Detection of N-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas aeruginosa*

Hong Wu, Zhijun Song, Morten Hentzer, Jens Bo Andersen, Arne Heydorn, Kalai Mathee, Claus Moser, Leo Eberl, Søren Molin, Niels Høiby and Michael Givskov

The pathogenesis of *Pseudomonas aeruginosa* is associated with expression of virulence factors, many of which are controlled by two N-acylhomoserine lactone (AHL)-based quorum-sensing systems. *Escherichia coli* strains equipped with a luxR-based monitor system expressing green fluorescent protein (GFP) in the presence of exogenous AHL molecules were used to detect the production of AHFs from *P. aeruginosa* in vivo. Mice were challenged intratracheally with alginate beads containing *P. aeruginosa* and *E. coli* and killed on different days after the challenge. By means of confocal scanning laser microscopy, GFP-expressing *E. coli* bacteria could be detected in the lung tissues, indicating production and excretion of AHFs in vivo by the infecting *P. aeruginosa*. AHF signals were detected mainly in lung tissues exhibiting severe pathological changes. These findings support the view that expression of AHFs by *P. aeruginosa* during infection coincides with its pathogenesis.

Keywords: AHL, quorum sensing, green fluorescent protein (GFP), mouse model, lung infection

INTRODUCTION

*Pseudomonas aeruginosa* is an opportunistic pathogen. Infection with *P. aeruginosa* may result in death of patients with burns (Richard et al., 1994) and nosocomial pneumonia in intubated (Bowton, 1999) and neutropenia cancer patients (Bergen & Shelhamer, 1996). The leading cause of mortality among patients with the autosomal recessive disorder cystic fibrosis (CF) is respiratory failure due to chronic lung infection with *P. aeruginosa* (Koch & Hoiby, 1993). The immune defence mechanisms against *P. aeruginosa* in CF patients are dominated by the infiltration of polymorphonuclear leukocytes (PMNs) into the lung tissues and the remarkable antibody response in serum (Hoiby et al., 1993). *P. aeruginosa* grown as a laboratory biofilm activates the oxidative burst of PMNs (Baltimore et al., 1989; Kharazmi et al., 1986) and the complement system (Jensen et al., 1993). An important factor behind the success of this organism is its ability to produce a number of extracellular products such as proteases (elastase, staphyloytic protease, alkaline protease), pigments (pyocyanin, pyoverdin), haemolysins, polysaccharides (lipopolysaccharide, alginate) and toxins. In recent years, it has been demonstrated that expression of a large number of these virulence factors in *P. aeruginosa* is regulated by quorum sensing (Van Delden & Iglewski, 1998).

Bacterial quorum-sensing systems enable the expression of target genes in response to culture density (Fuqua & Greenberg, 1998; Hardman et al., 1998; Hastings & Greenberg, 1999). Quorum-sensing systems exert their activities via small diffusible signal molecules which, in Gram-negative bacteria, are often N-acylhomoserine lactones (AHLs) (Fuqua & Greenberg, 1998; Fuqua et al., 1996; Salmond et al., 1995). AHFs are synthesized from precursors by a synthetase (LuxI homologue), and...
they interact with a transcription activator (LuxR homologue) to induce expression of target genes (Fuqua et al., 1996). When the bacteria reach a certain population size, the concentration of AHLs exceeds a threshold level. At this critical concentration, the AHL molecules are thought to induce a conformational change in the R-protein, which then initiates transcription of target genes (Fuqua et al., 1996). Short- and medium-chain AHL molecules are thought to freely pass the bacterial membrane whereas the long-chain molecules appear to require active efflux (Pearson et al., 1999).

In *P. aeruginosa* there are at least two different quorum-sensing systems, las (Gambello & Iglewski, 1991) and rhl (Ochsnr & Reiser, 1995), which consist of two signal-generating synthetases (LasI/RhlI) and two cognate transcriptional regulators (LasR/RhlR). The major products of LasI and RhlI are N-(3-oxododecanoyl)-homoserine lactone (OdDHL or 3OC12-HSL or PAI-1) (Pearson et al., 1994) and N-butanoylhomoserine lactone (BHL or C4-HSL or PAI-1) (Pearson et al., 1995; Winson et al., 1995), respectively. The lasR-encoded quorum-sensor system has been shown to modulate expression of *las* itself (Seed et al., 1995), *lasB* (elastase) (Passador et al., 1993; Pearson et al., 1997), *lasA* (staphylolytic protease) (Gambello et al., 1993), *apr* (alkaline protease) (Gambello et al., 1993), *scp* (secretion pathway) (Chapon-Herve et al., 1997) and *rhlR* (Latifi et al., 1996; Pesci et al., 1997). The rhlR-encoded quorum sensor modulates expression of *rhlB* itself (Latifi et al., 1996), *rhlAB* (rhamnolipid biosynthesis) (Ochsnr & Reiser, 1995; Pearson et al., 1997), *lasB* (Brind & Ohman, 1995; Pearson et al., 1995, 1997) and *rpoS*. (Latifi et al., 1996). Recently, the two quorum-sensing systems have been shown to regulate twitching motility (Glessner et al., 1999) and to be involved in the differentiation of biofilm (Davies et al., 1998). Biofilm differentiation is thought to protect *P. aeruginosa* from the host defence system and from the action of antibiotics (Costerton et al., 1987). But on top of that, OdDHL interferes with the host immune system, where it specifically down-regulates the production of the cytokines IL-12 and TNFα which support the bactericidal Th-1 milieu and protect the host (Telford et al., 1998).

