerance to antimicrobial measures. Microscopic investigations have revealed that biofilms develop commonly on inert surfaces of medical devices or on dead tissue, but they can also form on living tissues. When it comes to in-depth studies, the preferred model organism is undoubtedly *P. aeruginosa*. The genus *Pseudomonas* is one of the most diverse and ecologically significant groups of bacteria known. *P. aeruginosa* is also the most common bacterium found in nosocomial and life-threatening infections of immune compromised patients (Van Delden & Iglewski, 1998). As the understandings of pathogenicity, biofilm development and QS as well as the elucidation of the molecular mechanisms behind QS have gained momentum, researchers have conceived several strategies to block QS. Hospitalized patients acquire infections in connection with the use of medical devices such as arteriovenous shunts, pacemakers, urinary and other types of catheters, orthopaedic devices and mechanical heart valves. The biofilm lifestyle is believed to dominate in the above-mentioned chronic bacterial infections. Consequently, they share similar characteristics: they resist the activities of the host immune system and cannot be eradicated with antibiotic intervention regimes. New anti-biofilm measures must therefore be identified which efficiently decrease the biofilm-forming capability or persistence of the infectious bacteria. The central role of QS in virulence and biofilm development has made it easy (at least in academia) to see a direct application for QS-inhibitory drugs.

In vitro biofilms of *P. aeruginosa* in which the QS communication systems have been disabled by mutation are more prone to killing by tobramycin and hydrogen peroxide (Bjarnsholt *et al.*, 2005a), as well as ciprofloxacin, ceftizidime and kanamycin treatment (our unpublished results), shear forces, protozoan grazing and neutrophil phagocytosis, than biofilms formed by a wild-type counterpart (Allesen-Holm *et al.*, 2006; Bjarnsholt *et al.*, 2005b; Davies *et al.*, 1998; Hassett *et al.*, 1999; Hentzer *et al.*, 2002,

2003; Matz *et al.*, 2004; Rasmussen *et al.*, 2005a, b). Moreover, in animal models these mutants appear attenuated and are more readily cleared by the host organism (Bjarnsholt *et al.*, 2005a, b).

We have developed yet another animal model to study the effect of functional QS on biofilm susceptibility *in vivo*. By inserting implants pre-colonized with either the $\Delta lasR$ *rhlR* mutant or wild-type *P. aeruginosa* in the peritoneal cavity of mice it was shown that the former was cleared significantly faster than the latter from the implants both in BALB/c and NMRI mice. In contrast to the NMRI mice, BALB/c mice were eventually able to clear the biofilms present on the *P. aeruginosa* wild-type-colonized implants (Fig. 1). The pathophysiological mechanisms resulting in the different outcomes in the two mouse strains were not the focus of the present study, and will be investigated further in the future.

Buret *et al.* (1991) performed a foreign-body insertion experiment on rabbits, where silastic implants colonized with *P. aeruginosa* were inserted intraperitoneally. Their results showed a similar pattern to the one observed with the NMRI mice in the present study. In their study, bacterial counts decreased 13-fold from day 0 to day 4, but increased 20-fold in bacterial counts from day 4 to day 42 (Buret *et al.*, 1991). Likewise in the study performed by Buret *et al.* (1991), it was in one case observed that walling-off and necrosis of host and pathogen debris resulted in abscess formation, which was also observed in the present study upon removal of the wild-type-colonized implants from NMRI mice 15 days post-infection. The bacterial counts were also significantly higher than in cases where no abscess was seen. Due to the abscess formation and high number of bacteria adhering to the implants upon removal, it is likely that the *P. aeruginosa* wild-type was able to withstand the host immune system in the NMRI mice and establish a successful chronic infection, which was not the case in BALB/c mice.

Similar to the present study, Buret *et al.* (1991) found host cells attached to the implants. These cells were dominated by PMNs, but erythrocytes and macrophages were also observed (Buret *et al.*, 1991). When removing the implants from the mice it was observed that the bacterial cells had gained an altered morphology, as compared to the rod shape they possessed at insertion. This is a well-known phenomenon observed when Gram-negative cells such as *P. aeruginosa* adapt to environments which provide conditions that sustain only slow or no growth (Clegg *et al.*, 1996; Givskov *et al.*, 1994; Lappin-Scott *et al.*, 1988). Since this phenomenon was observed with both the QS mutant and wild-type strain, change in morphology does not seem to be the reason for the slower clearing of wild-type bacteria from the implants as compared to the mutant.

