Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound

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Novel molecular tools have been constructed which allow for *in situ* detection of N-acetyl homoserine lactone (AHL)-mediated quorum sensing in *Pseudomonas aeruginosa* biofilms. The reporter responds to AHL activation of LasR by expression of an unstable version of the green-fluorescent protein (Gfp). Gfp-based reporter technology has been applied for non-destructive, single-cell-level detection of quorum sensing in laboratory-based *P. aeruginosa* biofilms. It is reported that a synthetic halogenated furanone compound, which is a derivative of the secondary metabolites produced by the Australian macroalga *Delisea pulchra*, is capable of interfering with AHL-mediated quorum sensing in *P. aeruginosa*. It is demonstrated that the furanone compound specifically represses expression of a *PlasB-gfp* reporter fusion without affecting growth or protein synthesis. In addition, it reduces the production of important virulence factors, indicating a general effect on target genes of the las quorum sensing circuit. The furanone was applied to *P. aeruginosa* biofilms established in biofilm flow chambers. The Gfp-based analysis reveals that the compound penetrates microcolonies and blocks cell signalling and quorum sensing in most biofilm cells. The compound did not affect initial attachment to the abiotic substratum. It does, however, affect the architecture of the biofilm and enhances the process of bacterial detachment, leading to a loss of bacterial biomass from the substratum.

Keywords: biofilms, quorum sensing inhibitors, green-fluorescent protein

INTRODUCTION

Many host-associated bacteria use chemical signals to monitor their own species population density and to control expression of specific genes in response to population density. This type of gene regulation is termed quorum sensing (Fuqua *et al.*, 1997) and is a generic phenomenon described in many Gram-negative (Eberl, 1999; Greenberg, 1997) and Gram-positive bacteria (Kleerebezem *et al.*, 1997). Many Gram-negative bacteria capable of quorum sensing employ N-acetyl homoserine lactones (AHLs) as the signalling compound. The various AHL compounds described in Gram-negative bacteria differ from one another in length and substitutions on their acyl side chains. The signalling molecule is synthesized by a LuxI-type synthase and they bind to a cognate LuxR-type transcriptional activator protein to regulate expression of target genes. At low cell density, the signalling compound is synthesized at a low basal level and is thought to diffuse into the surrounding medium where it becomes diluted. During growth, the AHL accumulates in the medium until a critical threshold concentration is reached. At this concentration, the AHL binds to its cognate

Abbreviations: AHL, N-acetyl homoserine lactone; BHL, N-butanoyl-L-homoserine lactone; Gfp, green-fluorescent protein; OdDHL, N-[3-oxo-dodecanoyl]-L-homoserine lactone; ODHL, N-[3-oxo-hexanoyl]-L-homoserine lactone; OSCI, quorum-sensing-controlled; OSI, quorum sensing inhibitor; Rfp, red-fluorescent protein; SCLM, scanning confocal laser microscopy.
receptor, which in turn becomes activated and stimulates or represses transcription of target genes.

*Pseudomonas aeruginosa*, a Gram-negative opportunistic human pathogen, is responsible for persistent and often incurable infections in immunocompromised people and individuals with cystic fibrosis (Hoiby, 2000; Koch & Hoiby, 1993; Pollack, 1990). The list of *P. aeruginosa* quorum-sensing-controlled (QSC) genes and phenotypes is growing continuously (Glessner et al., 1999; Hassett et al., 1999) and classes of qsc genes are emerging (Whiteley et al., 1999; for reviews, see Passador & Iglewski, 1995; Pesci & Iglewski, 1997; Swift et al., 1996; Van Delden & Iglewski, 1998; Williams et al., 2000).

