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Identification of *N*-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients

Catherine E. Chambers [a,1], Michelle B. Visser [a,1], Ute Schwab [b], Pamela A. Sokol [a,*]

[a] *Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre, Calgary, Alta., Canada T2N 4N1*
[b] *Department of Food Science, Cornell University, Ithaca, NY 14853, USA*

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Abstract

Pseudomonas aeruginosa and species of the *Burkholderia cepacia* complex are the primary bacterial pathogens contributing to lung disease in patients with cystic fibrosis. Quorum sensing systems using *N*-acyl homoserine lactone (AHL) signal molecules are involved in the regulation of a number of virulence factors in these species. Extracts of mucopurulent respiratory secretions from 13 cystic fibrosis patients infected with *P. aeruginosa* and/or strains of the *B. cepacia* complex were fractionated using reverse-phase fast pressure liquid chromatography and analyzed for the presence of AHLs using a *traI–luxCDABE*-based reporter that responds to AHLs with acyl chains ranging between 4 and 12 carbons. Using this assay system, a broad range of AHLs were detected and identified despite being present at low concentrations in limited sample volumes. *N*-(3-oxo-dodecanoyl)-L-homoserine lactone, *N*-(3-oxo-decanoyl)-L-homoserine lactone and *N*-octanoyl-L-homoserine lactone (OHL) were the AHLs most frequently identified. OHL and *N*-decanoyl-L-homoserine lactone were detected in nanomolar concentrations compared to picomolar amounts of the 3-oxo-derivatives of the AHLs identified.

Keywords: *N*-Acylhomoserine lactone; *Burkholderia cepacia*; *Pseudomonas aeruginosa*; Quorum sensing; Cystic fibrosis

1. Introduction

Cystic fibrosis (CF) is due to a mutation in the cystic fibrosis transmembrane regulator (CFTR) which leads to lung disease characterized by altered fluid transport, increased mucus viscosity, and reduced mucociliary clearance resulting in persistent microbial colonization (reviewed in [1,2]). *Pseudomonas aeruginosa* and strains of the *Burkholderia cepacia* complex (Bcc) are the primary pathogens responsible for morbidity and mortality in CF patients [1,3,4].

Quorum sensing is a cell density-dependent mechanism used by bacteria to coordinate gene expression via diffusible signal molecules termed *N*-acylhomoserine lactones (AHLs). These systems consist of an AHL synthase belonging to the LuxI protein family as well as a transcriptional regulator belonging to the LuxR family [5]. *P. aeruginosa* has two inter-related AHL mediated signaling systems, *lasIR* and *rhlIR*, as well as a non-AHL signal 2-heptyl-3-hydroxy-4-quinolone (PQS for Pseudomonas quinolone signal) [6–9]. LasI directs the synthesis of *N*-(3-oxododecanoyl)-L-HSL (3-oxo-C_{12}-HSL) and *N*-(3-oxooctanoyl)-L-HSL (3-oxo-C_8-HSL)

* Corresponding author. Tel.: +1 403 220 6037; fax: +1 403 270 2772.
E-mail address: psokol@ucalgary.ca (P.A. Sokol).
[1] C.E.C. and M.B.V. contributed equally to this work.

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[8]. RhlI is responsible for the synthesis of *N*-butanoyl-L-HSL (C_4-HSL) and *N*-hexanoyl-L-HSL (C_6-HSL) [7]. Using microarray analysis, more than 400 genes in *P. aeruginosa* were shown to be regulated by the LasIR and RhlIR systems including many which may play a role in virulence [10,11]. Three sets of LuxRI homologs have been identified in the Bcc [12–14]. The *cepIR* genes are widely distributed [13,15]. CepI synthesizes *N*-octanoyl-L-HSL (C_8-HSL) and C_6-HSL [13,15,16]. Gene regulation by the *cepIR* system may be species dependent but includes the regulation of potential virulence factors such as proteases, ornibactin biosynthesis, motility, and biofilm formation [12,16–19]. The *cciIR* genes have recently been identified in *B. cenocepacia* ET12 strains and shown to be present on a genomic island that contains the *B. cepacia* epidemic strain marker [14]. The *bviIR* genes have only been detected in *B. vietnamiensis* [13,20].

Recent studies have provided evidence that AHLs are produced by *P. aeruginosa* and *B. cepacia* CF isolates in vivo. Evidence that AHLs are produced during lung infections was first provided using a mouse model [21]. AHLs were detected in mouse lung samples coinfected with *P. aeruginosa* and an *Escherichia coli luxR-gfp* reporter. AHLs have subsequently been isolated from the sputum of CF patients infected with *P. aeruginosa* or *B. cepacia* [22,23]. Long chain AHLs have been detected in bronchoalveolar lavage (BAL) fluid from lung transplant recipients [24], and both long and short chain AHLs detected directly in lung tissue samples from CF patients [25]. PQS has also been detected in sputum, BAL and mucopurulent fluid from distal airways of lungs at transplant [26]. Previously, we constructed a *traI–luxCDABE* gene reporter system and used it to detect AHLs in lung homogenates from mice infected with *B. cenocepacia* [27]. In the present study, we use this reporter system to detect and identify AHLs from mucopurulent respiratory secretions from CF lung transplant recipients colonized with *P. aeruginosa* and/or species of the *B. cepacia* complex. This sensitive reporter system can be used to detect and identify a broad range of AHLs at low concentrations and in limited sample volumes.