Quorum-sensing-regulated gene expression probably reflects the need for the invading pathogen to reach a critical population density sufficient to overwhelm host defences and thus establish infection. It has been suggested that rising AHL levels indicate increasing preparedness for assault. Current understanding of AHL quorum-sensing systems in bacteria is mainly based on in vitro data. A few studies, however, have demonstrated the relationship between components of the quorum-sensing systems and virulence. Tang et al. (1996) demonstrated that a *P. aeruginosa* lasR mutant is substantially less virulent in an acute pneumonia mouse model. In plant-pathogenic bacteria of the genus *Erwinia*, which causes soft rot disease, production of several extracellular enzymes that degrade cell walls, such as pectate lyase, pectin lyase, polygalacturonase, cellulase and protease, is subject to quorum-sensing-regulated gene expression (Jones et al., 1993; Pirhonen et al., 1993). In a simple but elegant ‘potato experiment’ the direct effect of exogenous AHL-induced virulence factors was demonstrated (Jones et al., 1993).

The ability to monitor the production of AHL molecules from pathogenic Gram-negative bacteria during infection will make an important contribution to the understanding of host and micro-organism interactions. In principle, the presence of exogenous AHL molecules can be detected by a reporter gene fused to any quorum-sensing target gene. The prototypic quorum sensor is encoded by the *Photobacterium fischeri* luxR–luxI operon (Fuqua & Greenberg, 1998; Salmond et al., 1995). The cognate AHL signal is N-(3-oxohexanoyl)homoserine lactone (OHHL). In order to perform on-line studies of AHL communication among bacteria, components of the Ph. fischeri quorum sensor encoded by *luxR-P**-lasI* have been fused to gfp genes encoding green fluorescent proteins (GFPs) with different half-lives (J. B. Andersen and others, unpublished). The sensitivity of the GFP reporter enables visualization of cell–cell communication at the single-cell level. The present study reports on this biological approach to detect AHLs produced by *P. aeruginosa* in mouse lung tissues.

**METHODS**

**Bacterial strains.** The *P. aeruginosa* strains used in the present study were PAO579 (a mucoid strain isolated from a CF patient) classified according to the International Antigenic Typing System as type O:2/5, and the prototypic nonmucoid strain PA01 (Holloway & Morgan, 1986). The E. coli strains used were JM105 [*tbt rpsL(Strr) end sbeB15 sbcC hsdR4 (r*<sup>−</sup> m*<sup>−</sup>)] *Δlac–proAB* (*F’ trd36 lac*<sup>r</sup> *ΔlacZ* Δ*lacY1*) *M15 proA* (Brint & Ochsnr, 1993), respectively. The unstable derivatives of MC1000 (ara–leu7697 Δlac, thi, hsdR). The &gt;10<sup>7</sup> genome copy number per cell. The unstable versions are useful for monitoring temporal expression of AHL molecules in *in vivo* (J. B. Andersen and others, unpublished). One such variant, GFP(ASV), containing alanine (A), serine (S) and valine (V) at the C-terminus, was used in our study. All GFP-based reporter plasmids carried the *P. fischeri* region encoding luxR and a fusion between *luxCDABE* and gfp. The plasmids pJB88 and pJB89 are pUC18NotI derivatives that contain this promoter fused to the stable gfpmut3<sup>®</sup> and the unstable gfp(ASV), respectively. The E. coli JM105 monitor strains containing these plasmids were denoted JB353 and JB357, respectively. The *luxR*, *luxCDABE–gfpmut3* and the *luxR*, *luxCDABE–gfp(ASV)* (unstable GFP) fusions were cloned into a stable broad-host-range vector pME6031 (pVS1 replicon, accession no. AF118811), giving rise to pJB130 and pJB132, respectively (J. B. Andersen and others, unpublished). The E.
coli MT102 monitor strains containing these plasmids were denoted JB524 and JB525, respectively. Henceforth, the monitor strains are referred to as JB353-gfpmut3, JB357-gfpmut3, JB524-gfpASV, and JB525-gfpASV. Two additional AHL monitor strains were used, JM105/pSB353 and JM105/pMH297, which encode bioluminescent AHL monitors. pSB353 carries the Aeromonas hydrophila abyR, PahY–luxAB fusion to detect exogenous BHL (Swift et al., 1997), whereas pMH297 carries the P. aeruginosa lasR, PlasB–luxAB fusion to detect OdDHL and N-(3-oxodecanoyl)homoserine lactone (ODHL).

**Extraction of AHL signals from culture supernatants.** Extraction was performed essentially as described by Shaw et al. (1997). A 100 ml culture of P. aeruginosa PA0579 was grown in Luria broth to an OD_{600} of 2.0. The culture was extracted with an equal volume of HPLC-grade ethyl acetate, and the aqueous phase was separated using a separating funnel. The organic phase was dried with anhydrous magnesium sulphate, filtered, and evaporated almost to dryness. The residue was then resuspended in 200 µl ethyl acetate.