Two AHL-mediated quorum sensing circuits have been identified in *P. aeruginosa*. The las system consists of *lasI*, an AHL synthase gene responsible for the synthesis of OdDHL [N-(3-oxo-dodecanoyl)-l-homoserine lactone; 3-oxo-C12-HSL; PAI-1] (Pearson et al., 1994), and *lasR* which encodes a LuxR-type transcriptional regulator protein (Gambello & Iglewski, 1991; Passador et al., 1993). The las system has been shown to regulate the expression of several virulence factors, such as extracellular enzymes (LasB elastase, LasA protease, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin), toxins (exotoxin A) and *lasI* itself. In the rhl system, the rhl gene product directs the synthesis of BHL (N-butanoyl-l-homoserine lactone; C4-HSL; PAI-2), which, in conjunction with the *rhlR* gene product, activates transcription of the *rhlAB* rhamnolipid biosynthesis genes and the *rhlI* gene itself. The rhl system is also involved in modulating the expression of several of the virulence factors controlled by the las system (Glessner et al., 1999; Pearson et al., 1995).

In the cystic fibrosis lung, *P. aeruginosa* grows primarily as biofilms (Hoiby, 1977; Lam et al., 1980; Singh et al., 2000), which provide protection from the host defence system and from the action of antibiotics (Koch & Hoiby, 1993). Biofilms are highly structured, surface-attached communities of cells enclosed in self-produced polymeric matrix. In laboratory-based systems, *P. aeruginosa* forms biofilms several hundred micrometres thick with tower- and mushroom-shaped microcolonies intervened by water channels and void spaces (Costerton et al., 1995; Davies et al., 1998). The current model is that biofilm formation proceeds through a series of programmed events. O'Toole & Kolter (1998) have demonstrated that flagellar motility and type IV pili-mediated twitching motility in *P. aeruginosa* is necessary for surface attachment and colonization. There is increasing evidence that cell-to-cell communication plays a crucial role for the maturation of biofilms, i.e. for the development of a characteristic three-dimensional biofilm architecture. For *P. aeruginosa* it has been demonstrated that the ability to form biofilms in flow chamber systems is affected by the las, but not the rhl quorum sensing system (Davies et al., 1998). While the wild-type formed characteristic microcolonies separated by water channels, the lastI mutant developed only flat, undifferentiated biofilms which exhibited greater sensitivity to the biocide SDS. These results argue in favour of functional overlaps between factors necessary for cell-to-cell signalling, biofilm maturation and bacterial pathogenesis.

Phenotypes regulated by cell-to-cell communication have been proven or suggested to be important for bacterial colonization of eukaryotes (Eberl et al., 1996; Givskov et al., 1996, 1997; Kjelleberg et al., 1999; Piper et al., 1993; von Bodman & Farrand, 1995). Given the widespread occurrence of AHL-mediated cell-to-cell communication systems, it has been hypothesized that higher organisms may have evolved specific means to interfere with bacterial communication and possibly escape colonization. The Australian marine macroalga *Delisea pulchra* has been suggested to possess such a countermeasure to bacterial processes (Kjelleberg et al., 1999). The alga produces a number of halogenated furanones (de Nys et al., 1993; Reichelt & Borowitzka, 1984) which display strong bacterial activities, including antifouling and antimicrobial properties (de Nys et al., 1995; Reichelt & Borowitzka, 1984). Most interestingly, recent reports indicate that some furanones possess AHL-antagonistic activity, which can likely be attributed to a structural similarity to AHLs (Givskov et al., 1996; Manefield et al., 1999, 2000). We, therefore, have initiated research to assess the potential of these furanone compounds as quorum sensing inhibitors (QSIs). This approach may lead to the development of novel non-antibiotic drugs which aim at the attenuation of bacterial virulence rather than killing the pathogen (Givskov et al., 1996).

