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# *N*-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms

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***Pseudomonas aeruginosa* and *Burkholderia cepacia* are capable of forming mixed biofilms in the lungs of cystic fibrosis patients. Both bacteria employ quorum-sensing systems, which rely on *N*-acylhomoserine lactone (AHL) signal molecules, to co-ordinate expression of virulence factors with the formation of biofilms. As both bacteria utilize the same class of signal molecules the authors investigated whether communication between the species occurs. To address this issue, novel Gfp-based biosensors for non-destructive, *in situ* detection of AHLs were constructed and characterized. These sensors were used to visualize AHL-mediated communication in mixed biofilms, which were cultivated either in artificial flow chambers or in alginate beads in mouse lung tissue. In both model systems *B. cepacia* was capable of perceiving the AHL signals produced by *P. aeruginosa*, while the latter strain did not respond to the molecules produced by *B. cepacia*. Measurements of extracellular proteolytic activities of defined quorum-sensing mutants grown in media complemented with AHL extracts prepared from culture supernatants of various wild-type and mutant strains supported the view of unidirectional signalling between the two strains.**

**Keywords:** quorum sensing, cross-talk, intergeneric communication, cystic fibrosis

## INTRODUCTION

Cystic fibrosis (CF) is the most common inherited lethal disease among Caucasians. The genetic lesion in CF leads to impaired epithelial chloride ion transport. This, in turn, leads to the production of a sticky dehydrated mucus in the ducts of exocrine glands, e.g. in the airways of the lungs. As a consequence, mucociliary and alveolar clearing are impaired and colonization of the lung epithelium by opportunistic bacterial pathogens leading to airway infections is facilitated. Early in life, CF patients are usually colonized by *Staphylococcus aureus* and non-capsulated *Haemophilus influenzae*, followed, later on, by mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*, which in most cases will establish chronic infections (Govan & Deretic, 1996; Tümmler & Kiewitz, 1999).

*P. aeruginosa* produces a wide variety of extracellular products, many of which contribute to its virulence. Expression of the majority of these virulence factors is not constitutive but is regulated in a cell-density-dependent manner. This form of gene regulation ensures that *P. aeruginosa* remains invisible to the immune system of the host until the opportunistic pathogen has reached a critical population density sufficient to overwhelm host defences and to establish the infection. Like many other Gram-negative bacteria, *P. aeruginosa* utilizes cell–cell communication systems that rely on

**Abbreviations:** AHL, *N*-acylhomoserine lactone; C4-, C6-, C8-, C10- and C-12-HSL, *N*-butanoyl, *N*-hexanoyl-, *N*-octanoyl, *N*-decanoyl- and *N*-dodecanoyl-L-homoserine lactone; 3-oxo-C10, 3-oxo-C12 and 3-oxo-C14-HSL, *N*-(3-oxodecanoyl)-, *N*-(3-oxododecanoyl)- and *N*-(3-oxotetradecanoyl)-L-homoserine lactone; CF, cystic fibrosis; CSLM, confocal scanning laser microscopy; DsRed, red fluorescent protein; Gfp, green fluorescent protein; QS, quorum sensing.

diffusible *N*-acylhomoserine lactone (AHL) signal molecules to monitor the size of the population in a process known as quorum sensing (for recent reviews see Van Delden & Iglewski, 1998; Williams *et al*., 2000; de Kievit & Iglewski, 2000). Typically, these communication systems depend on two proteins: an AHL synthase, usually a member of the LuxI family of proteins, and an AHL receptor protein, which belongs to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL via the activity of the AHL synthase. As the cell density increases, the diffusible AHL signal molecule accumulates in the growth medium. On reaching a critical threshold concentration, the AHL binds to the cognate LuxR-type receptor protein, which in turn leads to the induction/repression of target genes. Two quorum-sensing systems have been identified in *P. aeruginosa*: the *las* system, consisting of the transcriptional activator LasR and the AHL synthase LasI, which directs the synthesis of *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and the *rhl* system, consisting of RhlR and RhlI, which directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL). The two systems do not operate independently as the *las* system positively regulates expression of both *rhlR* and *rhlI*. Thus, the two quorum-sensing systems of *P. aeruginosa* are hierarchically arranged, with the *las* system being on top of the signalling cascade. In complex interplays with additional regulators, including Vfr, GacA, RsaL and RpoS, the quorum-sensing cascade regulates expression of a battery of extracellular virulence factors such as exoenzymes (elastase, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin) and toxins (exotoxin A). The importance of quorum sensing in the pathogenicity of *P. aeruginosa* has been demonstrated in a number of animal models including a *Caenorhabditis elegans* nematode model (Tan *et al*., 1999), the neonatal mouse model of pneumonia (Tang *et al*., 1996) and a burned mouse model (Rumbaugh *et al*., 1999). In all these animal models mutants defective in quorum sensing were substantially less virulent than the parent strains.

In recent years *B. cepacia* has emerged as another important pathogen in patients with CF (Govan & Deretic, 1996; Govan *et al*., 1996). In most cases infection with *B. cepacia* occurs in patients who are already colonized with *P. aeruginosa*. It has been suggested that *P. aeruginosa* produces an extracellular factor which modifies the epithelial cell surface of the lung in a way that facilitates attachment of *B. cepacia* (Saiman *et al*., 1990). Co-colonization can result in three clinical outcomes: asymptomatic carriage, slow and continuous decline in lung function, or, for approximately 20% of the patients, fulminant and fatal pneumonia, the so-called 'cepacia syndrome' (Isles *et al*., 1984).

Like *P. aeruginosa*, *B. cepacia* controls expression of various extracellular factors by an AHL-dependent quorum-sensing system, which consists of the AHL synthase CepI and the transcriptional regulator CepR (Lewenza *et al*., 1999; Gotschlich *et al*., 2001). The major signal molecule produced via CepI is *N*-octanoyl-L-homoserine lactone (C8-HSL). The *cep* system was shown to positively regulate production of extracellular proteolytic and chitinolytic activity and to repress synthesis of the siderophore ornibactin (Lewenza *et al*., 1999; Huber *et al*., 2001).

Recent work has presented strong evidence that *P. aeruginosa* cells exist as a biofilm in the CF lung (Singh *et al*., 2000). In the biofilm mode of growth the cells are embedded in a thick matrix of extracellular polymeric substances, can withstand host immune responses, and exhibit a dramatically increased resistance to antibiotics and biocides when compared to cells grown in liquid culture (Schierholz *et al*., 1999; Xu *et al*., 2000). Most interestingly, the formation of biofilms is a quorum-sensing-regulated process in *P. aeruginosa* as well as in *B. cepacia* (Davies *et al*., 1998; Huber *et al*., 2001). Both a *P. aeruginosa lasI* mutant and a *B. cepacia cepI* mutant form only flat and undifferentiated biofilms, suggesting that the respective quorum-sensing systems are in some way required for biofilm maturation. Importantly, the *P. aeruginosa lasI* mutant biofilm was also shown to be much more sensitive than the wild-type biofilm to the biocide sodium dodecyl sulfate.

During chronic co-infection *P. aeruginosa* and *B. cepacia* form mixed biofilms in the lungs of CF patients. Given that both bacteria utilize the same chemical language to control biofilm formation and expression of virulence factors it appears likely that not only are the two organisms capable of communicating with each other but that these interactions may also synergistically enhance the virulence of the consortium. In fact, McKenney *et al*. (1995) have shown that addition of spent culture supernatants of *P. aeruginosa* to the medium used for cultivation of *B. cepacia* enhances production of siderophores, lipase and protease of the latter species. The authors suggested that this stimulation of virulence factor production is caused by AHL molecules present in the *P. aeruginosa* supernatants.

In a recent study we analysed the AHL profiles, i.e. the types and amounts of AHL molecules, of sequential *P. aeruginosa* isolates from several chronically infected CF patients by TLC (Geisenberger *et al*., 2000). In one case the patient became transiently co-infected with an AHL-producing *B. cepacia* strain. During the co-infection period a dramatic reduction in the amounts of AHLs produced by the co-residing *P. aeruginosa* isolates was observed. However, 18 months after the last *B. cepacia*-positive sputum the initial *P. aeruginosa* AHL profile was regained. This observation led to the speculation that AHL-mediated cross-talk between the two pathogens may affect the virulence of the mixed consortium and that this change in pathogenic potential may in turn select for *P. aeruginosa* mutants producing lowered amounts of AHLs.