**Separation and identification of AHL signals by TLC.** Analytical TLC was done essentially as described by Shaw et al. (1997). C_{18}-reversed-phase TLC plates (aluminium sheets RP-18 F_{254} (20 × 20 cm), Merck Chrom line) were used to chromatograph 10 µl of the AL preparation with methanol/water (60:40, v/v). After separation, the solvent was evaporated, and the dried plates were overlaid with AHL monitor strains, prepared as follows. A 0.7 ml overnight culture was added to 450 ml Luria broth containing 1% agar at 42 °C. The media/culture mix was overlaid on top of the TLC plate, until the entire plate was covered. Following solidification, the plate was incubated overnight at 30 °C. The positions of AHL spots were visualized as described previously (Shaw et al., 1997; J. B. Andersen and others, unpublished). Chemically synthesized OHHL and OdDHL were obtained from P. Williams, University of Nottingham, UK. All other AHLs were from Fluka Chemie.

**Animals and experimental groups.** Two mouse strains, NMRI and CBA/J, were used. The strains were obtained from The Panum Institute, Copenhagen University, Denmark. The number of animals used, and the experimental protocols, are shown in Table 1. All animal experiments were performed after authorization from the National Animal Ethics Committee. Where appropriate, the animals were given subcutaneous injections of 200 µg ampicillin per g body weight once a day.

**Imobilization of bacteria in seaweed alginate beads.** Imobilization of P. aeruginosa and E. coli in seaweed alginate beads was performed as previously described (Pedersen et al., 1990). One millilitre of overnight culture grown in Luria broth was harvested, resuspended in fresh medium and mixed with 9 ml of sterile seaweed alginate (Protanal 10–20; Protan, Drammen, Norway). The mixture was forced with air through a channel into a solution of 0.1 M CaCl₂ in 0.1 M Tris/HCl buffer (pH 7.0). The suspension of P. aeruginosa or E. coli was adjusted to yield 1 × 10⁸ c.f.u. ml⁻¹. The alginate beads of P. aeruginosa and E. coli were mixed at 1:2 ratio when mixed inoculum was needed (Table 1). To verify that the monitor strains were non-fluorescent at the time of intratracheal challenge, mixed beads were inspected under the epifluorescence microscope.

**Challenge procedures.** Before challenge, all mice were anaesthetized by subcutaneous injection of a 1:1 mixture of etomidate (Janssen) and midazolam (Roche) at a dose of 10 ml per kg body weight and tracheotomized (Johansen et al., 1993; Moser et al., 1997). Intratracheal challenge with 0.04 ml (1.0 × 10⁸ c.f.u. ml⁻¹) of alginate beads was performed as described previously (Moser et al., 1997). The incision was sutured with silk and healed without any complications. The animals were killed on different days by administering 20% pentobarbital (DAK) at 2 ml per kg body weight.

**Freeze microtomy.** The lung tissues with pathological changes from each group (Table 1) were embedded with Tissue-Tek and frozen at −20 °C to −40 °C immediately after death. Frozen sections 40–50 µm thick were made at different levels of the lung tissues by freeze microtomy. The frozen sections were mounted with saline to prevent drying.

**Epifluorescence microscopy.** An axioplan epifluorescence microscope (Leitz ARISTOPLAN E Camera System, type 307–148.002) was used to visualize green fluorescence of the AHL monitor strains. The microscope was equipped with a 100 W mercury lamp, and filter set no. 10 (Carl Zeiss) to visualize GFP. A slow-scan charge-coupled device (CCD) camera CH250 (Photometrics) equipped with a KAF 1400 chip (pixel size 608 × 608 µm) was used for capturing digital images. The camera was operated at −40 °C, and the chip was read out in 12 bits (4096 intensity levels) at a rate of 200 kHz.

**Confocal scanning laser microscopy (CSLM).** Microscopic inspection and image acquisition of the frozen lung sections were performed by CSLM (model TC S4, Leica Lasertechnik) equipped with the I3 filter set (Leica Lasertechnik) for detection of green fluorescence emitted by the monitor bacteria. Simulated fluorescence projections and vertical cross-sections through the lung tissues were generated using the IMARIS (Bitplane AG) software package running on a Silicon Graphics Indy 2 workstation. Images were further processed for display using Adobe Photoshop software.

**Macroscopic pathology of the lungs.** The macroscopic lung pathology was expressed as the lung index of macroscopic pathology (LIMP) as described previously (Song et al., 1998) according to the following modified formula: LIMP = the lung area with pathological change divided by the area of the whole lung.

**Histopathology of the lungs.** Frozen sections (thickness 10 µm) of the lung samples from different days after challenge were chosen for haematoxylin/eosin staining to evaluate the severity of pathological changes by light microscopy. The pathology includes lung consolidation, where lung tissues and bronchi are filled by inflammatory cells and oedema or haemorrhage, and atelectasis, where the alveoli and airways have collapsed.