In the present study, we present new genetic tools that allow monitoring of cell–cell communication in live cells. The system utilizes the green-fluorescent protein (Gfp) of the jellyfish *Aequorea victoria* for non-invasive, real-time detection of gene expression at the single-cell level without the addition of chemical substrates. Moreover, since the Gfp used has a short half-life, the system allows for repeated measurements of the same cells to continually assess bacterial activity. This opens unprecedented possibilities for *in situ* studies of quorum sensing and quorum sensing inhibition in ecologically and clinically relevant scenarios (environments). We have applied the technology for on-line detection of quorum sensing in flow-cell-based *P. aeruginosa* biofilms. In addition, we present a novel halogenated furanone compound capable of interfering with *P. aeruginosa* cell-to-cell communication. We demonstrate that the furanone reduces QSC gene expression while having no effect on bacterial protein synthesis or growth. The halogenated furanone reduces the production of several QSC virulence factors and the compound interferes with *P. aeruginosa* biofilm formation.

**METHODS**

**Bacterial strains.** *Escherichia coli* and *P. aeruginosa* strains used in this study are listed in Table 1.
Table 1. Bacterial strains, plasmids and primers used in this study

<table>
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<th>Strain/plasmid/primer</th>
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<td>T. Hansen, Novo Nordisk A/S</td>
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<tr>
<td>CC118 zipr</td>
<td>(ara-leu) araD ΔlacX746 galE galK pkoΔ20 tbi-1 rps-1 rpoB argE</td>
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</tr>
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<td><strong>P. aeruginosa</strong></td>
<td></td>
<td></td>
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<tr>
<td>PAO1</td>
<td>Wild-type P. aeruginosa</td>
<td>Holloway (1955)</td>
</tr>
<tr>
<td>PAO-JP2</td>
<td>lasI rhl derivative of PAO1, HgR</td>
<td>Pearson et al. (1997)</td>
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<td><strong>Plasmids</strong></td>
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<td>J. B. Andersen</td>
</tr>
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<td>pJB27</td>
<td>Apβ; pUC18-NovI Pα1+Oα-3 RBSII-gfpmut3*-Tβ-T1</td>
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</tr>
<tr>
<td>pJB132Gm</td>
<td>TcR GmR; luxR Plux-gfp(ASV)</td>
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<td>pUCP22Not</td>
<td>Apβ GmR; Pseudomonas shuttle cloning vector, ori pRO1641</td>
<td>Herrero et al. (1990)</td>
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<td>pKT17</td>
<td>Apβ; lasB-lacZ Plac-lasR</td>
<td>Pearson et al. (1994)</td>
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<td>Apβ TcR; Tn5-based delivery plasmid</td>
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<td>Whiteley et al. (2000)</td>
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<td>Apβ; DsRed expression vector</td>
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<td>pMHLB</td>
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<td>pBADGfp</td>
<td>Apβ GmR; Pseudomonas shuttle vector with araC Pβ3mR16-gfp (ASV)</td>
<td>This study</td>
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<td>pRK600</td>
<td>CmR; ori ColE1 RK2-Mob+ RK2-Tr a+; helper plasmid in triparental conjugations</td>
<td>Kessler et al. (1992)</td>
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**Primers**

<table>
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<th>Primers</th>
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<th>Source or reference</th>
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<tr>
<td>lasB rev</td>
<td>5’-GCTGCTGATCAGGCTAGATGCCGCAAGCT-3’</td>
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<tr>
<td>lasR fwa</td>
<td>5’-GCGGATCCGGCCAGAAGCTTTTCGAC-3’</td>
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<tr>
<td>lasR rev</td>
<td>5’-GCGGATCCGGCCAGAAGCTTTTCGAC-3’</td>
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<td>araCP fwa</td>
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</tr>
<tr>
<td>araCP rev</td>
<td>5’-GGGTACGTGCAGACTGATCCATGATGCATCCGGCTCAAGCGG-3’</td>
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**Media.** The basic medium was either modified Luria–Bertani (LB) medium (Bertani, 1951) containing 4 g NaCl l⁻¹ or AB minimal medium [AB minimal medium (Clark & Maaloe, 1967) containing 2.5 mg thiamine l⁻¹]. Antimicrobial agents were added as appropriate at the following concentrations: gentamicin, 15 µg ml⁻¹ for E. coli and 60 µg ml⁻¹ for P. aeruginosa; ampicillin, 100 µg ml⁻¹ for E. coli; carbenicillin, 300 µg ml⁻¹ for P. aeruginosa; tetracycline, 60 µg ml⁻¹ for P. aeruginosa.