This study was initiated to investigate whether *P. aeruginosa* and *B. cepacia* are in fact capable of

communicating with each other using AHL signal molecules. Novel Gfp-based AHL sensor plasmids were constructed and these were used for *in situ* studies of cell–cell communication between the two organisms. Evidence is presented that in mixed biofilms intergeneric signalling only occurs in one direction, namely from *P. aeruginosa* to *B. cepacia*.

## METHODS

**Organisms and culture conditions.** *Escherichia coli*, *Burkholderia cepacia* and *Pseudomonas aeruginosa* strains used in this study are listed in Table 1. Strains were grown in modified Luria–Bertani medium (Bertani, 1951) containing 4 g NaCl $l^{-1}$ instead of 10 g NaCl $l^{-1}$ or ABt minimal medium [AB minimal medium (Clark & Maaløe, 1967) supplemented with 2·5 mg thiamin $l^{-1}$ and 1 mM glucose].

**AHL extraction and TLC.** AHL molecules were extracted with dichloromethane from culture supernatants (grown in minimal medium to an $OD_{600}$ of 1·0) and separated by TLC as described by Geisenberger *et al.* (2000). The AHL molecules were visualized by overlaying the TLC plates with soft agar seeded with the sensor strain *E. coli* MT102(pSB403) (Winson *et al.*, 1998). After overnight incubation at 30 °C bioluminescent spots were detected by exposure of an X-ray film. By comparing the obtained $R_F$ values of the spots with those of synthetic AHL standards a tentative identification of the AHLs was possible. Synthetic AHLs were either purchased from Fluka or were a generous gift from P. Williams, University of Nottingham, UK.

**Measurement of proteolytic activity.** Strains were grown overnight in LB medium in the presence or absence of 5 μM AHLs or dichloromethane extracts of spent culture supernatants from different strains. Proteolytic activity was measured as described by Ayora & Götz (1994). Briefly, 250 μl 2 % (w/v) azocasein (Sigma) in 50 mM Tris/HCl and 150 μl of the sterile filtered supernatants were incubated for 4 h at

***Table 1.*** Bacterial strains, plasmids and primers used in this study

| Strain, plasmid or primer | Relevant genotype and characteristics | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| MT102 | F$^-$ *thi araD139 ara–leu*Δ*7679* Δ(*lacIOPZY*) *galU gal'K* r$^-$ m$^+$ Sm$^R$ | T. Hansen, Novo Nordisk A/S |
| CC118 λpir | Δ(*ara–leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rps-1 rpoB argE*(Amp) *recA thi pro hsdRM*$^+$ RP4-2-Tc::Mu-Km::Tn*7* λ*pir* | Herrero *et al.* (1990) |
| ***P. aeruginosa*** | | |
| PAO1 | Wild-type *P. aeruginosa* | Holloway (1955) |
| PAO1-JP2 | *lasI rhlI* derivative of PAO1; Hg$^R$ Tc$^R$ | Pearson *et al.* (1997) |
| SH1 | Clinical isolate | Römling *et al.* (1994) |
| SH38 | Clinical isolate | Römling *et al.* (1994) |
| ***B. cepacia*** | | |
| H111 | Clinical isolate | Römling *et al.* (1994) |
| H111-I | *cepI* derivative of H111 | Huber *et al.* (2001) |
| **Plasmids** | | |
| pSB403 | Tc$^R$; broad-host-range AHL monitor plasmid | Winson *et al.* (1998) |
| pGEM-3Zf(+) | Ap$^R$; *lacZ*α, cloning vector | Promega |
| pBBR1MCS-5 | Gm$^R$; broad-host-range vector | Kovach *et al.* (1995) |
| pMHLAS | Ap$^R$ Gm$^R$; *Pseudomonas* shuttle vector carrying $P_{lasB}$–*gfp*(ASV) $P_{lac}$–*lasR* | Hentzer *et al.* (2002) |
| pKR-C12 | Gm$^R$; pBBR1MCS-5 carrying $P_{lasB}$–*gfp*(ASV) $P_{lac}$–*lasR* | This study |
| pAS-C8 | Gm$^R$; pBBR1MCS-5 carrying $P_{cepI}$–*gfp*(ASV) $P_{lac}$–*cepR* | This study |
| pUT-Tc-*dsred* | Ap$^R$ Tc$^R$; Tn5-based delivery plasmid, carrying $P_{lac}$-*dsred*-$T_0$-$T_1$ | Hentzer *et al.* (2002) |
| pUT-Tel-*dsred* | Ap$^R$ Tel$^R$; Tn5-based delivery plasmid, carrying $P_{lac}$-*dsred*-$T_0$-$T_1$ | M. Hentzer, unpublished |
| pUT-Gm-*dsred* | Ap$^R$ Gm$^R$; Tn5-based delivery plasmid, carrying $P_{lac}$-*dsred*-$T_0$-$T_1$ | M. Hentzer, unpublished |
| pRK600 | Cm$^R$; oriColE1 RK2-Mob$^+$ RK2-Tra$^+$; helper plasmid in triparental conjugations | Kessler *et al.* (1992) |
| **Primers** | | |
| cepI-fwd | 5′-CGGGATCCGACATCGGCATGTTGC-3′ | This study |
| cepI-rev | 5′-ACATGCATGCATGTCCTCGGATCTGTGC-3′ | This study |
| gfp(ASV)-fwd | 5′-ACATGCATGCGTAAAGGAGAAGAAC-3′ | This study |
| gfp(ASV)-rev | 5′-CCCAAGCTTATTAAACTGATGCAGC-3′ | This study |
| cepR-fwd | 5′-GGGGTACCGGATGAGCATGGAGAAAAGC-3′ | This study |
| cepR-rev | 5′-GGGGTACCAACCTGACAAGTATGACAGCG-3′ | This study |

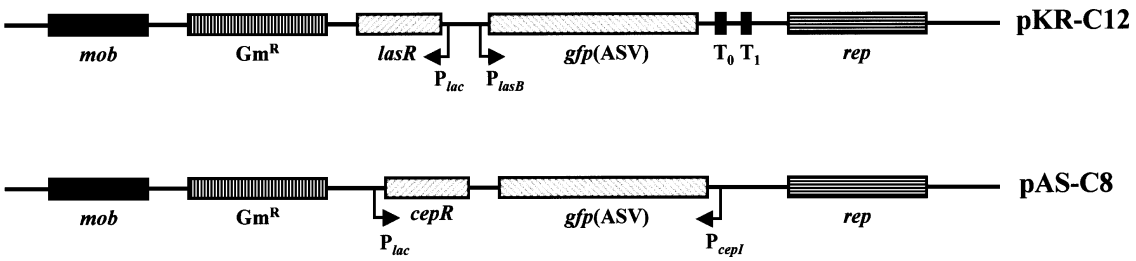


***Fig. 1.*** Schematic drawings (not to scale) of the *las*- and *cep*-based AHL sensor plasmids used in this study. Plasmid pKR-C12 contains a *lasB–gfp*(ASV) translational fusion together with the *lasR* gene placed under control of $P_{lac}$ on the broad-host-range plasmid pBBR1MCS-5. Plasmid pAS-C8 contains a *cepI–gfp*(ASV) translational fusion together with the *cepR* gene transcribed from the $P_{lac}$ promoter of the broad-host-range plasmid pBBR1MCS-5. The genetic components are: $P_{lasB}$, elastase promoter fragment of PAO1; *gfp*(ASV), gene encoding an unstable Gfp; $P_{cepI}$, promoter fragment of the C8-HSL synthase of H111; *lasR* and *cepR*, genes encoding the transcriptional activators LasR and CepR, respectively; $T_0$, transcriptional terminator from phage lambda; $T_1$, transcriptional terminator from *rrnB* operon of *E. coli*; $Gm^R$, gentamicin-resistance marker; *rep*, replication gene; *mob*, mobilization gene.

37 °C. After precipitation of undigested substrate with 1·2 ml 10 % (w/v) trichloroacetic acid for 15 min at room temperature, followed by 10 min centrifugation at 15 000 r.p.m., 1·4 ml 1 M NaOH was added to the supernatant. The absorbance ($A_{440}$) of the supernatant was measured and relative protease activities were calculated as $A_{440}/OD_{600}$.

**Plasmid and DNA manipulations.** Purification, cloning, electrophoresis, and other manipulations of DNA were performed using standard techniques (Sambrook *et al*., 1989). *P. aeruginosa* strains were tagged with the red fluorescent protein DsRed by inserting the hybrid transposon mini-Tn*5 dsred* into the chromosome of the strains using a three-factor mating procedure (Christensen *et al*., 1999). The plasmids used in this study are listed in Table 1.

