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

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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 AHLs 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 AHL molecules *in vivo* by the infecting *P. aeruginosa*. AHL signals were detected mainly in lung tissues exhibiting severe pathological changes. These findings support the view that expression of AHL molecules 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, staphylolytic protease, alkaline protease), pigments (pyocyanin, pyoverdine), 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). AHLs are synthesized from precursors by a synthetase (LuxI homologue), and

Abbreviations: AHL, *N*-acylhomoserine lactone (BHL, *N*-butanoyl-, HHL, *N*-hexanoyl-, OdDHL, *N*-(3-oxododecanoyl)-, ODHL, *N*-(3-oxodecanoyl)-, OHHL, *N*-(3-oxohexanoyl)-, OHL, *N*-octanoylhomoserine lactone); CF, cystic fibrosis; CSLM, confocal scanning laser microscopy; GFP, green fluorescent protein; PMN, polymorphonuclear leukocyte.

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* (Ochsner & Reiser, 1995), which consist of two signal-generating synthetases (LasI/RhII) and two cognate transcriptional regulators (LasR/RhIR). The major products of LasI and RhII 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 *lasIR*-encoded quorum-sensor system has been shown to modulate expression of *lasI* 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), *xcp* (secretion pathway) (Chapon-Herve *et al.*, 1997) and *rhIR* (Latifi *et al.*, 1996; Pesci *et al.*, 1997). The *rhIIIR*-encoded quorum sensor modulates expression of *rhII* itself (Latifi *et al.*, 1996), *rhIAB* (rhamnolipid biosynthesis) (Ochsner & Reiser, 1995; Pearson *et al.*, 1997), *lasB* (Brint & 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 TNF α 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* (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_{luxI}* 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 PAO1 (Holloway & Morgan, 1986). The *E. coli* strains used were JM105 [*thi rpsL*(Str^r) *end sbcB15 sbcC hsdR4* (r_K⁻ m_K⁻) Δ (*lac-proAB*) {F' *trad36 lacI*^r Δ (*lacZ*)M15 *proA*⁺B⁺}] (Yanisch-Perron *et al.*, 1985) and MT102, which is a restriction-minus derivative of MC1000 (*araD139* Δ (*ara-leu*)7697 Δ *lac*, *thi*, *hsdR*).

AHL monitor strains. The GFP derivatives GFPmut3* and its unstable variant were used as reporters (Andersen *et al.*, 1998). The *gfpmut3** gene encodes a 238 amino acid protein that emits fluorescent light at 511 nm when excited by light with wavelength around 488 nm (Cormack *et al.*, 1996). GFPmut3* requires trace amounts of oxygen to mature and does not need any substrate addition in order to fluoresce. A number of *gfpmut3** derivatives whose products have different half-lives are also available (Andersen *et al.*, 1998). The unstable versions are useful for monitoring temporal expression of AHL molecules *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 monitor plasmids carried the *P. fischeri* region encoding *luxR* and a fusion between *PluxI* and *gfp*. The plasmids pJBA88 and pJBA89 are pUC18NotI derivatives that contain this promoter fused to the stable *gfpmut3** and the unstable *gfp*(ASV), respectively. The *E. coli* JM105 monitor strains containing these plasmids were denoted JB353 and JB357, respectively. The *luxR*, *PluxI-gfpmut3** fusion and the *luxR*, *PluxI-gfp*(ASV) (unstable GFP) fusion were cloned into a stable broad-host-range vector pME6031 (pVS1 replicon, accession no. AF118811), giving rise to pJBA130 and pJBA132, 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-*gfp*(ASV), JB524-*gfpmut3** and JB525-*gfp*(ASV). Two additional AHL monitor strains were used, JM105/pSB536 and JM105/pMH297, which encode bioluminescent AHL monitors. pSB536 carries the *Aeromonas hydrophila* *ahyR*, *PahyI-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* PAO579 was grown in Luria broth to an OD₆₀₀ 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). C₁₈-reversed-phase TLC plates (aluminium sheets RP-18 F_{254s} (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.

Immobilization of bacteria in seaweed alginate beads. Immobilization 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 10/60; 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 1.0×10^9 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 etomidat (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 (1.0×10^9 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 TCS4D, 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 Indigo 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 RhII (Winson *et al.*, 1995). The monitor also detects OHHL, *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 *ahyR*, *Pahyl-luxAB* fusion of pSB536 is highly specific for BHL whereas the

Fig. 1. The AHL monitor plasmid pJBA89. At the top is the DNA nucleotide sequence showing the fusion between the *luxR*-*P_{luxI}*-RBSII fragment (similar in pJBA88, pJBA89, pJBA130 and pJBA132), the *gfpmut3** and the *gfp*(ASV). The ATG start codon is in bold letters. The arrow indicates direction of transcription of *gfp*. The positions of the Lux-box sequences are underlined, as are important restriction sites.

Fig. 2. (a) AHL molecules produced by *P. aeruginosa* PAO1 and PAO579. Thin-layer chromatogram with AHL monitor overlays of (panel A) *E. coli* JB357-*gfp*(ASV) and (panel B) *E. coli* JM105/pSB536 and JM105/pMH297. The position and identity of AHL molecules is indicated with arrows. Red areas indicate the strongest signal whereas dark blue areas indicate weak signal intensity. Extracts obtained from PAO1 and PAO579 and synthetic AHL compounds were applied to the TLC plate as indicated under the chromatogram. (b) Sensitivity of the *E. coli* JB357-*gfp*(ASV) monitor to various AHL molecules. An overnight culture was diluted fourfold in fresh Luria broth, distributed to the wells of a microtitre dish with series of twofold AHL dilutions and incubated at 37 °C. After 2 h, green fluorescence in each well was measured on a fluorimeter.

lasR, *PlasB-luxAB* fusion of pMH297 recognizes primarily long-chain AHL signals and identifies the presence of ODHL and OdDHL. This analysis demonstrates that PAO579 produces BHL, HHL, OHHL, OHL, ODHL 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 OHHL (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 fluorescence, cells induced with 32 nM OdDHL were 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-*gfpmut3** cells. In related studies, an approximately 45 min half-life was calculated for GFP(ASV) in exponentially growing cultures of *E. coli* (J. B. Andersen and others, unpublished).