**Plasmids and DNA manipulations.** The plasmids used in this study are listed in Table 1. DNA treatment with modifying enzymes and restriction endonucleases (Gibco BRL), ligation of DNA fragments with T4 ligase (Gibco BRL) and transformation of E. coli were performed using standard methods (Sambrook et al., 1989). Plasmid DNA was isolated from the Spin Miniprep kit (Qiagen) and DNA fragments were excised and purified from agarose gels using GFX DNA and the Gel Band Purification kit (Amersham Pharmacia Biotech). PCR was carried out on a Biometra T3 thermocyler using the Expand High Fidelity PCR kit (Boehringer Mannheim). Transformation of P. aeruginosa was performed according to the method described by Diver et al. (1990).

The transcriptional fusion vector pMHLB391 was constructed by inserting the 1765 bp NoI fragment containing the RBSII-gfp(ASV)-Tβ-T1 cassette of pJB25, as described by Andersen et al. (1998), into NoI-digested pUCP22Not.

A translational fusion between the N-terminal part of lasB and an unstable variant of the gfp gene was constructed. The first codon of the lasB gene was maintained and fused to the gfp(ASV) ORF devoid of the start codon (Andersen et al., 1998). The fusion retains the lasB promoter and the 5’ untranslated region of the lasB transcript and ensures that the native ribosome-binding site (RBS) and the spacing to the start
A vector for constitutive Gfp expression in *P. aeruginosa* was constructed by cloning the 2 kb *NotI* fragment of pJBA27 (Andersen *et al.*, 1998) containing the P_{AHL} fragment, and then inserting the resulting plasmid into the *NotI* digest of pUCP22Not. The resulting plasmid was termed pMH306 (Fig. 1c).

The araC-P_{BAD}-controlled gfp expression vector was constructed by PCR amplification of a 1658 bp fragment containing the araC-P_{BAD} region using the primers araCP fw and araCP rev (Table 1) and pBAD18 as template. The araC-P_{BAD} fragment was digested with *Bcl* and *Xba*I and ligated into the BamHI-XbaI site of pMH391, giving rise to pBADgfp (Fig. 1d). The araC-P_{BAD}-gfp expression cassette was subsequently excised as a *NotI* fragment and moved into the corresponding site of pTn5-Gm to give pTn5-BADgfp.

The plasmid used to provide a red-fluorescent colour tag on bacteria was constructed as follows. pDsRed was digested with *NotI*, polished with T4 DNA polymerase and digested with *PstI*. A 916 bp blunt-ended fragment containing the dsred gene under control of the lac promoter was ligated into the blunt-ended EcoRI–HindIII site of pMH391. This resulted in pMH210 with a translational stop codon in all three reading frames and two strong transcriptional terminators. The dsred expression cassette was excised as a 1926 bp *NotI* fragment and moved into the corresponding site of the pUTTc delivery vector to yield pTn5-Red. The lac promoter of *E. coli* acts as a constitutive promoter in *Pseudomonas* spp., due to the absence of lac repressor activity (Andersen *et al.*, 1998).

The reporter cassettes were inserted at random positions in the chromosomes of *P. aeruginosa* PA01 and PA0-JP2 by triparental mating. The selected transconjugants with random insertion of the mini-Tn5 elements showed no sign of phenotypic changes compared to the parental strains when tested in liquid medium or flow-chamber biofilms.