The broad-host-range 3-oxo-C12-HSL-sensor plasmid pKR-C12 (Fig. 1) was constructed as follows. A *Not*I cassette, containing divergently transcribed $P_{lac}$–*lasR* and $P_{lasB}$–*gfp*(ASV) translational fusions, was excised from plasmid pMHLAS (Hentzer *et al*., 2002), blunt-ended with Klenow fragment, and inserted into the unique *Sma*I site of the broad-host-range vector pBBR1MCS-5. The *cep*-based sensor pAS-C8 was constructed in a three-step cloning procedure. A 344 bp DNA fragment containing the start codon and upstream region of *cepI* was PCR amplified using the primers cepI-fwd and cepI-rev and chromosomal DNA of *B. cepacia* H111 (GenBank accession no. AF330025) as template. Following digestion with *Sph*I and *Bam*HI the PCR fragment was ligated into the corresponding sites of pGEM, giving rise to pAS1. The *gfp*(ASV) gene was amplified using the primer pair gfp(ASV)-1 and gfp(ASV)-2, and plasmid pMHLAS as template. The resulting 759 bp DNA fragment was digested with *Sph*I and *Hin*dIII and ligated into pAS1 cut with the same enzymes, yielding pAS2. In this construct the *cepI* promoter is translationally fused to *gfp*(ASV) at the start codon. This cassette was inserted into the broad-host-range plasmid pBBR1MCS-5 via the restriction sites *Bam*HI and *Hin*dIII. Next, the *cepR* gene of *B. cepacia* H111 (GenBank accession no. AF330020), which encodes the cognate C8-HSL receptor protein, was amplified using the primers cepR-fwd and cepR-rev. The resulting PCR product was digested with *Kpn*I and cloned into the corresponding site of pAS2. One clone, in which the *cepR* gene was placed downstream of the *lac* promoter of the plasmid, was chosen. This plasmid, which was designated pAS-C8, contains a $P_{lac}$–*cepR* transcriptional fusion together with a $P_{cepI}$–*gfp*(ASV) translational fusion transcribed in the opposite direction.

Both sensor plasmids, pKR-C12 and pAS-C8, were transferred to *P. aeruginosa* and *B. cepacia* strains by triparental mating (Christensen *et al*., 1999).

**Characterization of AHL monitor strains.** To determine the specificity and sensitivity of the different AHL monitor strains respective overnight cultures were diluted fourfold into fresh LB medium, incubated 1 h at 30 °C and then distributed in 200 µl aliquots into wells of a microtitre plate. C4-HSL, C6-HSL, 3-oxo-C6-HSL, C8-HSL, C10-HSL, 3-oxo-C10-HSL, C12-HSL and 3-oxo-C12-HSL were added to the wells at final concentrations of 5000, 2500, 1250, 600, 400, 100 and 25 nM. Following 6 h of incubation at 30 °C, green fluorescence of the monitor strains was measured using the microtitre plate reader Lambda fluoro 320 Plus (MWG Biotech) with an excitation wave length of 474 nm and emission detection at 515 nm. Data were processed with the KC4 software (Bio-Tek Instruments). Detection limits are defined as minimal AHL concentrations giving rise to at least 30 % of the activity of fully induced cultures.

**Cross-streaking experiments.** The monitor strain and the respective test strains were streaked close to each other to form a T. Following 24 h incubation at 30 °C, the plates were illuminated with blue light using an HQ 480/40 filter (F44–001; AHF-Analysentechnik) in combination with a halogen lamp (Intralux 5000-1; Volpi) as a light source. Illumination took place in a darkbox that was equipped with a light-sensitive camera (C2400-40; Hamamatsu) with a Pentax CCTV camera lens and an HQ 535/20 filter (F42-001; AHF-Analysentechnik). The Argus 20 image analysis system (Hamamatsu) was used for detection and documentation of green fluorescent areas within the monitor strain streak.

**Flow-chamber experiments.** Surface-attached mixed-species biofilms were cultivated in artificial flow chambers (Møller *et al*., 1998) with channel dimensions of $1 \times 4 \times 40$ mm. The substratum consisted of a microscope coverslip (Knittel, $24 \times 50$ mm; Knittel Gläser) and the flow chambers were supplied with a flow of ABt minimal medium containing 1 mM glucose. The flow system was assembled and prepared as described previously (Møller *et al*., 1998). To cultivate mixed biofilms of *B. cepacia* and *P. aeruginosa*, flow chambers

were inoculated with 350 µl of a 1:1 mixture of exponentially growing cultures diluted to an $OD_{600}$ of 0·1 in 0·9% NaCl. After inoculation, the medium flow was arrested for 1 h to allow efficient colonization of the glass surface. Medium flow was then started and the substrate was pumped through the flow chamber at a constant rate of 0·2 mm $s^{-1}$ using a peristaltic pump (Watson Marlow 205S). After 24–72 h of cultivation at 30 °C, the mixed-species biofilms were inspected by confocal scanning laser microscopy (CSLM).

***In situ* hybridization of biofilms.** Embedding of mixed biofilms and *in situ* hybridization were performed as previously described (Christensen *et al*., 1999). Specific rRNA probes were used to visualize cells of *B. cepacia* and *P. aeruginosa*: Bcv13b (5′-GCTCATCCCATTTCGCTC-3′ – 23S rRNA) labelled with CY3 for H111, and Paa1448 (5′-GTAACCGT-CCCCCTTGCG-3′ – 16S rRNA) labelled with CY5 for SH1 and SH38.

**Microscopy and image analysis.** All microscopic observations and image acquisitions of biofilms were performed on a confocal scanning laser microsope (TCS4D; Leica Lasertechnik) equipped with a detector and a filterset for monitoring green fluorescent and red fluorescent protein. In addition, a reflection detector for bright-field images was installed. Images were obtained with a 63×/1·32 oil objective and image scanning was carried out with the 488 nm laser line from an Ar/Kr laser. Simulated fluorescence projections, and sections through the biofilms, were generated using the IMARIS software package (Bitplane) running on a Silicon Graphics Indigo 2 workstation. Images were further processed with the Photoshop software (Adobe).

**Animal experiments.** The mouse strain NMRI was obtained from the Panum Institute, Copenhagen University, Denmark. All animal experiments were performed after authorization from the National Animal Ethics Committee. Immobilization of *P. aeruginosa* and *B. cepacia* strains in seaweed alginate beads was performed as previously described (Wu *et al*., 2000). Cultures of *P. aeruginosa* and *B. cepacia* were adjusted to a concentration of $1{\cdot}0 \times 10^8$ c.f.u. $ml^{-1}$ and a 2:3 mixture of these suspensions was used for immobilization. Intratracheal challenge with 0·04 ml of alginate beads was performed as described by Moser *et al*. (1997). 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). The animals were killed 1, 3 and 7 d after challenge by administering 20% pentobarbital (DAK) at 2 ml per kg body weight. Freeze microtomy of the lung tissue and CSLM of the 40–50 µm thick lung sections were performed as described previously (Wu *et al*., 2000).