2. Materials and Methods

2.1. Supernatants of mucopurulent material obtained from CF patients

Mucopurulent respiratory secretions were harvested from excess portions of lungs removed during lung transplant operations of 12 CF patients and an autopsy of a CF patient under the auspices of the University of North Carolina Institutional Committee on the Protection of The Rights of Human Subjects. Approximately 1–5 ml of mucopurulent material was collected with a syringe. The mucopurulent material was ultracentrifuged at 100,000 rpm (=543,000*g*) for 60 min in a TL-100 ultracentrifuge with a TL-100.4 fixed-angle rotor (Beckman Instruments, Fullerton, CA). The supernatants were stored at −20 °C prior to analyses. Table 1 lists the sputum culture results preceding the specimen procurement, and shows the culture data closest to the lung transplant date that included all the organisms that were found on repeated cultures.

2.2. Bacterial strains and culture conditions

AHLs were extracted for purification from *P. aeruginosa* strain PAO214 (pSL800) [28], which carries *lasI* on a plasmid. C_{10}-HSL, C_8-HSL and C_6-HSL were purchased from Fluka (Oakville, Ont., Canada). Two reporter strains were used for the detection of AHLs. *Agrobacterium tumefaciens* A136 (pCF218)(pMV26) [27] was used to detect AHLs in column fractions. *A. tumefaciens* A136 (pCF218)(pCF372) [29,30] was used to detect AHLs separated by thin layer chromatography. Both strains contain the plasmid pCF218 [31], which over expresses the TraR protein, which activates the *traI* fusions in response to AHLs. Two *traI* fusions were used for the detection of AHLs. pCF372 [30] has a *traI lacZ* fusion containing nucleotides from −143 to +359 with respect to the transcriptional start site. pMV26 [27] contains a *traI*-lux*CDABE* fusion. The *traI* promoter region from −143 to +68 and the Psa origin of replication were amplified by PCR from pCF372 [30] and inserted into the promoterless *luxCDABE* vector pCS26-Pac [32] to form PMV26 [27].

PAO214 (pSL800) was grown in one liter trypticase soy broth (TSB, DIFCO Laboratories, Detroit, MI) for 16 h at 37 °C for production of AHLs. *A. tumefaciens* strains were routinely grown at 30 °C in LB or on LB solidified with 1.5% agar supplemented with 25 μg ml^{-1} kanamycin, 4.5 μg ml^{-1} tetracycline and 50 μg ml^{-1} spectinomycin as required. Liquid medium was supplemented with 3 μg ml^{-1} tetracycline.

2.3. AHL isolation and assays

3-oxo-AHLs to be used as standards were extracted from one liter culture supernatant with two equal volumes of acidified ethyl acetate. The extract was dried and resuspended in 4 ml of 10% acetonitrile/0.1% trifluoracetic acid (TFA) and subjected to reverse-phase FPLC using a Seqhasil Peptide C18 12 μm ST 4.6/250 column fitted to the AKTA Explorer 900 FPLC system (Amersham Pharmacia Biotech). Analysis of the resulting chromatogram was performed using the UNICORN version 3.12.02 program (Amersham Pharmacia Biotech). The AHLs were injected in 500 μl aliquots and separated at a flow rate of 1 ml min^{-1} with a three stage

Table 1
Corresponding sputum culture results for mucopurulent respiratory secretion samples from cystic fibrosis patients

Sample	*P. aeruginosa*	*Burkholderia* spp.	Other[a]
32	Mucoid		
34	Mucoid, non-mucoid		*Brevundimonas vesicularis*
35	Mucoid, non-mucoid		
36	Mucoid		*Staphylococcus aureus*
37	Non-mucoid	*B. cenocepacia*	
38	Mucoid	*B. multivorans*	*S. aureus*
39	Non-mucid		*S. aureus*, *Alcaligenes xylosoxidans*
40[b]	Mucoid	*B. cenocepacia*	
41		*B. cenocepacia*	
42	Non-mucoid	*B. cenocepacia*	*S. aureus*
44	Mucoid, non-mucoid		
45	Mucoid		*A. xylosoxidans*
46	Mucoid		*A. xylosoxidans*

[a] Oral pharyngeal flora was also present in most of the samples.
[b] Respiratory secretions obtained at autopsy from patient diagnosed with cepacia syndrome [36].