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*

Expt	Mouse strain	No. of animals	Group	Bacterial strain(s) inoculated†	Comments
I	NMRI, female 9–11 weeks old, weight 25–30 g	10	Pa	<i>P. aeruginosa</i> PAO579	Animals in expt I were killed 3, 5, 7 and 14 d after challenge
		10	Ec	<i>E. coli</i> JB357- <i>gfp</i> (ASV)	
		10	Pa + Ec	PAO579 + JB357- <i>gfp</i> (ASV)	
II	NMRI, female 9–11 weeks old, weight 25–30 g	60	Pa	<i>P. aeruginosa</i> PAO579	Animals in expt II were killed 3, 5, 7 and 14 d after challenge
		60	Ec	<i>E. coli</i> JB524- <i>gfpmut3</i> *	
		60	Pa + Ec	PAO579 + JB524- <i>gfpmut3</i> *	
III	CBA/J, female 10–11 weeks old, weight 20–25 g	60	Pa	<i>P. aeruginosa</i> PAO579	Animals in expt III were killed 1, 3, 5, and 7 d after challenge
		60	Ec	<i>E. coli</i> JB525- <i>gfp</i> (ASV)	
		60	Pa + Ec	PAO579 + JB525- <i>gfp</i> (ASV)	

† *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.

Fig. 3. For legend see facing page.

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

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.

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

In experiment II, NMRI female mice were challenged with *P. aeruginosa* PAO579 and/or *E. coli* JB524-*gfpmut3**. In experiment III, CBA/J female mice were challenged with *P. aeruginosa* PAO579 and/or *E. coli* JB525-*gfp*(ASV). Statistical significance: in experiment II, compared to the Pa group, * $P < 0.001$; in experiment III, compared with the Pa group, ** $P < 0.01$.

Expt	Days after challenge	Pa group		Ec group		Pa + Ec group		
		Bacterial count, c.f.u. ml ⁻¹ (range)	GFP signal	Bacterial count, c.f.u. ml ⁻¹ (range)	GFP signal	Bacterial count, c.f.u. ml ⁻¹ (range)		GFP signal
						<i>P. aeruginosa</i>	<i>E. coli</i>	
II	3	1.2 × 10 ⁶ (1.6 × 10 ⁵ –3.8 × 10 ⁷)	—	5.5 × 10 ⁴ * (0–1.2 × 10 ⁵)	—	7.1 × 10 ⁵ (2.8 × 10 ⁴ –2.0 × 10 ⁶)	2.3 × 10 ⁵ (5.0 × 10 ³ –1.2 × 10 ⁶)	+
	5	4.4 × 10 ⁵ (3.0 × 10 ⁴ –6.3 × 10 ⁶)	—	0*	—	1.1 × 10 ⁴ (5.6 × 10 ³ –1.5 × 10 ⁵)	0	—
	7	1.4 × 10 ⁵ (0–5.8 × 10 ⁵)	—	0*	—	6.4 × 10 ³ (1.0 × 10 ² –7.5 × 10 ⁴)	0	—
	14	8.8 × 10 ⁴ (4.6 × 10 ⁴ –8.2 × 10 ⁵)	—	0*	—	2.2 × 10 ⁴ (0–1.2 × 10 ⁵)	0	—
III	1	9.0 × 10 ⁵ (1.1 × 10 ⁵ –3.3 × 10 ⁷)	—	3.4 × 10 ⁴ ** (2.0 × 10 ² –1.2 × 10 ⁵)	—	5.0 × 10 ⁴ (1.3 × 10 ⁴ –2.2 × 10 ⁷)	2.2 × 10 ⁴ (1.5 × 10 ⁴ –4.0 × 10 ⁵)	+
	3	2.9 × 10 ³ (3.5 × 10 ² –1.7 × 10 ⁴)	—	2.8 × 10 ³ (5–1.7 × 10 ⁴)	—	1.3 × 10 ³ (6–6.0 × 10 ³)	7.2 × 10 ² (0–2.6 × 10 ³)	+
	5	3.0 × 10 ³ (4.9 × 10 ² –1.4 × 10 ⁴)	—	0** (0–1.1 × 10 ²)	—	2.4 × 10 ¹ (0–7.2 × 10 ¹)	0	—
	7	7.3 × 10 ² (3.0 × 10 ¹ –7.1 × 10 ³)	—	0** (0–1.4 × 10 ¹)	—	5.9 × 10 ² (4.3 × 10 ² –1.2 × 10 ³)	0	—

fluorescent 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 this test demonstrated that the mice on day 3 carried functional monitor bacteria that were simply not turned on in the lung tissue (data not shown).

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* JB357-*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).

Table 3. Lung index of macroscopic pathology (LIMP)

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

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* JB357-*gfp*(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-*gfp*mut3* 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* PAO1 (*lasI rhII*) (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

a basis for the development of anti-AHL therapy, as postulated by Finch *et al.* (1998) and Hartman & Wise (1998).

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