**Furanone compound.** Furanone 56 was made by chemical synthesis as described by Manny *et al.* (1997).

**AHL and furanone bioassay.** Strains were grown exponentially in LB or A/B medium supplemented with 0.5% glucose at 30°C, shaking at 250 r.p.m. At an OD of 0.8, the cultures were diluted and split into subcultures in glass culture flasks. A/HLs and furanone 56 were added to appropriate concentrations and the cultures were further incubated at 30°C with vigorous shaking. Culture samples were retrieved at various time intervals and green fluorescence was measured with a fluorometer (model RF-1501; Shimadzu) at an excitation wavelength of 475 nm and emission wavelength of 515 nm. Relative fluorescence was calculated as green fluorescence normalized to 1 ml culture divided by OD.

**P. aeruginosa biofilms.** Biofilms were grown at 30°C in three-channel flow cells (Christensen *et al.*, 1999) with individual channel dimensions of 0.3 × 4 × 40 mm supplied with A/B minimal medium supplemented with 2% LB. The flow system was assembled and prepared as described by Christensen *et al.* (1999). The substratum consisted of a microscope glass coverslip (Knittel 24 × 50 mm; st1; Knittel Gläser). Cultures for inoculation of the flow channels were prepared as follows.
The chitinase activity assay was performed as described by the manufacturer (Loewe Biochemica). Supernatant (560 µl) of cultures prepared as described for the elastase assay was mixed with carboxymethyl/chitin/remazol brilliant violet (200 µl) and sodium phosphate buffer (40 µl, 1 M, pH 7.5) as the reaction mixture was incubated for 18 h at 40 °C in a water bath. The reaction was stopped by the addition of HCl (200 µl, 2 M) and kept for 15 min on ice. After centrifugation (10 min at 15 000 r.p.m.), the A550 of the supernatant was measured. Relative chitinase activity was calculated as A550/OD600 normalized to 1 ml supernatant.

**Scanning confocal laser microscopy (SCLM).** Microscopic inspection and image acquisition were performed on a scanning confocal laser microscope (model TCS4D; Leica Lasertechnik) equipped with a 63×/1.32-0.6 oil objective. The microscope was equipped with a motorized and programmable xy-stage which was used for monitoring single colonies during the biofilm experiments. At the beginning of each online experiment, the microscope was programmed to track single randomly selected microcolonies; the sensitivity of image scanning was adjusted and the laser intensity was thereafter kept constant throughout the duration of the experiments. Image scanning was carried out using the 488 and 568 nm lines of an Ar/Kr laser for detection of GFP and red-fluorescent protein (Rfp), respectively. Visualization of captured images was performed using the IMARIS software package (Bitplane) running on a Silicon Graphics Indigo 2 workstation.

P. aeruginosa strains were streaked on LB plates with the appropriate antibiotics and incubated for 24 h at 37 °C. From each plate a single colony was used for inoculation of 10 ml ABr with 10 % LB. The cultures were grown at 30 °C for 18 h before they were diluted to an OD600 of 0-1 in sterile 0-9 % NaCl and used for inoculation of the flow channels. Medium flow was kept at a constant rate of 3 ml h⁻¹, equivalent to a mean flow velocity of 0.7 mm s⁻¹, using a Watson–Marlow 205S peristaltic pump. Biofilms were grown for 24 h before being shifted to media containing AHL and furanone.

**Measurements of virulence factors.** PAO-JP2 was grown in LB medium at 37 °C with shaking at 250 r.p.m. to an OD600 of 1-0. The culture was divided into subcultures to which was added OdDHL (1 µM) and BHL (3 µM), individually or in combination, and 0, 3 or 5 µg furanone 56 ml⁻¹. The cultures were grown for an additional 4 h at 37 °C. Proteolytic activity was measured as described by Ayora & Götz (1994). Azocasein (250 µl, 2 % ; Sigma) in 30 mM Tris/HCl and sterile-filtered (0-2 µm pore size) supernatant (150 µl) were incubated for 4 h at 4 °C. After precipitation of undigested substrate with trichloroacetic acid (1 : 2 ml, 10 %) for 15 min, followed by 10 min centrifugation at 10 000 r.p.m., NaOH (1-4 ml, 1 M) was added to the supernatant. The relative protease activity was measured as A540 of the supernatant divided by the OD600 of the culture.

