Direct detection of N-acylhomoserine lactones in cystic fibrosis sputum

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Abstract

Pseudomonas aeruginosa and Burkholderia cepacia cause destructive lung disease in cystic fibrosis (CF) patients. Both pathogens employ 'quorum sensing', i.e. cell-to-cell communication, via diffusible N-acyl-l-homoserine lactone (AHL) signal molecules, to regulate the production of a number of virulence determinants in vitro. However, to date, evidence that quorum sensing systems are functional and play a role in vivo is lacking. This study presents the first direct evidence for the presence of AHLs in CF sputum. A total of 42 samples from 25 CF patients were analysed using lux-based Escherichia coli AHL biosensors. AHLs were detected in sputum from patients colonised by P. aeruginosa or B. cepacia but not Staphylococcus aureus. Furthermore, using liquid chromatography-mass spectrometry and thin layer chromatography, we confirmed the presence of N-hexanoylhomoserine lactone and N-(3-oxododecanoyl)homoserine lactone respectively in sputum samples from patients colonised by P. aeruginosa. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Cystic fibrosis (CF) is a genetic disease characterised by mucus hyper-secretion, chronic bacterial infection and airway inflammation. Microbial colonisation of the major airways of CF patients by Pseudomonas aeruginosa, Burkholderia cepacia and Staphylococcus aureus leads to destructive lung disease. This is the major cause of morbidity and mortality in CF patients (for review see [1]), requiring frequent physiotherapy and aggressive treatment with antibacterial drugs.

In recent years it has been established that many different bacteria co-ordinate gene expression in a cell density-dependent manner using small diffusible signalling molecules, a phenomenon termed 'quorum sensing' [2–4]. In Gram-negative bacteria, the most intensively studied quorum sensing systems utilise N-acylhomoserine lactones (AHLs) as inter-cellular signalling molecules, the synthesis of which is mediated by a LuxI-type protein. In these organisms, a transcriptional regulator protein (LuxR-type) acts in conjunction with the cognate AHL to regulate target gene expression. Of particular relevance, the two major CF pathogens P. aeruginosa [5–8] and B. cepacia [9] are both known to regulate virulence gene expression via AHLs in vitro.

P. aeruginosa possesses two quorum sensing systems employing the LuxRI homologues LasRI and RhlR (VsmRI), with the major cognate signal molecules being N-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C12-HSL), N-butanoyl-l-homoserine lactone (C4-HSL) and N-hexanoyl-l-homoserine lactone (C6-HSL) [7–13]. Importantly, cell-cell signalling in P. aeruginosa is involved in the regulation of a number of virulence determinants, such as elastase, which contribute to the deterioration of lung function in colonised CF patients [8,14,15]. In another important CF pathogen, B. cepacia, the production of both exoprotease and the siderophore, ornibactin, are regulated by CepR and its cognate AHL, N-octanoyl-l-homoserine lactone (C8-HSL). Interestingly, it has been
shown that CF isolates of *P. aeruginosa* and *B. cepacia* produce AHLs in vitro [16,17]. Furthermore, indirect evidence for AHL production in CF sputum containing *P. aeruginosa* was obtained by Singh et al. [18]. They used ^14^C-labelled methionine, which is incorporated into AHLs via S-adenosylmethionine during AHL biosynthesis, to show in cystic fibrosis sputa (incubated ex vivo for 4 h to stimulate de novo AHL biosynthesis), *P. aeruginosa* produces AHLs. More direct evidence for AHL production by *P. aeruginosa* in vivo has come from studies of lung infections in mice in which *P. aeruginosa* was co-inoculated with a recombinant *Escherichia coli* AHL biosensor carrying a *luxRI::gfp* reporter fusion [19].

In this paper we present direct evidence that AHLs are produced in CF sputum during *P. aeruginosa* and *B. cepacia* lung infections by using sensitive recombinant *E. coli*-based AHL biosensors. Furthermore, we have confirmed the nature of the AHLs present in CF sputum colonised by *P. aeruginosa* using thin layer chromatography (TLC) and high resolution mass spectrometry.

2. Materials and methods

2.1. Patient data

Patients were recruited from the Adult CF Unit (City Hospital, Nottingham, UK) between July 1998 and August 1999. All had elevated sweat sodium (> 60 mmol l^{-1}) and chloride (> 70 mmol l^{-1}) concentrations as well as clinical and radiological features consistent with the diagnosis of cystic fibrosis with a median FEV1 of 1.8 (range 0.55–4.00), FVC of 3.0 (0.9–5.1). The genotype of 15/25 patients was known: six patients were homozygous for ΔF508, seven patients had ΔF508 the other mutation being unknown and there were two patients with ΔF508/621+1 G>T and G551D/621+1 G>T respectively. A total of 42 sputum samples were taken from 25 CF patients (median age 22 years, range 17–37). Sputum samples were collected from stable CF patients attending out-patient clinic who were not taking oral or intravenous antibiotics. Patients were colonised with either *P. aeruginosa* (17 patients), *B. cepacia* (four patients) or *S. aureus* (four patients). Sputum samples were stored on ice during transportation and processed to determine the presence of AHL(s) on the same day they were obtained from the patient.