## RESULTS

### AHL profiles of strains used in this study

In a first step to assess the possibility of AHL-mediated intergeneric signalling between *P. aeruginosa* and *B. cepacia* we determined the AHL profiles of bacterial isolates from one CF patient who became co-infected with *B. cepacia* (Geisenberger *et al*., 2000). *P. aeruginosa* SH1, a strain that was isolated from the patient prior to co-infection with *B. cepacia*, produced large amounts of AHL molecules. Using the bioluminescent plasmid sensor pSB403 in combination with TLC six different AHL molecules could be detected. On the basis of their

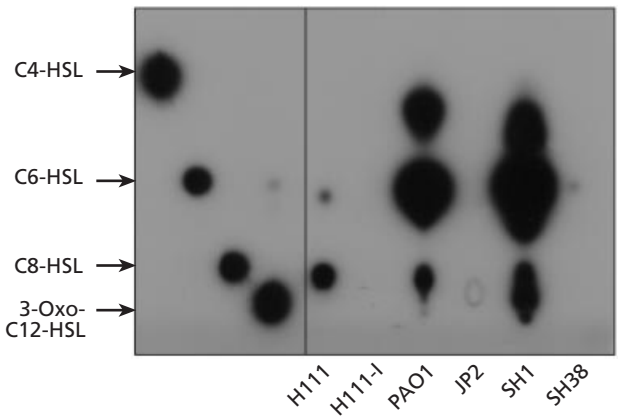


***Fig. 2.*** TLC analysis of AHLs produced by strains of *P. aeruginosa* and *B. cepacia* used in this study. Samples were chromatographed on $C_{18}$ reversed-phase thin-layer plates, developed with methanol/water (60:40, v/v) and spots were visualized by overlaying the TLC plates with *E. coli* MT102 harbouring the bioluminescent sensor plasmid pSB403. AHL standards were included as indicated.

mobilities ($R_F$ values) and by including appropriate reference compounds it was concluded that these molecules represent 3-oxo-C12-HSL, 3-oxo-C8-HSL, 3-oxo-C14-HSL, C6-HSL and C8-HSL (Fig. 2; Geisenberger *et al*., 2000). As a control we also included the well-characterized *P. aeruginosa* strain PAO1 in this analysis. For this strain a very similar AHL profile was determined, except that we were unable to detect 3-oxo-C14-HSL (Fig. 2) and that the levels of 3-oxo-C8-HSL and 3-oxo-C12-HSL were significantly lower than those of SH1. This result is in good agreement with previous studies that demonstrated that in PAO1 LasI directs the synthesis of primarily 3-oxo-C12-HSL together with small amounts of 3-oxo-C8-HSL and 3-oxo-C6-HSL (Pearson *et al*., 1994) and that RhlI directs the synthesis of C4-HSL and C6-HSL in a molar ratio of 15:1 (Winson *et al*., 1995). However, C4-HSL could not be detected by the aid of plasmid pSB403 since this AHL sensor is very insensitive to this signal molecule (Winson *et al*., 1998). The production of both C4-HSL and C6-HSL was easily visualized by the use of *Chromobacterium violaceum* CV026 as sensor (Geisenberger *et al*., 2000). This sensor is highly sensitive to unsubstituted short-chain AHL molecules but is quite insensitive to other AHLs (McClean *et al*., 1997).

In contrast to SH1, strain SH38, which was isolated during the co-infection period, only produced trace amounts of C6-HSL. This situation is similar to that found with the *lasI rhlI* double mutant PAO1-JP2 (Pesci *et al*., 1997), which, as expected, does not produce any AHL molecules.

The genomovar III *B. cepacia* strain H111 originates from the same patient from whom *P. aeruginosa* strains SH1 and SH38 were isolated. Strain H111 produces C8-

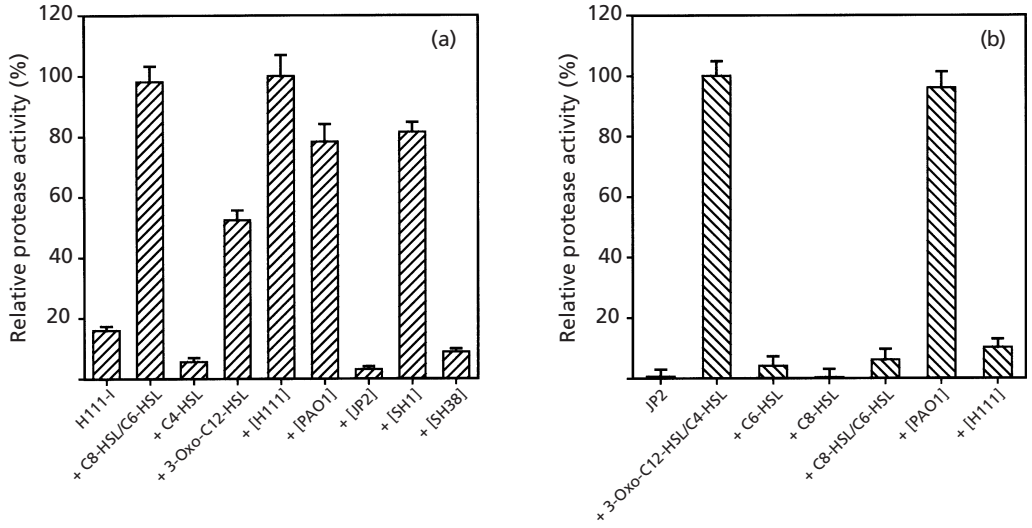


***Fig. 3.*** Proteolytic activities of culture supernatants of (a) *B. cepacia* H111-I and (b) *P. aeruginosa* PAO1-JP2. The strains were grown in the absence or presence of different AHL molecules (at concentrations of 1 μM) or of dichloromethane extracts of supernatants prepared from cultures of the *P. aeruginosa* strains PAO1, PAO1-JP2 and SH1, and the *B. cepacia* strains SH38 and H111 (addition of extracts of the different strains is indicated by the strain name in brackets). Sterile filtered supernatants were used for measurements of proteolytic activities. The data represent mean values of three independent experiments. Error bars represent the standard errors of the means.

HSL and C6-HSL in a molar ratio of approximately 10:1 (Fig. 2; Gotschlich *et al*., 2001). The AHL profile of this *B. cepacia* clone was found to remain unchanged during the entire co-infection period (data not shown). No AHLs were detected in the supernatants of a recently constructed *cepI* mutant of this strain, which was designated H111-I (Fig. 2; Huber *et al*., 2001).

These results show that *P. aeruginosa* SH1 and *B. cepacia* H111, which were isolated from the same patient at the onset of co-colonization, produce C6-HSL, although in both cases in relatively small amounts. However, given that LuxR-type AHL receptor proteins exhibit a considerable degree of flexibility, i.e. molecules that are similar but not identical to the natural AHL ligand are capable of activating the receptor, these data support the idea that in the case of the formation of mixed biofilms the quorum-sensing systems operating in the two bacteria may cross-react.

### *P. aeruginosa* supernatants restore protease production by *B. cepacia* H111-I

McKenney *et al*. (1995) showed that production of siderophores, lipase and protease of *B. cepacia* 10661 was stimulated when the growth medium was supplemented with concentrated spent culture supernatants of *P. aeruginosa* PAO1. They suggested that AHL molecules produced by PAO1 induce expression of extracellular products in *B. cepacia* 10661. However, the reported induction of exoproduct synthesis was relatively weak, being at best sevenfold for siderophore production. This only mild induction is, at least in part, due to the use of the *B. cepacia* wild-type strain, which itself produces AHLs, and thus complicates the analysis.

For a more detailed analysis we tested extracts of *P. aeruginosa* and *B. cepacia* wild-type strains for cross-stimulation of extracellular proteolytic activities of respective quorum-sensing-defective mutants. We chose to investigate effects on protease production as this phenotype is strictly regulated by quorum sensing in both bacteria (Passador *et al*., 1993; Lewenza *et al*., 1999). Accordingly, virtually no proteolytic activities were observed with the *cepI* mutant of *B. cepacia* or with the *lasI rhlI* double mutant of *P. aeruginosa* when compared with the wild-type strains (Fig. 3). Addition of a mixture of C8-HSL and C6-HSL (1 μM each) or extracts of H111 culture supernatants to the growth medium completely restored protease production of H111-I. The presence of 1 μM C4-HSL showed no effect, and the presence of 1 μM 3-oxo-C12-HSL partially complemented the defect. More importantly, extracts of the AHL-producing *P. aeruginosa* strains PAO1 and SH1 also restored protease production, while extracts of the AHL-negative strains PAO1-JP2 and SH38 did not. Conversely, extracts of H111 did not stimulate protease production of the *lasI rhlI* double mutant PAO1-JP2. Neither did the presence of C4-HSL, C8-HSL, C6-HSL, or a mixture of C8-HSL and C6-HSL, affect the results. Restoration of the defect was only observed when the medium was supplemented with 1 μM 3-oxo-C12-HSL, a mixture of 3-oxo-C12-HSL and C4-HSL (1 μM each), or an extract of the spent culture supernatant of PAO1. These data suggest that in principle *P. aeruginosa* and *B. cepacia* are capable of communicating with each other,