The chitinase activity assay was performed as described by the

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**Fig. 2.** Characterization of lasB-based quorum sensing reporter. (a) Induction of pMHLAS in E. coli MT102 by different AHL compounds, all at 1000 nM. The relative green fluorescence emitted by the cells was calculated as the fluorescence at 515 nm divided by OD600. The AHL compounds assayed were: OdDHL, ODHL [N-(3-oxo-decanoyl)-l-homoserine lactone], DHL (N-decanoyl-l-homoserine lactone), OOHL [N-(3-oxo-octanoyl)-l-homoserine lactone], OHHL (N-octanoyl-l-homoserine lactone), OHHL, HHL (N-hexanoyl-l-homoserine lactone) and BHL. The results are mean values ± SEM of three independent experiments. (b) OdDHL-mediated induction of the Plas-gfp (ASV) Plas-lasR reporter cassette on a mini-Tn5 transposon integrated into the chromosome of PAO-JP2. The results are mean values ± SEM of three independent experiments. (c) Phase-contrast and epifluorescence microphotographs of OdDHL-induced PAO-JP2 cells containing the mini-Tn5-based reporter system. The OdDHL concentrations used were 10 (I), 100 (II) and 1000 nM (III).
RESULTS

Construction and characterization of lasB-based AHL monitor

Our genetic construct for detection of AHL signal molecules relies on the availability of a promoter that is transcriptionally controlled by an AHL-activated LuxR-type receptor protein. Several target genes of the las and rhl quorum sensing systems of P. aeruginosa have been identified (Ochsner & Reiser, 1995; Passador et al., 1993; Pearson et al., 1993; Whiteley et al., 1999; Winson et al., 1995). For the purpose of OdDHL detection, we have chosen the well-characterized and tightly regulated lasB promoter. Several regulatory elements of the lasB promoter such as putative regulatory sequences have been described (Anderson et al., 1999; Fukushima et al., 1997; Gray et al., 1994; Rust et al., 1996). Previous studies using a PlasB-lacZ transcriptional fusion in E. coli MG4 have demonstrated a 63-fold induction of the promoter in response to OdDHL addition (Gray et al., 1994).

We have constructed a reporter system consisting of a translational fusion of the lasB promoter to a gene encoding an unstable variant of Gfp, Gfp(ASV) (Andersen et al., 1998). Expression of the reporter is controlled by LasR from P. aeruginosa in conjunction with OdDHL. Several plasmid-based systems which feature high as well as low copy numbers have been used to accommodate the present reporter cassette in P. aeruginosa. These include the pUCP series of Pseudomonas shuttle cloning vectors (Bloemberg et al., 1997; West et al., 1994) and mini-Tn5 transposon systems for chromosomal integration (de Lorenzo et al., 1990). The copy number of each system in P. aeruginosa is 10 and 1, respectively (de Lorenzo et al., 1990; Schweizer, 1991).

Initially, the lasB-gfp(ASV) translational fusion (OdDHL sensor) was thoroughly characterized with respect to its sensitivity and specificity. A culture of E. coli hosting the pMHLAS monitor plasmid was diluted and split into several subcultures which were then supplemented with AHLs at concentrations ranging from 0 to 1000 nM. Not surprisingly, the most efficient inducer of the monitor was OdDHL, the cognate signal molecule of the las quorum sensing system. A closely related analogue, ODHL (3-oxo-C10-HSL), also activated lasB expression, albeit at a lower level. The
remaining AHL compounds did not induce significant expression of the reporter gene at a concentration of 1 µM (Fig. 2a). When the pMHLAS-based reporter system was hosted by PAO-JP2, the OdDHL concentration required for half-maximal activation of the lasB-gfp expression was 8 nM (data not shown). When the PlasB-gfp reporter system was inserted in the chromosome of PAO-JP2 in single copy, the OdDHL concentration required for half-maximal activation of lasB expression was approximately 250 nM (Fig. 2b). Green-fluorescent cells were visible by epifluorescence microscopy at a minimal OdDHL concentration of 50 nM (Fig. 2c).