2.2. Bacterial strains and growth conditions

*P. aeruginosa* were isolated from sputum by streaking on Pseudomonas Isolation Agar (PIA) (Difco, US) whereas *S. aureus* and *B. cepacia* were isolated on Luria–Bertani agar (LB). All isolates were then incubated at 37°C overnight. AHL biosensor strains were grown at 30°C overnight in LB broth. For plasmid maintenance *E. coli* JM109 (pSB401) and *E. coli* JM109 (pSB1075) were grown in LB broth containing 10 μg ml^{-1} tetracycline.

2.3. Detection of AHLs in sputum

Sputum samples were diluted to 10 ml with sterile phosphate-buffered saline (PBS) pH 7.4, centrifuged (10 000 rpm for 10 min; Beckman Avanti 30 centrifuge, rotor CO650), to remove any debris. The resulting diluted sputum supernatants were extracted using a modified protocol of Câmara et al. [20]. Essentially, supernatants were extracted twice using equal volumes of dichloromethane and evaporated to dryness using a rotary evaporator (Buchi R-114, Switzerland). The extract was reconstituted in 50 μl of acetonitrile and stored at −20°C. Two *E. coli* biosensor strains containing *lux*-based bioluminescence AHL reporter plasmids were used to detect the presence of AHLs in concentrated solvent extracts. The reporter plasmid pSB401 [21], contains the *Vibrio fischeri luxR* gene and *luxI* promoter fused to luxCDABE from *Photorhabdus luminescens*. When introduced into *E. coli* JM109, this construct responds preferentially to the presence of exogenously added AHLs with acyl chains from six to eight carbons in length such as *N*-(3-oxohexanoylhomoserine) lactone (3-oxo-C_{6}-HSL) and C_{6}-HSL and C_{9}-HSL by the emission of light. For detection of long chain AHLs such as 3-oxo-C_{12}-HSL, *E. coli* JM109 harbouring the reporter plasmid pSB1075 [21] was used. This reporter contains the *P. aeruginosa* lasR gene and *lasI* promoter fused to luxCDABE from *P. luminescens* and preferentially responds to AHLs with acyl chains of 10–14 carbons in length. In all cases, light emission was detected using a Berthold LB980 photon video camera (E.G. and G. Berthold UK Ltd, Milton Keynes, UK). AHL bioassays, using the *E. coli* *lux*-based reporters, were performed in microtitre well plates as described previously [20]. Briefly, 10 μl of the sputum extract was added to 90 μl of the sensor strain culture, after which doubling dilutions were performed in a microtitre well plate, using LB as the diluent. Similar doubling dilutions were performed on synthetic standards (10 μl of 10 μg ml^{-1} synthetic 3-oxo-C_{6}-HSL or 3-oxo-C_{12}-HSL) and used as positive controls. Light emission was observed following overnight incubation at 30°C. The investigator performing the AHL assay was blinded to the patient clinical data.

2.4. Identification of AHLs

TLC was employed to separate AHLs and overlaid with soft top agar seeded with the appropriate biosensor strain as described by McClean et al. [22]. Briefly, for analysis of short chain AHLs, reverse phase aluminium-backed RP18 F_{254S} TLC plates (20 cm × 20 cm; Merck) and a mobile phase of 60% v/v methanol in water were employed. Long chain AHLs were analysed on aluminium-backed Silicagel 60 F_{254} normal phase TLC plates (20 cm × 20 cm; Merck)
using a 45:55% v/v hexane:acetone mix as the mobile phase.

AHLs detected by TLC were purified and characterised as described [5,20,23]. Essentially, AHLs from sputum extracts were purified by semi-preparative reverse-phase high pressure liquid chromatography (HPLC) (Kromasil KR100-5C8 [250×8 mm] column; Hichrom, Reading, UK). Fractions were eluted with a linear gradient of acetonitrile in water (20–95%) over a 30-min period at a flow rate of 2 ml min\(^{-1}\) and monitored at 210 nm. Six fractions (F1–F6) were collected, covering 5-min intervals, and assayed for activity using the \(E. coli\) lux-based biosensors. Following preparative HPLC, the active fractions were analysed by HPLC-mass spectrometry (LC-MS) (Micromass Instruments, Manchester, UK) as described previously [24]. The spectra obtained were compared with those for synthetic AHL standards subjected to the same LC-MS conditions.