acetonitrile gradient using water/0.1% TFA as buffer A and 98% acetonitrile/0.1% TFA as buffer B. The gradient consisted of 3 column volumes of 10% buffer B, a linear gradient of 10–95% buffer B over nine column volumes and a final gradient of 95–100% buffer B over three column volumes. Fifty microliters from each were analyzed using the TLC assay with *A. tumefaciens* A136 (pCF218)(pCF372) as a reporter [13,33] to identify fractions containing AHLs. Fractions containing AHLs were pooled, dried and further purified by reverse phase chromatography using 10% MeOH/0.1% TFA for 3-oxo-C_6-HSL and 3-oxo-C_8-HSL and 25% MeOH/0.1% TFA for 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL as the eluent. Five μl of each fraction was tested for *luxCDABE* expression with *A. tumefaciens* A136 (pCF218)(pMV26) as the reporter. Fractions with activity were pooled, dried down and the product weighed. AHLs were reconstituted in 20% acetonitrile and stored at −20 °C. The identities of the AHLs were confirmed by TLC assay by comparison of the migration patterns of AHLs detected in the column fractions with the migration of synthetic C_6-HSL, C_8-HSL, C_{10}-HSL, and C_{12}-HSL and observation of tailing effects characteristic of AHLs possessing a 3-oxo substituent in the acyl side chain.

AHLs were extracted from 50 μl to 0.5 ml supernatants of mucopurulent secretions three times with dicholoromethane (2:1 vol/vol). Samples were centrifuged (2000 rpm, 10 min) and the organic layers removed and pooled. The solvent was removed by evaporation and the residue resuspended in 600 μl of 10% acetonitrile and 500 μl chromatographed using the three stage acetonitrile gradient described above. Each fraction was dried down and analyzed for the presence of AHLs using the *A. tumefaciens* (pCF218)-(pMV26) *traI–luxCDABE* reporter system as previously described [27]. Assays were performed in 96 well microtiter plates incubated for 18 h. Luminescence was measured using a Wallac Trilux luminescence counter (Perkin–Elmer Life Sciences). AHLs were identified by comparing the retention time of each positive fraction to the retention time of the standards 3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL, C_{10}-HSL, C_8-HSL, 3-oxo-C_8-HSL, 3-oxo-C_6-HSL and C_6-HSL. The concentration of each AHL present was estimated by comparing the cpm to that obtained using a standard curve of known concentrations of the respective AHL.

2.4. Sensitivity assays

Stock solutions of AHLs were prepared in 20% acetonitrile. C_4-HSL, C_6-HSL, C_8-HSL, C_{10}-HSL and docecanoyl-L-HSL (C_{12}-HSL) (Fluka) stock solutions were prepared at a concentration of 250 μM, and 3-oxo-C_{12}-HSL, 3-oxo-C_{10}-HSL, 3-oxo-C_8-HSL, and 3-oxo-C_6-HSL were prepared at a concentration of 2 μM. Assays to determine the sensitivity of *A. tumefaciens* A136 (pMV26)(pCF218) to various AHLs were performed using concentrations ranging from 2500 to 0.0025 nM for unsubstituted AHLs and 20,000–0.002 pM for 3-oxo-substituted AHLs.

3. Results

3.1. Sensitivity of A. tumefaciens A136(pMV26)(pCF218) reporter to AHLs

The sensitivity of *A. tumefaciens* A136(pMV26)-(pCF218), which contains the *traI–luxCDABE* fusion, to AHLs with acyl chains ranging in length from 4 to 12 carbons was determined by measuring luminescence. Concentrations tested ranged from 2500 to 0.0025 nM for unsubstituted AHLs with acyl chain lengths of 6–12 carbons, from 25 mM to 2.5 nM for C_4-HSL, and from 20 nM to 0.002 pM for 3-oxo-substituted AHLs. This reporter was able to detect all AHLs tested with

Table 2
Sensitivity of *Agrobacterium tumefaciens* A136(pMV26)(pCF218) to various AHLs

N-Acylhomoserine lactone	Minimum concentration detected[a]
C_4-HSL	25 μM
C_6-HSL	250 nM
C_8-HSL	0.25 nM
C_{10}-HSL	25 nM
C_{12}-HSL	250 nM
3-Oxo-C_6-HSL	20 pM
3-Oxo-C_8-HSL	0.2 pM
3-Oxo-C_{10}-HSL	0.02 pM
3-Oxo-C_{12}-HSL	0.02 pM

[a] Minimum concentration detectable in two independent assays performed in triplicate.

acyl chain lengths ranging from 4 to 12 carbons, although it was consistently more sensitive to the 3-oxo-substituted AHLs (Table 2). It was more responsive to AHLs with longer acyl chain lengths and detected 0.02 pM 3-oxo-C_{10}-HSL and 3-oxo-C_{12}-HSL, which was the lowest concentration of any AHL detected.