Furanone-mediated inhibition of quorum sensing

Furanone compounds produced by the Australian macroalga *S. pulchra* have been shown to possess quorum sensing inhibitory properties as well as interfering with complex surface-dependent phenomena such as swarming motility and biofilm formation of *S. liquefaciens* (Givskov et al., 1996; Lindum et al., 1998; Manefield et al., 1999, 2000). Natural furanone compounds have a rather limited effect on *P. aeruginosa* when tested individually (data not shown). However, natural QSI compounds can be further modified by means of combinatorial chemistry which is a highly efficient method of generating a large number of analogues for screening purposes. One such synthetic furanone compound, termed furanone 56, is characterized by a lack of side chain at position 3 on the furanone ring. This compound only contains one bromine substitution at the methylene group and no bromine substitution on the furanone ring (Fig. 3a).

To investigate whether the furanone compound efficiently inhibited the las quorum sensing system, planktonic cultures of PAO-JP2 cells harbouring the PlasB-gfp reporter were subjected to a range of furanone 56 and OdDHL concentrations. At a concentration of 1.25 µg ml⁻¹ (7.1 µM) furanone 56 inhibited lasB-gfp expression at a wide range of OdDHL concentrations (Fig. 3b). In the presence of 100 nM OdDHL about 2 µg furanone 56 ml⁻¹ (11.4 µM) was required to reduce fluorescence by more than 50%. However, complete inhibition was not attained at any of the tested concentrations. It is noteworthy that the inhibitory effect of furanone 56 was relieved at increased concentrations of OdDHL. These results clearly demonstrate that lasB-gfp expression is stimulated by OdDHL, while furanone 56 antagonizes this activation.

The PlasB-gfp reporter was inserted into the chromosome of wild-type *P. aeruginosa*. Expression of lasB-gfp (ASV) induction corresponded to a cell density-slight cell density slightly above OD₆₀₀ of 1.0, which is in agreement with other reports (Brumlik & Storey, 1992). The data show that 5 µg furanone ml⁻¹ (28.5 µM) caused a 40% reduction in lasB-gfp expression in wild-type *P. aeruginosa*; 10 µg furanone ml⁻¹ caused a 60% reduction.

To determine if the furanone compound worked specifically on the las quorum sensor and not indirectly by disruption of primary metabolic functions, we followed growth as optical density of *P. aeruginosa* PAO1 in the presence of furanone 56. Fig. 3(d) shows that lasB-gfp expression was induced in a cell-density-dependent manner. The quorum size for lasB-gfp induction corresponded to a cell density of 10⁻⁶ to 10⁻⁴ M. Gfp expression (fluorescence/OD₆₀₀) was unaffected throughout the growth cycle by the presence of the furanone compound (data not shown).

Effect of the furanone in a heterologous background

The direct regulation exerted by the las regulon on lasB expression has been well described in numerous studies (Gambello & Iglewski, 1991; Pearson et al., 1994, 1997). Regulatory complexity is added by the observation that the las quorum sensing circuit itself is subject to global regulators (Albus et al., 1997; Whiteley et al., 2000) and that lasB expression is also controlled by regulators other than LasR (Brumlik & Storey, 1992; Pesci et al., 1999; Schlichtman et al., 1995). It was therefore important...
to rule out the possibility that the QSI effect observed is not caused by furanone interaction with higher levels of control. Since there is no AHL-based quorum sensing system present in *E. coli* (Williams et al., 2000), this bacterium provides an unbiased and well defined genetic background for studying the direct effects of the furanone on the *P. aeruginosa* las quorum sensing system. We repeated the above-described experiments using *E. coli* MT102 as a heterologous host for the reporter system. The QSI activity of the furanone was observed in this background as well (data not shown). The *E. coli* strain harbouring the lasB reporter showed increased responsiveness to OdDHL (approx. 10-fold; see Fig. 2). This is likely to be attributed to the increased copy number of the reporter plasmid. The furanone had no effect on the growth of *E. coli* MT102 (data not shown).