2.5. Synthesis of AHLs

The synthesis of AHLs was done as previously described [20,25].

3. Results

3.1. Detection of AHL signalling molecules in sputum from CF patients colonised by \(P. aeruginosa\) and \(B. cepacia\)

To obtain direct evidence that AHLs are produced in vivo in the CF lung of patients colonised by \(P. aeruginosa\) and \(B. cepacia\), sputum samples were extracted with dichloromethane and assayed for the presence of AHLs using the \(luxR\)-based biosensor strains \(E. coli\) (pSB401) and \(E. coli\) (pSB1075) in microtitre well plates. The use of these strains allows detection of most AHLs which have so far been described in Gram-negative bacteria including those of \(P. aeruginosa\) and \(B. cepacia\) [17,21]. The emission of light by both \(E. coli\) (pSB401) and \(E. coli\) (pSB1075) when incubated with different sputum samples indicates the presence of AHLs, with different acyl side chain lengths (Fig. 1; Table 1).

Using \(E. coli\) (pSB401) AHLs were detected in 30 out of 42 samples (71%) (Table 1) whereas only 26 samples (61%) activated \(E. coli\) (pSB1075). Similarly, sputum extracts from \(B. cepacia\)-colonised patients activated \(E. coli\) (pSB1075) in four out of eight samples (50%). An example of a typical microtitre plate bioassay result, obtained using a photon imaging camera, is shown in Fig. 1. As anticipated, solvent extracts of sputum obtained from patients colonised by \(S. aureus\) did not activate either biosensor as this organism has not been shown to produce AHLs in vitro [3]. Therefore, sputum samples from \(S. aureus\)-colonised patients acted as an appropriate negative control in this study.

![Fig. 1. Photon camera image of extracts of sputum from patients colonised by \(P. aeruginosa\) and \(B. cepacia\) screened for the presence of AHLs using (A) the \(luxR\)-based biosensor \(E. coli\) (pSB401) and (B) the \(lasR\)-based AHL biosensor \(E. coli\) (pSB1075). Doubling dilutions of sputum extracts were performed from left to right in a microtitre well plate and then incubated with 100 \(\mu\)l of either \(E. coli\) (pSB401) or \(E. coli\) (pSB1075). 1, 2 and 4: sputum extracts from \(P. aeruginosa\)-colonised patients; 3: sputum extract from a \(B. cepacia\)-colonised patient. +ve (positive control): 10 \(\mu\)l of 10 \(\mu\)g ml\(^{-1}\) 3-oxo-C\(_{6}\)-HSL (A); 10 \(\mu\)l of 10 \(\mu\)g ml\(^{-1}\) 3-oxo-C\(_{12}\)-HSL (B); -ve (negative control): 10 \(\mu\)l of acetonitrile in LB broth.](image)

<table>
<thead>
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<th>Colonising organism</th>
<th>Number of patients</th>
<th>Number of samples</th>
<th>Number of samples activating bioassay</th>
</tr>
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<tbody>
<tr>
<td>(P. aeruginosa)</td>
<td>17</td>
<td>42</td>
<td>30/42</td>
</tr>
<tr>
<td>(B. cepacia)</td>
<td>4</td>
<td>8</td>
<td>7/8</td>
</tr>
<tr>
<td>(S. aureus)</td>
<td>4</td>
<td>6</td>
<td>0</td>
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The \(E. coli\) (pSB401) and \(E. coli\) (pSB1075) biosensors are used to detect AHLs with short and long acyl side chains respectively.

3.5. Synthesis of AHLs

The synthesis of AHLs was done as previously described [20,25].
AHLs, were pooled and concentrated. AHLs in the pooled extract were first separated by HPLC and the subsequent fractions assayed for activity against the AHL biosensors. Active fractions were then subjected to LC-MS analysis. The LC-MS spectrum obtained for the active molecule present in fraction 3 (Fig. 3), in which synthetic C6-HSL is expected to elute, revealed a molecular ion of 200 (M+1), which is consistent with C6-HSL. Furthermore, peaks at 102 and 99 were observed which correspond to the homoserine lactone ring moiety and the hexanoyl side chain respectively. For absolute confirmation, C6-HSL was chemically synthesised, as described in Section 2, and shown to possess the same LC-MS fragmentation pattern (Fig. 3). We were unable to identify molecular ions corresponding to either C4-HSL or 3-oxo-C12-HSL.