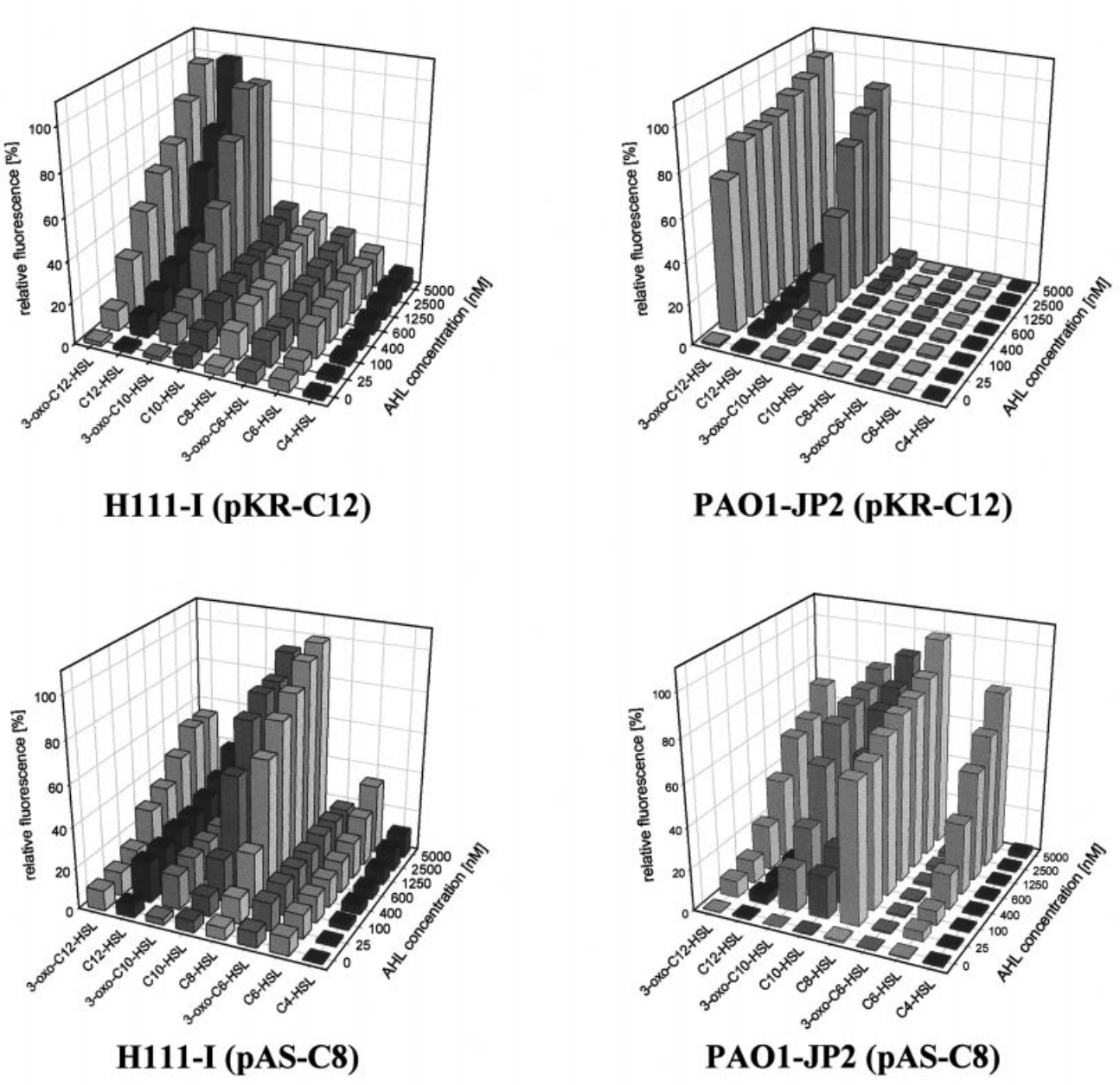


***Fig. 4.*** Characterization of the AHL monitor strains. The monitor strains were grown in the presence of different AHL compounds at concentrations of 0–5000 nM. Green fluorescence of the cultures was recorded by a microtitre plate reader at a wavelength of 515 nm. Maximal induction levels of the monitor strains were set to 100 %.

albeit in an unidirectional manner, i.e. while *B. cepacia* is capable of perceiving AHL molecules from *P. aeruginosa*, the latter organism cannot utilize the signals produced by *B. cepacia* for triggering quorum sensing.

### Construction and characterization of Gfp-based sensors for 3-oxo-C12-HSL and C8-HSL

To generate more direct evidence for communication between cells of *P. aeruginosa* and *B. cepacia* we constructed two Gfp-based AHL sensor plasmids as described in Methods (see also Fig. 1). The sensor plasmid pKR-C12 contains a translational fusion of the *lasB* elastase gene of *P. aeruginosa* to *gfp*(ASV), encoding an unstable version of the Gfpmut3* protein (Andersen *et al.*, 1998). Furthermore, the sensor contains the *lasR* gene, which encodes the cognate 3-oxo-C12-HSL receptor protein under control of a *lac*-type promoter. Since expression of *lasB* is controlled by the *las* quorum-sensing system, this sensor is expected to be most sensitive for 3-oxo-C12-HSL and related long-chain AHLs. The second sensor plasmid, pAS-C8, is based on the *cep* genes of *B. cepacia* and contains a translational *cepI–gfp*(ASV) fusion together with the *cepR* regulator

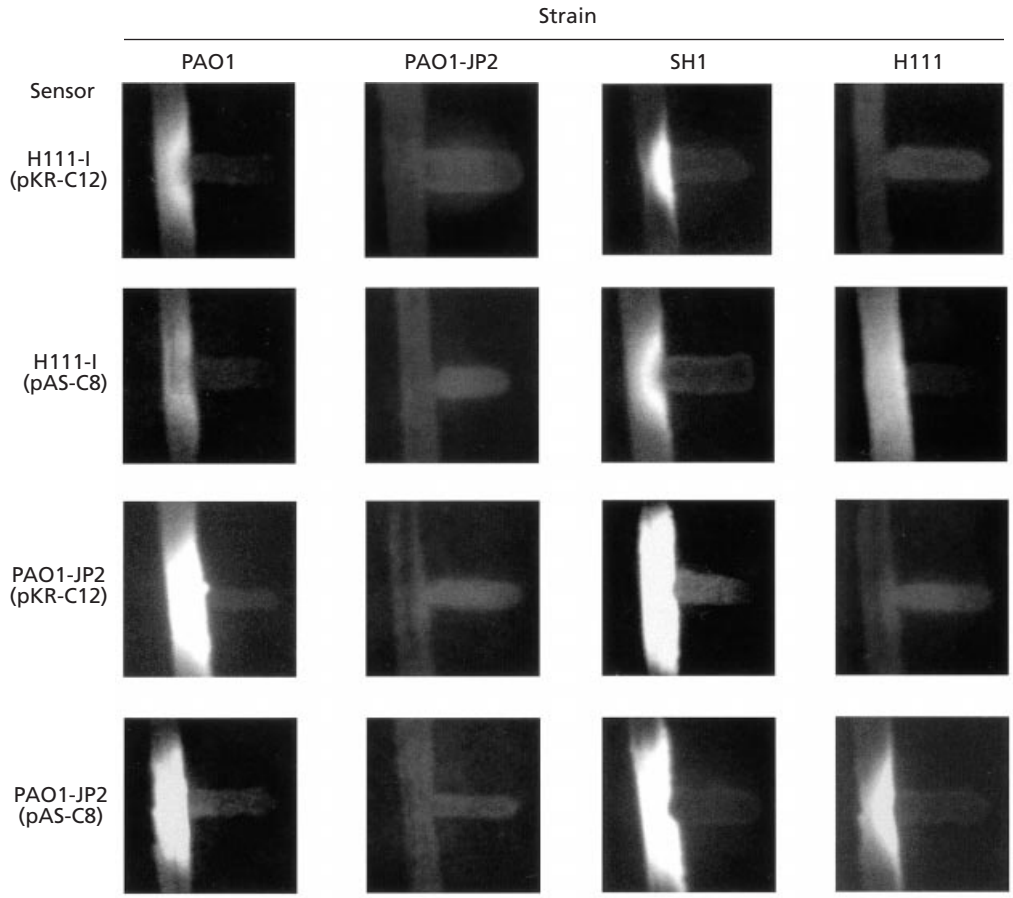


***Fig. 5.*** Activation of the AHL monitor strains in cross-streak experiments. The four monitor strains H111-I(pKR-C12), H111-I(pAS-C8), PAO1-JP2(pKR-C12), PAO1-JP2(pAS-C8) and the test strains PAO1, PAO1-JP2, SH1 and H111 were cross-streaked on LB agar plates as described in Methods. Following 24 h of incubation at 30 °C production of Gfp(ASV) by the monitor strains was visualized by exciting the plates with blue light.

gene placed under control of $P_{lac}$. Since expression of *cepI* is auto-regulated (Lewenza *et al*., 1999; Huber *et al*., 2001), this sensor plasmid should be most sensitive for C8-HSL.