**Lung bacteriology.** Lung samples from 10 of the animals in each group were prepared for quantitative bacteriological examination as previously described (Johansen et al., 1993). Lungs were homogenized in 5 ml PBS, and appropriately diluted samples were plated on ‘Blue agar plates’ (a solid medium for Gram-negative rods containing lactose, pH 7.0; State Serum Institute, Copenhagen, Denmark) to determine the number of c.f.u. after 20–24 h incubation at 37 °C. Bacteria from lung tissues containing mixed inocula of P. aeruginosa and E. coli (Pa+Ec group) were discriminated based on the colony colour on the blue agar plates combined with the ability of E. coli colonies to express green fluorescence in the presence of exogenous AHLs.

**Statistical analysis.** The Kruskal–Wallis test was used to compare the data of macroscopic lung pathology between three groups, and the Mann–Whitney U test was employed to compare the data of lung bacteriology and macroscopic lung pathology between two groups in the study.
RESULTS

Detection of AHL production in vitro

AHL-producing micro-organisms can be detected by the use of the live AHL monitor strains E. coli JB357-gfp(ASV), JB524-gfpmut3* and JB525-gfp(ASV) (J. B. Andersen and others, unpublished). The AHL detection capability of these strains is due to the presence of plasmids which carry luxR and the P_luxI promoter fused to the gene encoding GFP (Fig. 1). In response to passive uptake (diffusion) of AHL molecules from the surroundings these monitor strains fluoresce (J. B. Andersen and others, unpublished).

We determined if P. aeruginosa PAO579, by analogy with PAO1, produced the expected BHL and ODdHL signal molecules. Signal molecules were extracted from PAO1 and PAO579 cultures and separated by TLC as described in Methods. For the detection of AHL signals, E. coli JB357-gfp(ASV) supplemented agar was used as a TLC overlay (Fig. 2a, panel A). This monitor is practically blind to BHL but sensitive to the presence of N-hexanoylhomoserine lactone (HHL) (see Fig. 2b). Both AHLs are the products of RhlI (Winson et al., 1995). The monitor also detects OHL, N-octanoylhomoserine lactone (OHL) and ODdHL (products of LasI) (Pearson et al., 1994). In Fig. 2(a), panel B, a mixture of E. coli strains carrying pSB536 and pMH297 was used as TLC overlay. The abyR, Pabyl-luxAB fusion of pSB536 is highly specific for BHL whereas the lasR, PlasB-luxAB fusion of pMH297 recognizes primarily long-chain AHL signals and identifies the presence of ODdHL and ODdHL. This analysis demonstrates that PAO579 produces BHL, HHL, OHL, OHL, ODdHL and ODdHL, similar to PAO1 when grown in Luria broth. It should be emphasized that this type of analysis is far from quantitative. Due to diffusion constraints (from the TLC plate to the overlay) the signal intensity is strongly biased for short-chain molecules, but it offers a rough estimate of the AHL diversity.
In order to obtain quantitative data on the sensitivity of the gfp-based monitor JB357-gfp(ASV) liquid cultures were incubated with chemically synthesized AHLs. As can be seen from Fig. 2(b), the monitor is extremely sensitive to OHL (saturated at 10 nM), approximately 10-fold less sensitive to HHL, OHL and OdDHL, and at least 100-fold less sensitive to BHL. It was of particular interest to determine the minimum concentration of OdDHL (the cognate autoinducer for the lasR quorum sensor) that would allow for visual single-cell detection of GFP expression. We therefore examined the cells from Fig. 2(b) by epifluorescence microscopy. In the absence of any AHL the monitor cells appeared completely ‘dark’ (non-fluorescent); however, 4 nM OdDHL rendered approximately 50% of the monitor cells green fluorescent whereas 32 nM OdDHL resulted in 100% bright, green fluorescent cells (Fig. 3a). In order to determine the decay of green fluorescent, cells induced with 32 nM OdDHL where washed and shifted to Luria broth without OdDHL. Cells were examined by epifluorescence microscopy (Fig. 3b). The decay in signal intensity of JB357-gfp(ASV) was obvious after 1 h. After 4 h, 99% of the cells had lost the fluorescent signal, in sharp contrast to the JB357-gfp(ASV) liquid cultures (incubated in Tris buffer at 37 °C) for 48 h.

Detection of AHL production in vivo

To determine whether AHL molecules are produced in vivo during pulmonary infection, mouse strains were challenged intratracheally with alginate beads containing P. aeruginosa PAO579 and the E. coli monitor JB357-gfp(ASV) (Table 1). The strains were mixed in alginate beads and epifluorescence microscopic analysis was performed prior to intratracheal injection. No green fluorescent cells could be detected in the alginate beads, indicating that the monitors were not induced before the challenge (data not shown). To rule out the possibility that the entrapped monitor cells would respond to the presence of P. aeruginosa cells, we followed these beads in vitro (incubated in Tris buffer at 37 °C) for 48 h. Microscopic and CSLM inspection revealed that the cells were small and round, indicating exposure to nutrient starvation. In accordance with that, green fluorescent cells could not be detected (data not shown). Frozen lung tissues were analysed by epifluorescence microscopy and CSLM (Fig. 4). From day 1 to 3 post-intratracheal challenge, green fluorescent bacteria could be detected by means of epifluorescence microscopy in the lumen of the bronchi and in the lung tissues containing mixed inocula of P. aeruginosa and the E. coli monitor strain (Table 2). Microscopy and CSLM (Fig. 4) revealed the presence of alginate beads surrounded by numerous PMNs. Single, strongly green fluorescent as well as non-fluorescent bacteria were found in the beads (Fig. 4b, c). Green fluorescent cells were never found in the mice challenged with the E. coli monitor alone (Fig. 4d, e). Despite the sensitivity of the present AHL monitor the high-copy-number pUC vehicle was found to be highly unstable in vivo (data not shown). Plasmid loss was reversed by the administration of ampicillin to the mice.