3.2. Fractionation of AHLs from mucopurulent respiratory secretions

3-oxo-substituted AHLs purified from *P. aeruginosa* and commercially available unsubstituted AHLs were separated by reverse-phase FPLC. Fractions containing activity were identified using the *A. tumefaciens* A136(pMV26)(pCF218) reporter assay and their identities confirmed by their migration in the TLC assay. Using this assay, we were able to separate and detect AHLs with acyl chains ranging from 6 to 12 carbons (Fig. 1(a)). Detection of C_4-HSL in the patient samples was not attempted since the minimum concentration detectable with this reporter assay is in the range of 25 μM (Table 2).

Extracts from respiratory secretions were separated using the same conditions and fractions containing activity identified by measuring luminescence activity in the reporter assay. The AHLs present in the respiratory secretion extracts were identified by comparison of the fractions containing activity to the elution profiles of the AHL standards used in Fig. 1(a). A representative chromatogram is shown in Fig. 1(b). C_{10}-HSL, C_8-HSL, C_6-HSL, 3-oxo-C_8-HSL, and 3-oxo-C_6-HSL were identified in the sample shown.

3.3. Detection and quantitation of AHLs in mucopurulent respiratory secretions

Respiratory secretions from 12 CF patients at the time of lung transplant and one at autopsy were analyzed for the presence of AHLs (Table 3). AHLs were detected in nine specimens. At least two AHLs were identified in all of the specimens containing activity. 3-oxo-C_{12}-HSL and 3-oxo-C_{10}-HSL were the most frequently identified AHLs and were detected in 7 of 13 samples. C_8-HSL was detected in 6 of 13 samples. Although C_8-HSL is the predominant AHL produced by strains of the *B. cepacia* complex its presence or absence did not correlate with the ability to culture *B. cepacia* complex strains from the corresponding patient's sputum. C_8-HSL and C_{10}-HSL were generally present in higher concentrations than the 3-oxo-derivatives since nanomolar amounts were detected compared to picomolar for the 3-oxo-derivatives. C_6-HSL was only detected in sample 41, which was the only specimen from a CF patient not colonized with *P. aeruginosa* (Table 1 and 3). The four specimens that did not contain detectable AHL activity were from patients with *P. aeruginosa* present in their sputum culture. One of four was also culture positive for *B. multivorans*, and one was culture positive for *B. cenocepacia* and had been diagnosed with "cepacia syndrome".

4. Discussion

A variety of reporter systems have been used for the detection of AHLs produced in vivo. Erickson et al. [23] used *lasR–lacZ* and *rhlA–lacZ* fusions to detect 3-oxo-C_{12}-HSL and C_4-HSL, respectively, in sputum from CF patients. Although the reporters used in this study are most sensitive to these AHLs they can detect a range of acyl chain lengths. In fact, when CF sputum from a single patient was extracted and analyzed using the *traI–lacZ* TLC bioassay reporter system, significant amounts of 3-oxo-C_{10}-HSL and 3-oxo-C_8-HSL were also detected in addition to 3-oxo-C_{12}-HSL [23]. Identification of individual AHLs using this assay required the pooling of sufficient sputum samples from a patient to achieve a 30 ml volume for extraction. An *E. coli* (pSB401) *luxI-luxCDABE* reporter that responds to AHLs with acyl chains from six to eight carbons and an *E. coli* (pSB1075) *lasI-luxCDABE* reporter was used to detect AHLs with acyl chains of 10-14 carbons in CF sputum from patients infected with either *P. aeruginosa* or *B. cepacia* [22]. 3-oxo-C_{12}-HSL was detected in sputum from *P. aeruginosa* infected patients using a TLC overlay assay with *E. coli* (SB1075). Limited amounts of sputum prevented the identification of AHLs in the sputum from *B. cepacia* infected patients. LC–MS was used to identify C_6-HSL in multiple pooled sputum samples from a single patient but it was not possible to detect 3-oxo-C_{12}-HSL in this sample with LC–MS analysis. The *E. coli* (SB1075) reporter was also used to detect AHLs in bronchoalveolar lavage fluid from lung transplant recipients, but individual AHLs were not identified [24]. The *traI–luxCDBAE*-based reporter