NMRI and BALB/c mice carrying wild-type-colonized implants were treated with furanone C-30 given as intraperitoneal injections. Two individual experiments

with NMRI mice and one experiment with BALB/c mice were carried out, and it was shown that the furanone C-30-treated groups had a significantly lower amount of bacteria adhering to the implants when they were removed from the mice, as compared to the placebo-treated groups. This confirms that treatment of furanone C-30 induces antimicrobial conditions leading to bacterial clearing, as previously shown in an *in vivo* pulmonary infection model (Hentzer *et al.*, 2003).

It is likely that the difference in clearance observed in the present study is due to the *P. aeruginosa* wild-type elimination of the PMNs and we have recently found that the causative agent is rhamnolipid (Jensen *et al.*, 2007). Interestingly, we found that an ethyl acetate extract of the bronchoalveolar lavage (BAL) fluid of *P. aeruginosa*-infected cystic fibrosis patients was capable of lysing freshly isolated PMNs. Furthermore, it has been shown that treatment with furanone C-30 of a growing batch culture of the *P. aeruginosa* wild-type resulted in blocking of the development of PMN necrotic and haemolytic effects (Jensen *et al.*, 2007). In a recent study of the gene-expression profile of *P. aeruginosa* exposed to freshly isolated PMNs, several QS-regulated genes, which contribute to virulence and immune modulation, were further upregulated in the presence of PMNs (M. Alhede and others, unpublished). Among these were the genes encoding PQS, pyocyanin, rhamnolipid and lectin production, a result also found by Hentzer *et al.* (2003). Previous analysis has shown that expression of those genes was inhibited by furanone C-30. Taken together, these data strongly suggest that the ability of *P. aeruginosa* to kill the PMNs has been impaired in the furanone C-30-treated groups, thereby resulting in a faster clearance of the bacteria on the implants in both BALB/c and NMRI mice. This is in favour of a model in which administration of furanone C-30 could possibly increase the survival potential of the PMNs, by concomitantly reducing expression of the *P. aeruginosa* PMN-devastating products. Furthermore, furanone C-30 treatment was shown to have an impact on the bacterial clearance in both an inbred and an outbred mouse strain, which is of great advantage. This implies that the treatment effect of the compound is independent of the mouse strain, and hence it offers a guarded optimism that QS inhibitors can play a role in the treatment of various biofilm-based infections in humans.

In the present investigation, the biofilms that were allowed to develop on the silicone implants were formed in 20 h, and thus were very young. Despite this relatively young state, a transcriptomic analysis by Hentzer *et al.* (2005) on *in vitro* biofilms showed that the majority of QS-controlled genes were already switched on. Furthermore, this condition is likely to resemble a foreign-body infection in its early stages. In the future we aim to investigate mature and fully developed biofilms adhering to the implants. This condition would be expected to be medically relevant for foreign bodies that have been colonized for a longer period due to the fact that biofilm-growing bacteria are able to

withstand the host immune system and exposure to most antibiotics (Bjarnsholt *et al.*, 2005a; Costerton *et al.*, 1999; Hentzer *et al.*, 2003; Høiby *et al.*, 2001; Middleton *et al.*, 2002; Singh *et al.*, 2002).

In conclusion, the present *in vivo* model showed that functional QS plays a key role in the ability of mice to clear a *P. aeruginosa* foreign-body infection. The results indicate that the antimicrobial efficacy of the defence mechanisms to biofilm-growing *P. aeruginosa* on foreign bodies is improved when the bacteria are treated with QS-inhibitory drugs. Accordingly, the present investigation supports a model in which *P. aeruginosa* employs QS-regulated gene expression to circumvent the host response during the initial stages of infection. This model will be suitable for testing and calibrating the concentration of future pharmaceutically relevant QS-inhibitory drugs aiming at the prevention or treatment of *P. aeruginosa* biofilm infections.

ACKNOWLEDGEMENTS

M. G. received financial support from the Danish Research Council FTP supported 'A new approach to the control of microbial activity', the Biomedical Consortium 'Biomed', Mölnlycke Health Care, AB Wound Care R&D, Sweden, and the German Mukoviszidose e.v. T. B. received financial support from The Carlsberg Foundation and Lundbeck Foundation 'The implication of bacterial biofilms in chronic infections'.

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Edited by: P. Cornelis