4. Discussion

In this study we provide direct evidence that AHLs are present in sputum samples from CF patients colonised by P. aeruginosa and B. cepacia. Furthermore, we report the identification of the AHLs produced by P. aeruginosa in these samples. By developing a rapid and reliable microtitre plate bioassay for screening concentrated solvent extracts of sputum samples, we have shown that sputum from patients infected with P. aeruginosa contains both short chain AHLs and also a molecule which co-migrates with 3-oxo-C12-HSL on thin layer chromatograms. However, we were only able to confirm the presence of C6-HSL in sputum samples from patients colonised with P. aeruginosa, which was unexpected since, in vitro, this molecule is produced in much smaller quantities than C4-HSL [8] or 3-oxo-C12-HSL [16]. Nonetheless, P. aeruginosa strains isolated from the sputum produced 3-oxo-C12-HSL, C4-HSL and C6-HSL in vitro. The fact that the reporter plasmid pSB1075 is much more sensitive to low concentrations of 3-oxo-C12-HSL than LC-MS is likely to account for our inability to detect this signalling molecule using the latter. Alternatively, AHL production by P. aeruginosa in the CF lung may be different to that in vitro, perhaps due to the contrasting growth conditions encountered in the CF lung compared to those in laboratory culture conditions. Interestingly Singh et al. [18] have reported that, with respect to de novo synthesis of AHLs in sputum colonised by P. aeruginosa, the ratio of C4-HSL to 3-oxo-C12-HSL levels is the reverse of that observed for the laboratory strain PA01 when grown in laboratory medium, yet reflects that seen within in vitro biofilms of the sputum isolates. As a consequence the authors conclude that P. aeruginosa exists predominantly as a biofilm within CF sputum. Furthermore, in vitro analysis of strains isolated from the sputum samples showed for one strain that C6-HSL was, in fact, the predominant AHL produced. Importantly, transcripts of lasR and lasI, from P. aeruginosa, have previously been detected in CF spu-
Moreover, the accumulation of these transcripts correlates with the accumulation of transcripts of target genes which are regulated by lasRI [27]. Although this suggests that the production of AHLs in the CF lung is highly likely, the presence of AHLs in CF sputum had, until now, not been determined. Taken together, this demonstrates that for *P. aeruginosa*, AHL-mediated quorum sensing systems, which regulate the production of virulence factors that contribute to the deterioration in lung function seen in colonised CF patients, are functional within the CF lung. Of note, sputum extracts from CF patients colonised by *S. aureus* did not activate any of the biosensors which is consistent with the fact that this pathogen has not been shown to produce AHLs in vitro.

The fact that AHLs are produced in vivo is particularly noteworthy since certain AHLs possess both pharmacological significance and therapeutic potential. The identification of C6-HSL in sputum from *P. aeruginosa*-colonised patients by LC-MS. Seven extracts of sputum from patients colonised by *P. aeruginosa* were pooled and purified by HPLC. Subsequent LC-MS analysis confirmed the presence of C6-HSL. The figure shows (i) synthetic C6-HSL and (ii) C6-HSL from the pooled sample. The peaks indicated by the arrows correspond to the m/z for the molecular ion (M+1: 200) (A), the homoserine lactone moiety (102) (B) and the hexanoyl side chain (99) (C). The structure of C6-HSL and its MS fragmentation pattern are shown alongside. The vertical wavy lines show the point of fragmentation in the C6-HSL molecule. Numbers correspond to the masses (m/z) of the fragments (A, B, C) in the direction shown by the arrow.
logical and immunomodulatory activities and so may function as virulence determinants per se. Specifically, 3-oxo-C12-HSL has been shown to modulate T cell and macrophage functions [28] and to and exhibit potent pharmacological activity [29,30]. Consequently, 3-oxo-C12-HSL may not only play a role in the regulation of virulence factor production by *P. aeruginosa*, but also affect host cell functions to increase the availability of nutrients, whilst down-regulating host defence mechanisms, in order to promote its own survival and growth in vivo.

We are still far from fully understanding how *P. aeruginosa* and *B. cepacia* employ quorum sensing to survive and control the onset of disease in the CF lung. Recently, Geisenberger et al. [16] compared the AHL profiles of CF clinical isolates grown in vitro and claimed that both the amounts and types of AHLs produced by *P. aeruginosa* in vitro did not change during chronic colonisation. However, these isolates were grown under laboratory culture conditions. We have found, in some preliminary studies, a possible correlation between the levels of AHLs in vivo and the clinical status of patients (data not shown). Further work, with the inclusion of a larger number of subjects, using the assays described in this paper, is still required in order to draw any conclusions from these observations.

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References