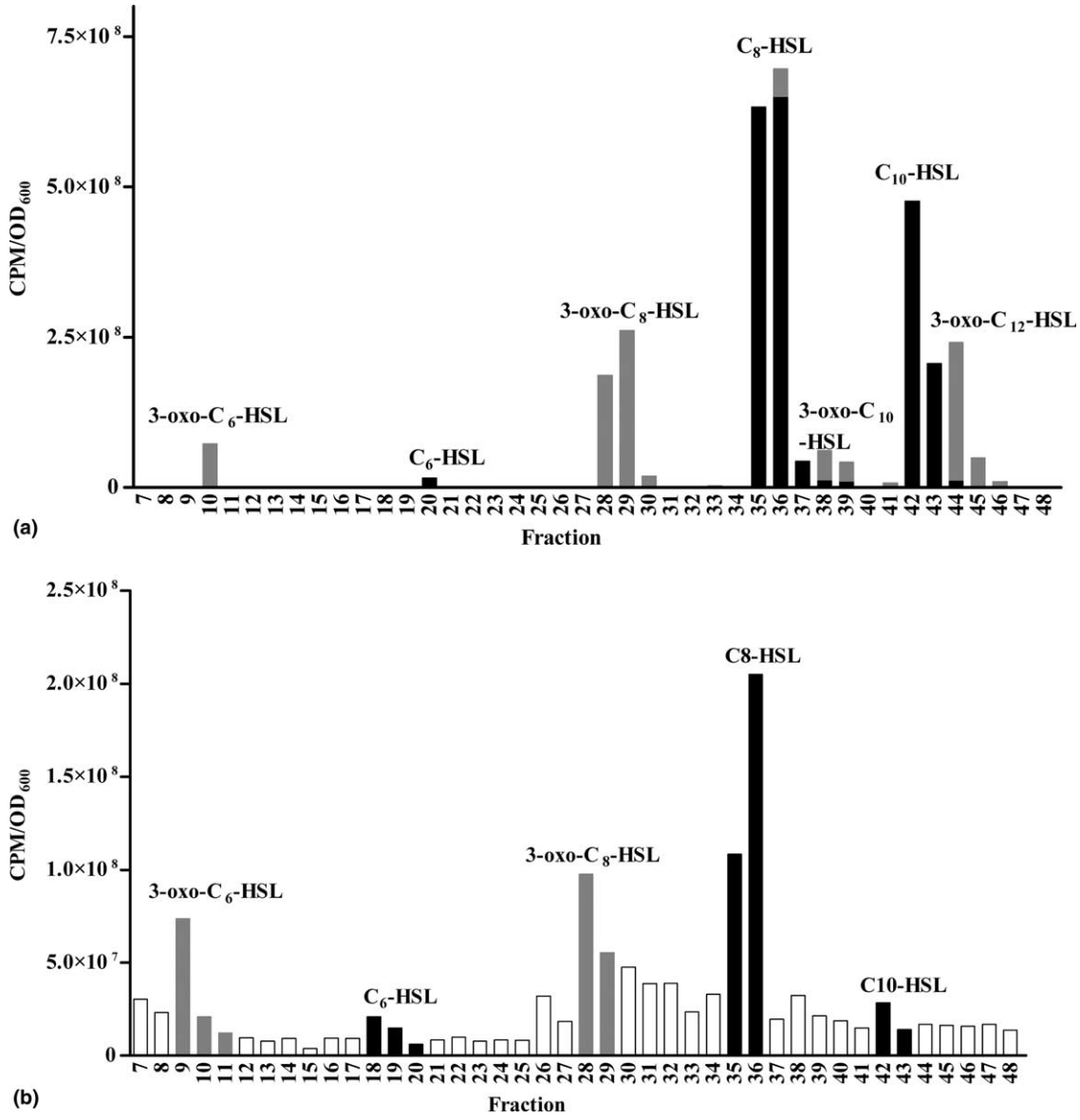

Fig. 1. Separation of AHLs by reverse-phase FPLC and detection using A136 (pCF218)(pMV26). (a) Separation of AHL standards. Mixtures of 3-oxo substituted AHLs and unsubstituted AHLs were chromatographed and assayed separately. The amounts of each AHL applied to the column were as follows: 25 nmol C_6-HSL, 7.5 nmol C_8-HSL, 75 nmol C_{10}-HSL, 75 nmol C_{12}-HSL, 100 pmol 3-oxo-C_6-HSL, 10 pmol 3-oxo-C_8-HSL, 10 pmol 3-oxo-C_{10}-HSL, and 10 pmol 3-oxo-C_{12}-HSL. One milliliter fractions were collected and 5 μl and 50 μl of the 3-oxo-substituted and unsubstituted AHLs, respectively, were assayed using the lux reporter. (b) Separation of extract from mucopurulent respiratory secretions from specimen 41. Fractions containing activity at least twice that of the background were considered positive for AHL activity. Using these criteria, C_{10}-HSL, C_8-HSL, C_6-HSL, 3-oxo-C_8-HSL, and 3-oxo-C_6-HSL were identified in the sample shown. In both panels the grey bars represent the 3-oxo-substituted AHLs, the black bars represent unsubstituted AHLs, and the open bars are used to indicate fractions that did not have activity greater than twice that of the background obtained with the buffers used to generate the acetonitrile gradients.

assay described in the present study when used in combination with reverse phase FPLC is able to detect, identify and quantitate small amounts of AHLs in clinical specimens. With the exception of C_4-HSL, this system may be used to identify individual AHLs in respiratory secretions and likely other samples from CF patients.