To circumvent these problems, two additional AHL monitor systems were employed. Green fluorescent cells of E. coli JB524-gfpmut3* could be detected under epifluorescence microscopy in the lumen of the bronchi and in the lung tissues containing mixed inocula of P. aeruginosa and the E. coli monitor strain 3 d post-intratracheal challenge (Table 2). Similar to JB357-gfp(ASV), CSLM revealed the presence of alginate beads surrounded by PMNs, and single, strongly green fluorescent as well as non-fluorescent bacteria were found in the beads (data not shown). Green fluorescent cells were never detected in the control experiments.

**Table 1. Protocols used in animal experiments for detection of AHLs in vivo**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Mouse strain</th>
<th>No. of animals</th>
<th>Group</th>
<th>Bacterial strain(s) inoculated†</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NMRI, female 9–11 weeks old, weight 25–30 g</td>
<td>10</td>
<td>Pa</td>
<td>P. aeruginosa PAO579</td>
<td>Animals in expt I were killed 3, 5, 7 and 14 d after challenge</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>Ec</td>
<td>E. coli JB357-gfp(ASV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>Pa + Ec</td>
<td>PAO579 + JB357-gfp(ASV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>Pa</td>
<td>P. aeruginosa PAO579</td>
<td>Animals in expt II were killed 3, 5, 7 and 14 d after challenge</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>Ec</td>
<td>E. coli JB524-gfpmut3*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>Pa + Ec</td>
<td>PAO579 + JB524-gfpmut3*</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>NMRI, female 9–11 weeks old, weight 25–30 g</td>
<td>60</td>
<td>Pa</td>
<td>P. aeruginosa PAO579</td>
<td>Animals in expt III were killed 1, 3, 5, and 7 d after challenge</td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>Ec</td>
<td>E. coli JB525-gfp(ASV)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>60</td>
<td>Pa + Ec</td>
<td>PAO579 + JB525-gfp(ASV)</td>
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</tr>
</tbody>
</table>

† P. aeruginosa and E. coli were immobilized in seaweed alginate beads and mice were challenged intratracheally as described in Methods. When a mixed inoculum was used, the alginate beads of P. aeruginosa and E. coli were mixed at 1:2 ratio.

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where the mice were challenged with the *E. coli* monitors alone (data not shown). Since these low-copy-number plasmids were stable *in vivo* no antibiotic treatment of the mice was required (data not shown). Therefore, the mice could be followed for an extended period of time, allowing prolonged surveillance of lung bacteriology, etc. One drawback of JB524-gfpmut3 is the stability of the GFP reporter: once formed it is stable for days, which renders it a poor on-line monitor of fluctuations in the local AHL concentration. The stability problem was circumvented by means of the GFP reporter encoded by the *E. coli* JB525-gfp(ASV) monitor. The green
Detection of AHLs in lung tissues

**Fig. 3.** (a) Single-cell detection of green fluorescence. Cells obtained from the wells in Fig. 2(b) supplemented with OdDHL were examined for production of green fluorescence. Panel A, phase-contrast microscopy; panel B, epifluorescence microscopy; OdDHL concentrations as indicated. (b) Cells induced with 32 nM OdDHL were washed and resuspended in fresh Luria broth without OdDHL and further incubated at 37 °C. Samples were analysed at the time points (hours after the shift) indicated at the left. Panel A, *E. coli JB357-gfp* (ASV) epifluorescence microscopy (left) and phase-contrast microscopy (right); panel B, *E. coli JB353-gfp*mut3* epifluorescence microscopy.
**Fig. 4.** For legend see facing page.
flourescent signals were weak in the lung tissues challenged with a mixture of P. aeruginosa and E. coli JB525-gfp (ASV) (data not shown). Although the fluorescent signal from E. coli JB525-gfp (ASV) could be detected under the strong excitable laser in the CSLM, the intensity was significantly reduced compared with the monitors used in the two previous experiments (data not shown).

**Lung bacteriology**

To determine the number of bacteria and verify if the lung tissues harboured functional monitor strains (especially in the control experiments where mice were challenged with the E. coli strains alone), we examined the lungs on different days after intratracheal challenge (Table 2). In experiments II and III, the E. coli JB524-gfpmut3* or JB525-gfp (ASV) counts were significantly reduced after day 3. P. aeruginosa PAO579 remained more stable in the lungs than did the E. coli strains. In experiment II (lungs with a single inoculum), bacterial clearance with the AHL monitor strains (Ec group) was significantly faster compared to the P. aeruginosa strain (Pa group) (P < 0.001). In experiment III, the bacterial counts in the Ec group were significantly reduced compared to the Pa group except for day 3 (P < 0.01). The experiments showed that it was hard to detect E. coli cells in the lung samples 5 d post-challenge (Table 2).