The presence of the two sensor cassettes on the mobilizable broad-host-range vector pBBR1MCS-5 (Kovach *et al*., 1995) enabled us to transfer the constructs to the AHL-negative strains PAO1-JP2 and H111-I, giving rise to the four monitor strains PAO1-JP2(pKR-C12), PAO1-JP2(pAS-C8), H111-I(pKR-C12) and H111-I(pAS-C8). We next tested the performance of these monitor strains with respect to their sensitivity for different AHL molecules. This was accomplished by measuring Gfp fluorescence of cultures exposed to various AHL concentrations. As expected, H111-I(pKR-C12) and PAO1-JP2(pKR-C12) exhibited the highest sensitivity to 3-oxo-C12-HSL and both sensors responded well to 3-oxo-C10-HSL (Fig. 4). Interestingly, while H111-I(pKR-C12) also responded to C12-HSL, PAO1-JP2(pKR-C12) was very insensitive to this molecule. The two monitor strains based on plasmid pAS-C8 were highly sensitive to C8-HSL and C10-HSL. However, both sensors were also responsive to a variety of related molecules, albeit with reduced sensitivity (Fig. 4).

### Assessment of interspecies communication in 'cross-streaking' experiments

To test the different monitor strains for their applicability to visualize cell–cell communication *in situ*, the monitor strains were cross-streaked against the wild-type and mutant strains of *P. aeruginosa* and *B. cepacia*. In this simple assay AHL-mediated signalling can be monitored by the production of Gfp in the monitor strain. The results of these experiments are shown in Fig. 5. Strong green fluorescence was observed when SH1 or PAO1 was cross-streaked against the monitor strains H111-I(pKR-C12) and PAO1-JP2(pKR-C12). As expected, green fluorescence was somewhat weaker when PAO1 was cross-streaked against the monitor strains H111-I(pAS-C8) and PAO1-JP2(pAS-C8). On the other hand, when the *P. aeruginosa*-based monitor strains PAO1-JP2(pKR-C12) and PAO1-JP2(pAS-C8) were used to detect production of AHLs by H111, only the latter sensor gave rise to weak signals. Given that *lasB* is a quorum-sensing-regulated target gene, these

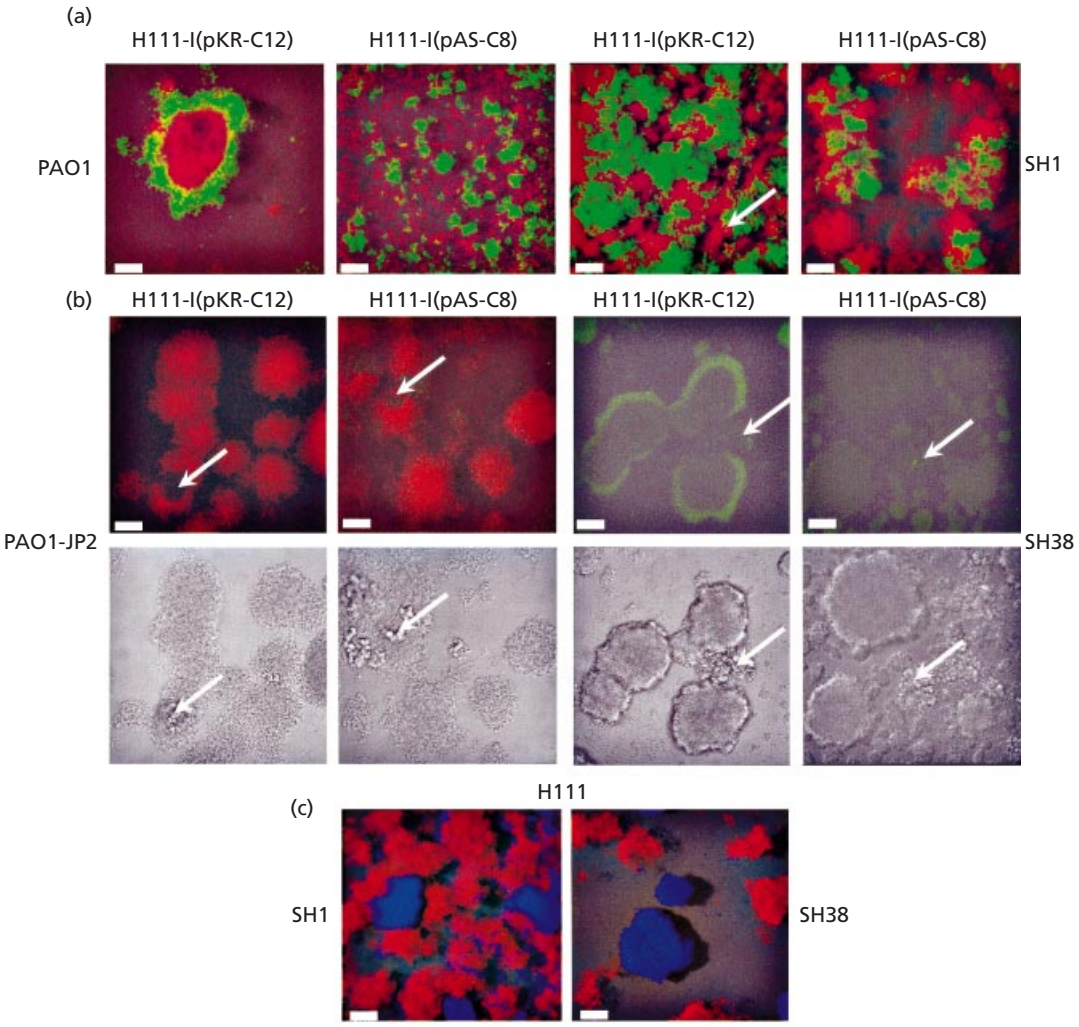


***Fig. 6.*** Intergeneric communication between *P. aeruginosa* and *B. cepacia* in biofilms cultured in flow chambers. (a) Mixed biofilms of H111-I harbouring the sensor plasmids pKR-C12 (left panel) or pAS-C8 (right panel), and *dsred*-tagged derivatives of PAO1 and SH1. Perception of AHL molecules by the *B. cepacia* monitor strain is indicated by the appearance of green fluorescent cells; the distribution of *P. aeruginosa* cells is visualized by their red fluorescence. Simulated fluorescence projections were generated by CSLM 72 h post-inoculation. (b) Mixed biofilms of H111-I harbouring the sensor plasmids pKR-C12 (left panel) or pAS-C8 (right panel), and *dsred*-tagged derivatives of PAO1-JP2 and the untagged but auto-fluorescent strain SH38. White arrows indicate microcolonies of the *B. cepacia* monitor strain. Upper panels show epifluorescence images; lower panels are reflection images of the same microscopic field. (c) CSLM photograph of mixed biofilms formed by *B. cepacia* H111 and *P. aeruginosa* SH1 (left panel) or SH38 (right panel). *B. cepacia* was hybridized with the CY3-labelled (red) probe bcv13b, and *P. aeruginosa* was hybridized with probe paa1448 labelled with CY5 (blue). Bars, 20 μm.

results indicate that PAO1 may be unable to respond to the AHL signal molecules produced by H111. This result is consistent with our observation that extracts of *B. cepacia* H111 do not affect protease production of PAO1-JP2, while extracts of PAO1 did restore protease production by H111-I.