Since it is possible for AHL degradation products and some non-AHL molecules to activate *traI*-based reporters, it is important to use this reporter in conjunction with careful measurement of retention times of AHL standards on reverse phase FPLC. Although definitive identification of AHLs would require mass spectrometry analysis, this has previously been shown to require significantly higher volumes of clinical specimens [22].

Singh et al. [34] reported that C_4-HSL was present in sputum in higher concentrations than 3-oxo-C_{12}-HSL

Table 3
Detection of AHLs in mucopurulent respiratory secretions from cystic fibrosis patients at the time of lung transplant[a]

Sample	*B. cepacia* complex[b]	3-Oxo-C_6-HSL (pM)	3-Oxo-C_8-HSL (pM)	3-Oxo-C_{10}-HSL (pM)	3-Oxo-C_{12}-HSL (pM)	C_6-HSL (nM)	C_8-HSL (nM)	C_{10}-HSL (nM)
32	–	0	0	0	0	0	0	0
34	–	0	0.9	4.8	1.9	0	4.7	11.9
35	–	0	0	0	0.8	0	0	26.0
36	–	0	2.6	2.5	1.5	0	5.3	28.0
37	*B. cenocepacia*	0	0	2.4	0	0	7.0	0
38	*B. multivorans*	0	0	0	0	0	0	0
39	–	206	0	4.3	1.0	0	4.2	66.5
40	*B. cenocepacia*	0	0	0	0	0	0	0
41	*B. cenocepacia*	389	5.0	0	0	88	8.3	47.1
42	*B. cenocepacia*	0	0	30.1	6.3	0	22.7	0
44	–	0	0	0	0	0	0	0
45	–	0	6.3	29.3	50	0	0	0
46	–	0	0	1.6	0.5	0	0	0

[a] The concentration of each AHL present was estimated by comparing the cpm to that obtained using a standard curve of known concentrations of the respective AHL.
[b] Corresponding sputum culture results. All patients except for #41 also had *P. aeruginosa* cultured from their sputum.

using a radiometric technique. It is difficult to compare the sensitivity of the radiometric assay to the *traI–luxCDBAE* reporter system since the actual amounts of the two AHLs detected were not reported, although presumably the radiometric assay would detect lower amounts of C_4-HSL than the *traI–luxCDBAE* reporter assay.

Most studies to detect *P. aeruginosa* AHLs in vivo have focused on the detection of 3-oxo-C_{12}-HSL and C_4-HSL using reporters that are not completely specific for these AHLs. In our study, we determined that C_8-HSL and C_{10}-HSL were present in at least 100-fold higher amounts in the respiratory secretions than either 3-oxo-C_{12}-HSL or the other 3-oxo-derivatives. In one sputum sample in which the AHL profile was determined, Erickson et al. [23] found significant amounts of 3-oxo-C_8-HSL and 3-oxo-C_{10}-HSL. Middleton et al. [22] reported that the predominant AHL present in one sputum sample was C_6-HSL, which would also be detected by the same reporters used to detect C_4-HSL. These studies together suggest that it is important to identify specific AHLs present in clinical specimens rather than relying on reporters that detect a range of AHLs and interpreting the data obtained based on the predominant AHLs produced in vitro. In our study, seven different AHLs were identified in the nine positive specimens.

The ratio of C_4-HSL to 3-oxo-C_{12}-HSL has been reported to be higher in sputum [34] and lung tissue samples [25] than in vitro broth cultures, although similar ratios have been found in biofilm cultures [34]. Differences in AHL profiles in vivo compared to broth cultures may be due to differences in growth conditions as suggested by Singh et al. [34], differences in substrate availability for the AHL synthases, or the degradation of AHLs by human airway epithelial cells [35]. Unfortunately, the *P. aeruginosa* and *B. cepacia* complex strains previously cultured from the sputa of patients in this study were not available for comparison of their in vitro AHL profiles.

Interestingly, we did not detect any AHLs in specimen #40 taken at the time of autopsy from a patient colonized with *B. cenocepacia* and diagnosed with cepacia syndrome, which is characterized by high fever, severe respiratory failure and bacteremia infection [36]. Although this sample was taken within hours of death, it is possible that AHLs might have degraded. It is also possible that *B. cenocepacia* had spread to the blood or liver and that the numbers of *B. cenocepacia* in the lung at the time of death were not significant. *B. cenocepacia* has been shown to be invasive in both wild type and $cftr^{(-/-)}$ mice [27,37] as well as CF lung tissues [38].

The number of AHLs detected in the mucopurulent respiratory secretions ranged from two to five in the positive samples, regardless of whether the patients were infected with *P. aeruginosa* and/or *B. cenocepacia*. The ability of *P. aeruginosa* and *B. cenocepacia* to respond to AHLs produced by the other species has been demonstrated in vitro and in experimental infections [28,39]. Our studies suggest that interspecies communication could certainly occur in vivo during infection in CF patients since C8-HSL and C10-HSL are the predominant AHLs detected in these specimens and both *P. aeruginosa* and *B. cenocepacia* can respond to these AHLs to regulate virulence factor genes. *B. cenocepacia* CepR is most responsive to these two AHLs [28] and therefore, prior colonization of the lungs by *P. aeruginosa* may provide an advantage to *B. cenocepacia* in that AHLs needed to regulate specific virulence factors may already be present in the respiratory secretions.