The presence of functional AHL monitor bacteria in the lungs of mice challenged with E. coli alone (the Ec group) was confirmed by plating and incubation on agar plates supplemented with 10 nM OHHL (data not shown). As judged from the expression of green fluorescence signal (ASV) alone, 3 d post-challenge (ASV) alone, 3 d post-challenge (ASV) alone, 3 d post-challenge (ASV) alone (the Ec group) was confirmed by plating and incubation on agar plates supplemented with 10 nM OHHL (data not shown). The blue arrow points to an area with green fluorescent monitor bacteria.

**Fig. 4. In vivo detection of AHL production in mouse lung tissues.** (a) Photomicrograph showing inflammation of mouse lung tissues due to P. aeruginosa PAO579 infection. Frozen sections of the mouse lung tissues containing mixed inocula of P. aeruginosa PAO579 and E. coli JB537-gfp (ASV) were stained with haematoxylin and eosin. The white arrow points to an alginate bead surrounded by numerous PMNs in small bronchia 3 d post-challenge. (b) CSLM of mouse lung tissue containing alginate beads, with mixed inocula of P. aeruginosa PAO579 and E. coli JB357-gfp (ASV), 3 d post-inoculation. The blue arrow points to an area with green fluorescent monitor bacteria. (c) Epifluorescence superimposed on reflection image (computer generated) showing the presence of multiple green fluorescent monitor cells (blue arrow) and non-fluorescent PAO579 (black arrows). The borderline of the alginate bead is clearly seen (red arrows) surrounded by PMNs (white arrows). (d) CSLM of mouse lung tissue containing alginate beads with E. coli JB357-gfp (ASV) alone, 3 d post-inoculation. Lung tissues. (e) Epifluorescence superimposed on reflection image (computer generated) showing the absence of green fluorescent monitor cells (open arrows).

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**Table 2. Lung bacteriology and expression of green fluorescence signal in lung tissues of animals**

<table>
<thead>
<tr>
<th>Expt</th>
<th>Days after challenge</th>
<th>Pa group</th>
<th>Ec group</th>
<th>Pa + Ec group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacterial count, c.f.u. ml⁻¹ (range)</td>
<td>GFP signal</td>
<td>Bacterial count, c.f.u. ml⁻¹ (range)</td>
<td>GFP signal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P. aeruginosa</td>
</tr>
<tr>
<td>II</td>
<td>3</td>
<td>1·2 × 10⁶</td>
<td>—</td>
<td>5·5 × 10⁴⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1·6 × 10⁴–3·8 × 10⁴⁰)</td>
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<td>(0·1–2 × 10⁴⁰)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>4·4 × 10³</td>
<td>—</td>
<td>0*</td>
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<td></td>
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<td>(3·0 × 10⁴–6·3 × 10⁴⁰)</td>
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<td></td>
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<td>(0·5–8 × 10³⁰)</td>
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<tr>
<td></td>
<td>14</td>
<td>8·8 × 10³</td>
<td>—</td>
<td>0*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(4·6 × 10³–8·2 × 10³⁰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>9·0 × 10³</td>
<td>—</td>
<td>3·4 × 10⁴⁰</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(1·1 × 10³–3·3 × 10³⁰)</td>
<td></td>
<td>(2·0 × 10³–1·2 × 10⁴⁰)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2·9 × 10³</td>
<td>—</td>
<td>2·8 × 10³</td>
</tr>
<tr>
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<td></td>
<td>(3·5 × 10³–1·7 × 10³⁰)</td>
<td></td>
<td>(5–1·7 × 10³)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>3·0 × 10³</td>
<td>—</td>
<td>0*</td>
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<tr>
<td></td>
<td></td>
<td>(4·9 × 10³–1·4 × 10³⁰)</td>
<td></td>
<td>(0–1·1 × 10³)</td>
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<tr>
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<td>7</td>
<td>7·3 × 10³</td>
<td>—</td>
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<td></td>
<td>(3·0 × 10³–7·1 × 10³⁰)</td>
<td></td>
<td>(0–1·4 × 10³)</td>
</tr>
</tbody>
</table>
Table 3. Lung index of macroscopic pathology (LIMP)