### Visualization of intergeneric communication in biofilms grown in flow chambers

To investigate whether cell–cell communication would occur in mixed biofilms of *P. aeruginosa* and *B. cepacia* we used artificial flow chambers for culturing biofilms consisting of a monitor strain and an appropriate partner strain. Since our results suggest that *B. cepacia* is capable of responding to the AHLs produced by *P. aeruginosa* but not vice versa, we used the monitor strain H111-I(pKR-C12) in combination with different *P. aeruginosa* strains. To be able to easily monitor the *P. aeruginosa* strains in these experiments they were tagged with the red fluorescent protein DsRed. When mixed biofilms of H111-I(pKR-C12) and one of the AHL-producing strains, SH1 or PAO1, were inspected by CSLM bright green fluorescent cells were detected (Fig. 6a). By contrast, no green fluorescent *B. cepacia* cells were observed in mixed biofilms containing the AHL-

***Table 2.*** Intergeneric communication between *P. aeruginosa* and *B. cepacia* in mouse lung tissue

In each of experiments I–IV, NMRI female mice were challenged with mixtures of *B. cepacia* H111-I harbouring an AHL sensor plasmid and different *dsred*-tagged *P. aeruginosa* strains. Three mice were killed on each of days 1, 3 and 7 after infection, and CLSM was employed to inspect tissue samples for green fluorescent cells. ++, Strong induction; +, induction; −, no induction of the monitor strain.

| Expt | Bacterial strains inoculated (ratio 2:3 c.f.u. $ml^{-1}$) | Day | Gfp signal |
|---|---|---|---|
| I | SH1 (DsRed) + H111-I(pKR-C12) | 1 | + |
| | | 3 | ++ |
| | | 7 | ++ |
| II | PAO1-JP2 (DsRed) + H111-I(pKR-C12) | 1 | − |
| | | 3 | − |
| | | 7 | − |
| III | SH1 (DsRed) + H111-I(pAS-C8) | 1 | + |
| | | 3 | ++ |
| | | 7 | + |
| IV | PAO1-JP2 (DsRed) + H111-I(pAS-C8) | 1 | − |
| | | 3 | − |
| | | 7 | − |

negative strains PAO1-JP2 and SH38 (Fig. 6b). Similar results were obtained when the monitor strain H111-I(pAS-C8) was used instead of H111-I(pKR-C12) (Fig. 6a). These results show that in mixed biofilms *B. cepacia* is capable of perceiving the AHL signals produced by most, but not all, *P. aeruginosa* strains.

During the course of these experiments we further noticed that the various mixed biofilms investigated exhibited significant structural differences. In biofilms consisting of *B. cepacia* H111-I and AHL-producing *P. aeruginosa* strains the microcolonies of the two species were often closely associated (Fig. 6a) while in mixed consortia of H111-I and AHL-negative *P. aeruginosa* strains the microcolonies were more separated (Fig. 6b).

In a previous study it was shown that the *las* quorum-sensing system is directly involved in the regulation of biofilm formation (Davies *et al*., 1998). When compared with the wild-type, a *lasI* mutant of *P. aeruginosa* formed only flat and undifferentiated biofilms, suggesting that the *las* system is in some way required for the maturation of biofilms. However, we were unable to detect notable differences in the structures of single-species biofilms formed by PAO1 and PAO1-JP2 (data not shown). This apparent discrepancy may be attributable to the different media used in the two studies and/or to different experimental settings for growing biofilms.

To investigate the role of AHL production by *P. aeruginosa* in the structure of mixed biofilms in more detail we analysed the structures of biofilms formed by *B. cepacia* H111 and either the AHL producing *P. aeruginosa* strain SH1 or the AHL-negative strain SH38 (Fig. 6c). The spatial distribution of the bacteria in the consortium was investigated by CSLM after visualizing cells by fluorescent *in situ* hybridization. In biofilms formed by H111 and SH38 the two strains tended to grow in well-separated microcolonies. By contrast, in biofilms formed by H111 and SH1 the association of microcolonies was much tighter, and mixed microcolonies were observed, which were never observed in H111/SH38 biofilms. These results lend further support to the hypothesis that AHL production by *P. aeruginosa* plays an important role in determining the structure of the mixed consortium.

### Evidence for intergeneric cell–cell communication in the lung tissue of infected mice

By the use of alginate-entrapped *P. aeruginosa* cells chronic lung infections can be established in mice (Moser *et al*., 1997). This animal model has recently been used in combination with a Gfp-based AHL monitor strain to show that *P. aeruginosa* produces AHL signal molecules when colonizing the lung tissue (Wu *et al*., 2000). To investigate whether AHL-mediated communication between *P. aeruginosa* and *B. cepacia* occurs during the course of a co-infection, mice were challenged with alginate beads containing a *B. cepacia* monitor strain together with different *dsred*-tagged *P. aeruginosa* strains. The mice were killed on day 1, 3 and 7 post-intratracheal challenge and the lung tissue was inspected by CSLM. The results of these investigations are summarized in Table 2. When the monitor strains H111-I(pKR-C12) or H111-I(pAS-C8) were used for co-infection together with the AHL-producing *P. aeruginosa* strain SH1, bright green fluorescent cells were

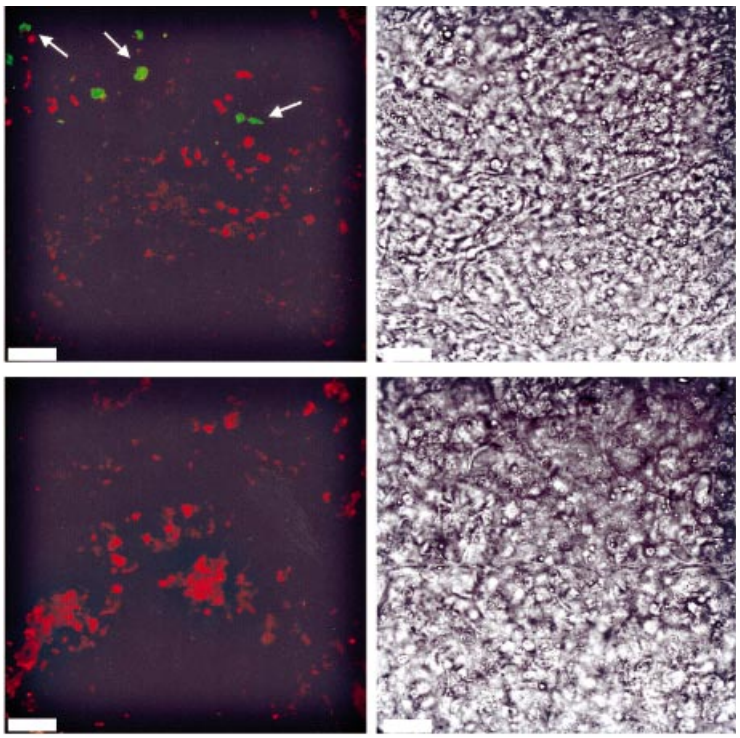

***Fig. 7.*** Intergeneric communication between *P. aeruginosa* and *B. cepacia* in mouse lung tissue. Simulated fluorescence projections generated by CSLM of mouse tissue infected with alginate beads containing a mixture of H111-I(pAS-C8) and *dsred*-tagged derivatives of SH1 (top panel) and PAO1-JP2 (bottom panel) 72 h post-inoculation. Reflection images of the same microscopic views are shown on the right. Perception of AHL molecules by the *B. cepacia* monitor strain is indicated by the appearance of green fluorescent cells (as indicated by white arrows); the distribution of *P. aeruginosa* cells is visualized by their red fluorescence. Bars, 20 μm.

detected in the lung tissues (Fig. 7). As expected, no green fluorescent cells were detected in cases where the *B. cepacia* monitor strains were used together with the AHL-negative strain PAO1-JP2. These data provide strong evidence that unidirectional signalling between *P. aeruginosa* and *B. cepacia* does in fact occur during co-infection of mouse lung tissue.

## DISCUSSION

Over the past few years evidence has emerged that quorum sensing is a common phenomenon in bacteria. To date, a large number of highly diverse signal molecules have been identified that are utilized by bacteria to sense their own population densities. Among Gram-negative bacteria, the most intensively investigated and probably the most widespread signal molecules are AHLs (for reviews see Swift *et al*., 1994; Fuqua *et al*., 1996; Salmond *et al*., 1995; Hardman *et al*., 1998; Eberl, 1999). Given that the vast majority of Gram-negative bacteria are capable of producing AHLs and that they often colonize the same environmental niche it is very tempting to assume that AHL signal molecules are used not only as cell-density sensors of one species but also for communication between cells of different species. A clinically highly relevant example of such a scenario is represented by mixed biofilms of *P. aeruginosa* and *B. cepacia* in the lungs of some CF patients. In most of these cases, the persons are already chronically colonized by *P. aeruginosa* before they become co-infected with *B. cepacia* (Govan & Deretic, 1996; Tümmler & Kiewitz, 1999). As both species employ AHL-dependent quorum-sensing systems to link biofilm formation with the expression of pathogenic traits (Davies *et al*., 1998; Parsek & Greenberg, 1999; Huber *et al*., 2001), intergeneric communication by the aid of AHLs may be of profound importance for the virulence of the mixed consortium.