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References

[1] Lyczak, J.B., Cannon, C.L. and Pier, G.B. (2002) Lung infections associated with cystic fibrosis. Clin. Microbiol. Rev. 15, 194–222.
[2] Ratjen, F. and Doring, G. (2003) Cystic fibrosis. Lancet 361, 681–689.
[3] Hutchison, M.L. and Govan, J.R. (1999) Pathogenicity of microbes associated with cystic fibrosis. Microbes Infect. 1, 1005–1014.
[4] Mahenthiralingam, E., Baldwin, A. and Vandamme, P. (2002) *Burkholderia cepacia* complex infection in patients with cystic fibrosis. J. Med. Microbiol. 51, 533–538.
[5] Miller, M.B. and Bassler, B.L. (2001) Quorum sensing in bacteria. Annu. Rev. Microbiol. 55, 165–199.
[6] Pesci, E.C., Milbank, J.B., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P. and Iglewski, B.H. (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 96, 11229–11234.
[7] Winson, M.K., Camara, M., Latifi, A., Foglino, M., Chhabra, S.R., Daykin, M., Bally, M., Chapon, V., Salmond, G.P. and Bycroft, B.W., et al. (1995) Multiple *N*-acyl-L-homoserine lactone signal molecules regulate production of virulence determinants and secondary metabolites in *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA 92, 9427–9431.
[8] Pearson, J.P., Gray, K.M., Passador, L., Tucker, K.D., Eberhard, A., Iglewski, B.H. and Greenberg, E.P. (1994) Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. Proc. Natl. Acad. Sci. USA 91, 197–201.
[9] De Kievit, T.R., Gillis, R., Marx, S., Brown, C. and Iglewski, B.H. (2001) Quorum-sensing genes in *Pseudomonas aeruginosa* biofilms: their role and expression patterns. Appl. Environ. Microbiol. 67, 1865–1873.
[10] Wagner, V.E., Bushnell, D., Passador, L., Brooks, A.I. and Iglewski, B.H. (2003) Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. J. Bacteriol. 185, 2080–2095.
[11] Schuster, M., Lostroh, C.P., Ogi, T. and Greenberg, E.P. (2003) Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. J. Bacteriol. 185, 2066–2079.
[12] Lewenza, S., Conway, B., Greenberg, E.P. and Sokol, P.A. (1999) Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI. J. Bacteriol. 181, 748–756.
[13] Lutter, E., Lewenza, S., Dennis, J.J., Visser, M.B. and Sokol, P.A. (2001) Distribution of quorum-sensing genes in the *Burkholderia cepacia* complex. Infect. Immun. 69, 4661–4666.
[14] Baldwin, A., Sokol, P.A., Parkhill, J. and Mahenthiralingam, E. (2004) The *Burkholderia cepacia* epidemic strain marker is part of a novel genomic island encoding both virulence and metabolism-associated genes in *Burkholderia cenocepacia*. Infect. Immun. 72, 1537–1547.
[15] Gotschlich, A., Huber, B., Geisenberger, O., Togl, A., Steidle, A., Riedel, K., Hill, P., Tummler, B., Vandamme, P., Middleton, B., Camara, M., Williams, P., Hardman, A. and Eberl, L. (2001) Synthesis of multiple *N*-acylhomoserine lactones is wide-spread among the members of the *Burkholderia cepacia* complex. Syst. Appl. Microbiol. 24, 1–14.
[16] Lewenza, S. and Sokol, P.A. (2001) Regulation of ornibactin synthesis and *N*-acyl-L-homoserine lactone production by CepR in *Burkholderia cepacia*. J. Bacteriol. 183, 2212–2218.
[17] Aguilar, C., Friscina, A., Devescovi, G., Kojic, M. and Venturi, V. (2003) Identification of quorum-sensing-regulated genes of *Burkholderia cepacia*. J. Bacteriol. 185, 6456–6462.
[18] Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S. and Eberl, L. (2001) The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. Microbiology 147, 2517–2528.
[19] Riedel, K., Arevalo-Ferro, C., Reil, G., Gorg, A., Lottspeich, F. and Eberl, L. (2003) Analysis of the quorum-sensing regulon of the opportunistic pathogen *Burkholderia cepacia* H111 by proteomics. Electrophoresis 24, 740–750.
[20] Conway, B.A., Venu, V. and Speert, D.P. (2002) Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J. Bacteriol. 184, 5678–5685.
[21] Wu, H., Song, Z., Hentzer, M., Andersen, J.B., Heydorn, A., Mathee, K., Moser, C., Eberl, L., Molin, S., Hoiby, N. and Givskov, M. (2000) Detection of *N*-acylhomoserine lactones in lung tissues of mice infected with *Pseudomonas aeruginosa*. Microbiology 146 (Pt. 10), 2481–2493.
[22] Middleton, B., Rodgers, H.C., Camara, M., Knox, A.J., Williams, P. and Hardman, A. (2002) Direct detection of *N*-acylhomoserine lactones in cystic fibrosis sputum. FEMS Microbiol. Lett. 207, 1–7.
[23] Erickson, D.L., Endersby, R., Kirkham, A., Stuber, K., Vollman, D.D., Rabin, H.R., Mitchell, I. and Storey, D.G. (2002) *Pseudomonas aeruginosa* quorum-sensing systems may control virulence factor expression in the lungs of patients with cystic fibrosis. Infect. Immun. 70, 1783–1790.
[24] Ward, C., Camara, M., Forrest, I., Rutherford, R., Pritchard, G., Daykin, M., Hardman, A., de Soyza, A., Fisher, A.J., Williams, P. and Corris, P.A. (2003) Preliminary findings of quorum signal molecules in clinically stable lung allograft recipients. Thorax 58, 444–446.
[25] Favre-Bonte, S., Pache, J.C., Robert, J., Blanc, D., Pechere, J.C. and van Delden, C. (2002) Detection of *Pseudomonas aeruginosa* cell-to-cell signals in lung tissue of cystic fibrosis patients. Microb. Pathog. 32, 143–147.
[26] Collier, D.N., Anderson, L., McKnight, S.L., Noah, T.L., Knowles, M., Boucher, R., Schwab, U., Gilligan, P. and Pesci, E.C. (2002) A bacterial cell to cell signal in the lungs of cystic fibrosis patients. FEMS Microbiol. Lett. 215, 41–46.
[27] Sokol, P.A., Sajjan, U., Visser, M.B., Gingues, S., Forstner, J. and Kooi, C. (2003) The CepIR quorum sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections. Microbiology 149, 3649–3658.
[28] Lewenza, S., Visser, M.B. and Sokol, P.A. (2002) Interspecies communication between *Burkholderia cepacia* and *Pseudomonas aeruginosa*. Can. J. Microbiol. 48, 707–716.
[29] Zhu, J., Beaber, J.W., More, M.I., Fuqua, C., Eberhard, A. and Winans, S.C. (1998) Analogs of the autoinducer 3-oxooctanoyl-homoserine lactone strongly inhibit activity of the TraR protein of *Agrobacterium tumefaciens*. J. Bacteriol. 180, 5398–5405.
[30] Fuqua, C. and Winans, S.C. (1996) Conserved cis-acting promoter elements are required for density-dependent transcription of *Agrobacterium tumefaciens* conjugal transfer genes. J. Bacteriol. 178, 435–440.
[31] Fuqua, W.C. and Winans, S.C. (1994) A LuxR–LuxI type regulatory system activates *Agrobacterium* Ti plasmid conjugal transfer in the presence of a plant tumor metabolite. J. Bacteriol. 176, 2796–2806.