<table>
<thead>
<tr>
<th>Expt</th>
<th>Days after challenge</th>
<th>Pa group median (range)</th>
<th>Ec group median (range)</th>
<th>Pa + Ec group median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>3</td>
<td>0.31 (0.07–0.32)*</td>
<td>0.21 (0.03–0.32)</td>
<td>0.33 (0.15–0.42)*</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.18 (0.06–0.05)*</td>
<td>0.07 (0.05–0.13)</td>
<td>0.16 (0.12–0.50)*</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.14 (0.04–0.33)*</td>
<td>0.07 (0.04–0.10)</td>
<td>0.17 (0.10–0.50)*</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.14 (0.02–0.17)*</td>
<td>0.02 (0.01–0.05)</td>
<td>0.09 (0.00–0.10)*</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>0.10 (0.00–0.23)</td>
<td>0.10 (0.06–0.26)</td>
<td>0.10 (0.08–0.16)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.14 (0.07–0.28)</td>
<td>0.12 (0.08–0.20)</td>
<td>0.15 (0.07–0.21)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0.13 (0.05–0.30)</td>
<td>0.08 (0.05–0.23)</td>
<td>0.08 (0.06–0.20)</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>0.11 (0.07–0.15)</td>
<td>0.11 (0.08–0.15)</td>
<td>0.10 (0.06–0.15)</td>
</tr>
</tbody>
</table>

In experiment II, compared with the Ec group, *P < 0.02.

Macroscopic lung pathology

In experiment II, the lung index of macroscopic pathology on day 14 was significantly milder compared to day 3 (P < 0.05) in all three groups. The differences among the three groups were significant (P < 0.001) on day 3, 5, 7 and 14 after challenge. The pathological changes in the Ec group were all remarkably milder than in the other two groups (P < 0.02) on the four different days (Table 3), whereas in experiment III the three groups did not significantly differ from each other in macroscopic lung pathology. In general, the macroscopic lung pathology in the Pa + Ec group was not significantly different from the Pa group in either experiment II or experiment III (Table 3). Introduction of alginate beads without bacteria did not lead to any significant pathological changes (data not shown).

Histopathology

On days 1 and 3, the alginate beads containing bacteria were found mainly in the upper bronchi or lower bronchia surrounded by numerous PMNs (Fig. 4). Lung oedema, congestion and large areas of lung tissue consolidation were seen, and bronchial walls were observed to be partly destroyed by inflammation (data not shown). On day 7, lung tissue oedema began to decline. On day 14, the lung inflammatory reaction was significantly milder and lung atelectasis became the main pathological change, although beads with PMNs blocking the bronchi were still seen in some of the mice (data not shown).

DISCUSSION

CF patients are susceptible to pulmonary infection with *P. aeruginosa* (Govan & Deretic, 1996; Hoiby, 1995). The virulence of *P. aeruginosa* depends on a number of cell-associated and extracellular factors. The quorum-sensing systems operating in *P. aeruginosa* control the production of many of these virulence factors (Van Delden & Iglewski, 1998). Tang et al. (1996) demonstrated that a functional lasR is required for virulence. However, the co-operation of the signal-generating components of the quorum sensors in vivo remains to be confirmed. In the present study we employed a biological method for monitoring the production of AHL molecules in an animal model. This method relies on freeze microtomy in combination with AHL monitor bacteria equipped with the luxR/PluxI-encoded quorum sensor of *Ph. fischeri*, which functions in a number of bacterial hosts. In the present AHL monitor systems the LuxR-regulated luxI promoter (PluxI) drives expression of a GFP reporter, either the highly stable gfpmut3* or the unstable gfp(ASV). This combination of sensor and reporter was used for three reasons. First, cloned in *E. coli* on high-copy-number vectors this quorum sensor expresses one of the most sensitive and broad-spectrum AHL detectors to date (Fig. 2). Second, GFP appears non-toxic to the bacterial cells and the ability to monitor its expression non-destructively at the single-cell level renders it a powerful reporter of exogenous AHL molecules in vivo. Third, the employment of the unstable version allows for on-line investigations of bacterial communication.

The use of alginate-entrapped bacteria enables the establishment of a chronic lung infection (Pedersen et al., 1990; Moser et al., 1997) and in combination with the GFP-based monitor systems it makes the study of bacterial cross-talk in the lungs of experimental animals possible. In the course of a *P. aeruginosa* co-infection, all versions of the AHL monitor strains [*E. coli* JB357gfp(ASV), JB524-gfpmut3* and JB525-gfp(ASV)] were induced to express green fluorescence. From 1 to 3 d after intratracheal bacterial challenge, production of AHLS from *P. aeruginosa* was detected by the monitor cells as expression of green fluorescence when frozen sections of the lung tissue were observed under the epifluorescence microscope. In experiment II, fully induced, green fluorescent bacteria could easily be found in the lung tissues exhibiting severe pathological changes (large areas of consolidation) but were rarely found in the lung tissues exhibiting minor pathological changes. The lung bacteriology showed that both *P. aeruginosa* and *E. coli* JB524-gfpmut3* or JB525-gfp(ASV) were...
found at high densities up to day 3. Five days after bacterial challenge, GFP-expressing bacteria could not be detected in the lung tissues. Taken together with colony counts, and since *E. coli* JB524-gfpmut3*<sup>®</sup> cells express stable GFP (signal stable for days), this shows that the monitor strain is cleared from the lung tissues. Two of the monitor strains, *E. coli* JB357-gfp(ASV) and JB525-gfp(ASV), express an unstable variant of GFP which is rapidly degraded in exponentially growing as well as in stationary-phase cells. In the absence of exogenous AHL molecules, the fluorescent signals of the monitor cells will be below detection level after 4 h (Fig. 3b). These results indicate that AHL molecules are being produced during infection until both types of bacteria are cleared. Although we have no data on the actual half-life of GFP in bacteria infecting the lungs, our results suggest that the inoculated *P. aeruginosa* produced AHL molecules from at least 24 h to 3 d of infection. In addition to functioning as AHL indicators *in vivo*, the *E. coli* monitor strains directly demonstrate that intercellular communication and quorum sensing take place in the infected animal lung.