To investigate whether AHL-mediated communication between *P. aeruginosa* and *B. cepacia* in mixed biofilms does occur we constructed two novel Gfp-based sensor plasmids, which are suitable for visualizing intergeneric communication at the single-cell level. The two plasmids respond to different spectra of AHL molecules, depending on the components used for their construction. The sensor plasmid pKR-C12 is based on components of the *P. aeruginosa* PAO1 *las* system and contains a *lasB–gfp*(ASV) translational fusion together with the *lasR* gene placed under control of $P_{lac}$ on the broad-host-range plasmid pBBR1MCS-5 (Fig. 1). In good agreement with previous studies (Passador *et al*., 1993; Hentzer *et al*., 2002) this plasmid sensor exhibited the highest sensitivity for 3-oxo-C12-HSL irrespective of whether *P. aeruginosa* PAO1-JP2 or *B. cepacia* H111-I was used as host. However, the strain background had a significant effect on the detection limits. In the *P. aeruginosa* background we determined a detection limit for 3-oxo-C12-HSL of less than 10 nM for single cells (assessed by epifluorescence microscopy) and of less than 25 nM for measurements in microtitre assays, while the respective detection limits in the *B. cepacia* background were approximately 50 nM and 100 nM, respectively. Noteworthy in this context is the fact that an unstable variant of Gfp, Gfp(ASV) (Andersen *et al*., 1998), was used for the construction of the sensor to enable detection of transient bacterial communication. This Gfp variant carries a C-terminal peptide tag, which

makes the protein prone to degradation by housekeeping/intracellular tail-specific proteases (Clp). As constitutive expression of Gfp(ASV) from a $P_{lac}$-type promoter also results in significantly lower fluorescence levels in *B. cepacia* than in *P. aeruginosa* (unpublished result) we speculate that the Gfp variant may have a shorter half-life in *B. cepacia*.

With slightly reduced sensitivities both monitor strains also responded to 3-oxo-C10-HSL, but only in the *B. cepacia* background was the sensor stimulated by C12-HSL. At present the reason for this strain difference is unclear. Recent investigations concerning the uptake and efflux of 3-oxo-C12- and C4-HSL in *P. aeruginosa* revealed that cells are only freely permeable for the short-chain AHL. The long-chain AHL 3-oxo-C12-HSL was shown to be actively transported out of the cells by the *P. aeruginosa* MexAB–OprM multidrug efflux system (Evans *et al*., 1998; Pearson *et al*., 1999). Thus, variations in the presence and/or specificity of long-chain AHL transporters in the two strains may account for the observed strain-dependent difference in sensitivity for C10-HSL. Alternatively, variations in the copy number of the sensor plasmid in the two strains may be responsible for the different sensitivities for C10-HSL, as it has been demonstrated previously that the amount of TraR protein present in the cell determines the range of AHL molecules that are bound by the receptor (Zhu *et al*., 1998).

For the sensitive detection of C8-HSL we constructed a novel AHL sensor plasmid, designated pAS-C8, which is based on components of the *cep* quorum-sensing system of *B. cepacia* H111. This plasmid contains a *cepI–gfp*(ASV) translational fusion together with the *cepR* gene, which is transcribed from the $P_{lac}$ promoter of the broad-host-range plasmid pBBR1MCS-5. As expected, this sensor plasmid responded most efficiently to C8-HSL, with a lower detection limit of less than 5 nM for single-cell analysis and of less than 25 nM in microtitre plate assays when the sensor plasmid was present in the *P. aeruginosa* PAO1-JP2 background. As for pKR-C12, the detection limits were higher in the *B. cepacia* H111-I background, namely 50 nM and 150 nM, respectively. The sensor was, with a lower efficiency, also stimulated by related molecules including C6- and C10-HSL. In contrast to pKR-C12, the strain background did not significantly influence the spectrum of AHL molecules detected by the sensor plasmid.

The AHL sensor plasmid pAS-C8, which is most sensitive for AHL molecules with a $C_8$ acyl side chain, nicely extends the range of AHLs that can currently be detected with the aid of Gfp-based AHL sensors. Beside *las*-based sensors, which are particularly suitable for detection of long-chain AHLs (Hentzer *et al*., 2002; this study), sensors for the detection of short-chain AHLs have been described. These sensors are based on components of the *lux* quorum-sensing system of *Vibrio fischeri* and were demonstrated to be highly sensitive for 3-oxo-C6-HSL (with a detection limit of 5 nM in an *E. coli* background) and other short-chain AHLs (Andersen *et al*., 2001). This series of Gfp-based sensors may prove to be highly valuable molecular tools for *in situ* visualization of AHL-mediated communication between individual bacterial cells in various natural habitats. Previously, we have used these sensors for detection of AHL signal molecules in the lung tissues of mice infected with *P. aeruginosa* (Wu *et al*., 2000), for visualization of interspecies communication in swarming colonies of *Serratia liquefaciens* (Eberl *et al*., 1999; Andersen *et al*., 2001), and for the analysis of quorum-sensing inhibition by halogenated furanone compounds in *P. aeruginosa* biofilms (Hentzer *et al*., 2002).

In this study we employed the C8- and 3-oxo-C12-HSL specific sensors to investigate the possibility of inter-generic communication between clinical isolates of *P. aeruginosa* and *B. cepacia*. Mixed consortia were either cultured in artificial flow chambers, which represent an artificial but highly controllable aquatic model system, or in the lungs of mice using alginate-entrapped bacteria. The detection of green fluorescent cells in both model systems as well as in cross-streak experiments provided evidence that the two bacteria utilize AHL molecules to interact with each other. Moreover, our data clearly show that communication between the two bacteria only occurs in one direction, namely from *P. aeruginosa* to *B. cepacia* and not vice versa.

It could be argued that the sensor plasmids used are artificial constructs that are valuable for detection of AHLs but do not necessarily indicate whether these AHLs are in fact perceived by the bacteria. In this respect it is important to bear in mind that both sensor plasmids used in these experiments are based on the promoter sequences of the genes *lasB* and *cepI*, which are controlled by the quorum-sensing systems of *P. aeruginosa* and *B. cepacia*, respectively. Hence, stimulation of these AHL sensor plasmids indeed indicates activation of target gene expression in the respective host bacterium. To further substantiate that production of AHLs by *P. aeruginosa* stimulates expression of target genes in *B. cepacia* we determined the effects of extracts of *P. aeruginosa* supernatants as well as of pure AHL compounds on restoration of extracellular proteolytic activity of the *cepI* mutant *B. cepacia* H111-I. Previous work has shown that production of an extracellular protease is tightly controlled by the *cep* quorum-sensing system of *B. cepacia* (Lewenza *et al*., 1999; Huber *et al*., 2001). Extracts of *P. aeruginosa* PAO1 and, more importantly, of SH1, the strain that colonized the lungs of the CF patient at the onset of co-infection with *B. cepacia* H111, restored protease production. By contrast, extracts of *B. cepacia* H111 supernatants did not stimulate protease production of the AHL-negative *P. aeruginosa* derivative PAO1-JP2, strongly supporting the view of unidirectional signalling between the two bacteria. Conceivably, this one-sided communication is a consequence of differences in the specificities of the AHL-binding R-homologues present in the two bacteria. The two AHLs produced by *B. cepacia* H111, C8- and C6-HSL, are very poor activators of the quorum-sensing systems of *P. aeruginosa* (Fig. 4), which

primarily utilize C4-HSL and 3-oxo-C12-HSL, respectively. On the other hand, *P. aeruginosa* produces C6- and 3-oxo-C8-HSL (Pearson *et al.*, 1994; Winson *et al.*, 1995; Geisenberger *et al.*, 2000; Fig. 2), two AHL molecules that are capable of activating the *cep* quorum-sensing system of *B. cepacia* at low concentrations (Fig. 4). Recent work has shown that most strains of the *B. cepacia* complex, which currently comprises six genomic species, produce C8- and C6-HSL (Gotschlich *et al.*, 2001). In this study it was further demonstrated that some strains belonging to the genomovar V (*Burkholderia vietnamiensis*) produce additional AHL molecules with acyl side chains ranging from $C_{10}$ to $C_{14}$. These *B. vietnamiensis* strains are capable of stimulating the *P. aeruginosa* monitor strain PAO1-JP2(pKR-C12) in cross-streaking experiments (data not shown), indicating that in these cases intergeneric communication may occur in both directions. Work is currently under way to determine the role of AHL-mediated communication between *P. aeruginosa* and *B. cepacia* for the pathogenicity of the mixed consortium.

## ACKNOWLEDGEMENTS

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