[32] Bjarnason, J., Southward, C.M. and Surette, M.G. (2003) Genomic profiling of iron-responsive genes in *Salmonella enterica* serovar typhimurium by high-throughput screening of a random promoter library. J. Bacteriol. 185, 4973–4982.
[33] Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, Jr., J.E., Rinehart, K.L. and Farrand, S.K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. Proc. Natl. Acad. Sci. USA 94, 6036–6041.
[34] Singh, P.K., Schaefer, A.L., Parsek, M.R., Moninger, T.O., Welsh, M.J. and Greenberg, E.P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms. Nature 407, 762–764.
[35] Chun, C.K., Ozer, E.A., Welsh, M.J., Zabner, J. and Greenberg, E.P. (2004) Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Proc. Natl. Acad. Sci. USA 101, 3587–3590.
[36] Isles, A., Maclusky, I., Corey, M., Gold, R., Prober, C., Fleming, P. and Levison, H. (1984) *Pseudomonas cepacia* infection in cystic fibrosis: an emerging problem. J. Pediatr. 104, 206–210.
[37] Cieri, M.V., Mayer-Hamblett, N., Griffith, A. and Burns, J.L. (2002) Correlation between an in vitro invasion assay and a murine model of *Burkholderia cepacia* lung infection. Infect. Immun. 70, 1081–1086.
[38] Sajjan, U., Corey, M., Humar, A., Tullis, E., Cutz, E., Ackerley, C. and Forstner, J. (2001) Immunolocalisation of *Burkholderia cepacia* in the lungs of cystic fibrosis patients. J. Med. Microbiol. 50, 535–546.
[39] Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S. and Eberl, L. (2001) *N*-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiology 147, 3249–3262.