The presence of fully induced *E. coli* JB357-gfp(ASV) cells indicates that the AHL concentration locally exceeds a minimum of 5 nM, but due to sensitivity limitations of the TLC overlay technique we were unable to determine which AHL molecule is the major species in the lungs of the infected animals. An analysis of this is in progress. It is likely that the production as well as the variety of AHLs from *P. aeruginosa* is affected by many factors such as specific antibodies, phagocytes and other host-defence mechanisms. The conditions in the lung may also affect the sensitivity of the *E. coli* monitor strains. The monitor bacteria present inside the lung tissues were covered by alginate, lung tissue and blood cells, etc. Thus, the induction of GFP in the monitor bacteria might require a much higher AHL level *in vivo* than *in vitro*. Although all three monitors functioned well and allowed detection of GFP-expressing bacteria at the single-cell level, *E. coli* JB357-gfp(ASV), producing the unstable version of GFP from a high-copy-number vector, gave the strongest signal compared to the other two monitor strains, which contained stabilized, low-copy-number vectors. The usefulness of the JB357-gfp(ASV) system in chronic infections is somewhat limited since persistence of plasmid-carrying cells in the lungs required administration of ampicillin to the mice.

The histopathological changes seen in the mouse lung tissues were similar to what we found in the lungs of CF patients, i.e. inflammation was dominated by the presence of PMNs as demonstrated in Fig. 4 (Hoiby et al., 1993). The lung bacteriology and pathology results indicate that the AHL monitor strains inside the alginate beads were cleared more easily and induced less severe pathological changes compared with *P. aeruginosa* PAO579. Macroscopic lung pathology studies showed that changes were still observed on days 7 and 14 in the *E. coli* monitor group even though the lung bacteriology indicated that the monitor strains had been cleared by day 5. Histopathology showed that lung atelectasis and beads blocking the bronchi were the major changes observed from day 5. Therefore mechanical blocking by the beads might be partly responsible for the lung atelectasis in the Ec group after day 5.

In favour of using the *E. coli* strains as AHL monitor is the fact that these strains induce minor lung pathological changes compared to *P. aeruginosa*. Furthermore, the *E. coli* monitor system is not controlled by a number of complex regulatory factors and media effects, as are the quorum sensors of *P. aeruginosa*. *E. coli* can therefore be employed as a useful AHL monitor strain in short-term experiments of mouse lung infections with *P. aeruginosa*. We also found that the *E. coli* monitors are 50- to 100-fold more sensitive to AHL molecules compared with the JP2 strain of *P. aeruginosa* PA01 (lasR rhlI) (Pearson et al., 1997) harbouring pJB130 and pJB132 (data not shown). However, for extended studies more appropriate monitor strains will have to be employed and work is in progress to construct modified *P. aeruginosa* and *Burkholderia cepacia* strains with inactivating mutations in the AHL signal-generating systems carrying lasR, PlasB-based monitors in combination with unstable versions of GFP. The ideal strain would be expected to persist for a long time in the lungs co-infected with virulent *P. aeruginosa* strains.

Geisenberger et al. (2000) recently reported that neither the amounts nor the AHL profiles of *P. aeruginosa* isolates (when grown under standard *in vitro* conditions) from five CF patients who were monitored over periods of up to 11 years changed significantly during long-term colonization. These results suggest that phenotypic adaptations to the CF lung environment during chronic colonization do not affect the ability to synthesize AHLs. The present study provides direct evidence of AHL expression *in vivo* in mouse lung tissues using live AHL monitor strains. This is in accordance with the finding of Hardman et al. (1999) that the sputum of CF patients infected with *P. aeruginosa* activated AHL monitor strains, strongly suggesting that AHL signals are being produced during infection. Expression of the *P. aeruginosa* virulence factors is modulated by AHL molecules. Hence it was not very surprising to detect the presence of AHL mainly in the lung tissues exhibiting severe pathological changes. This correlation between AHL exposure and severity of infection supports the view of Tang et al. (1996) that quorum sensing plays a critical role in the virulence of *P. aeruginosa*. In addition, Telford et al. (1998) suggested that OdDHL not only functions to regulate bacterial virulence gene expression through cell–cell communication but also, by virtue of its immuno-modulatory properties, it may be a virulence determinant *per se*. There is clearly a role for live AHL monitors for *in vivo* studies with quorum-sensing inhibitors. Furanone compounds produced by the macro-alga *Delisea pulchra* are powerful inhibitors of quorum-sensing systems (Givskov et al., 1996; Manefield et al., 1999) and their antagonist activity *in vivo* can be judged from their ability to switch off AHL-controlled GFP expression in the lung. This may provide...
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REFERENCES


Detection of AHLs in lung tissues


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