**Effect of furanone 56 on virulence factor production**

The experiments presented above utilized a reporter fusion to the lasB promoter to study the effect of OdDHL and furanone 56. An obvious limitation to this approach is the restriction of analysis to the level of transcription. We therefore investigated the effect of the furanone directly on production of the QSC virulence factors elastase and chitinase (Passador et al., 1993; Winson et al., 1995). Fig. 4 demonstrates that 1 µM OdDHL induced elastase and chitinase activity in *P. aeruginosa* PAO-JP2. Virulence factor production was further increased by simultaneous addition of 3 µM BHL. The addition of BHL alone did not induce elastase or chitinase expression. The presence of 3 or 5 µg furanone 56 ml⁻¹ reduced the elastase and chitinase activity.

**Inhibition of AHL-mediated signalling in* P. aeruginosa* biofilms**

The lasB-gfp(ASV) reporter was integrated into the chromosome of PAO-JP2 to ensure stable segregation and a constant gene dosage of the reporter system. The strain was grown in flow cells for 24 h in ABt-LB medium and a 10–15 µm thick biofilm developed. The medium was subsequently switched to ABt-LB containing the appropriate AHL and furanone concentrations. The development of green fluorescence was monitored on-line by SCLM for 8 h. Fig. 5 shows that...
the microcolonies were non-fluorescent prior to the switch of medium. When switched to medium containing 40 nM OdDHL, expression of the lasB-gfp(ASV) reporter fusion was induced and was visible in single cells within 4 h. Switching to a medium containing 40 nM OdDHL and 2 µg furanone 56 ml⁻¹ did not lead to induction of green fluorescence in the time course of the experiment. However, green fluorescence was induced by 80 nM OdDHL and 2 µg furanone 56 ml⁻¹. Induction of green fluorescence was abolished in medium containing 80 nM OdDHL and 4 µg furanone ml⁻¹ (data not shown), but was observed in the presence of 150 nM OdDHL and 4 µg furanone 56 ml⁻¹. No green fluorescence was observed in the presence of 4 µg furanone 56 ml⁻¹ alone (data not shown).

To determine if the furanone-mediated inhibition of green fluorescence is due to subtle non-specific effects on protein synthesis when the bacteria are growing in a biofilm, we examined expression of green fluorescence in cells harbouring the \textit{araC-P\_BAD\_gfp(ASV)} cassette induced by suboptimal levels of 1- arabinohe (0-2 %). The cells became green-fluorescent within 2 h after induction. The presence of furanone 56 at concentrations below 10 µg ml⁻¹ had no effect on Gfp expression (data not shown).

**Furanone 56 represses lasB expression in wild-type \textit{P. aeruginosa} biofilms**

Wild-type \textit{P. aeruginosa} (PAO1) carrying a chromosomally integrated lasB-gfp(ASV) reporter system was grown in flow cells similar to the PAO-JP2-based reporter strain. We focused on studying the effect of furanone 56 in long-term biofilm experiments. In favour of this approach is the observation by Davies et al. (1998) that quorum sensing is involved in maturation of \textit{P. aeruginosa} biofilms (up to 2 weeks old). To support such long-term cultivation, the biofilm medium was modified to contain 0-3 mM glucose instead of 2 % LB as carbon source. In addition, the recently available Rfp derived from the Indopacific sea anemone Discosoma was employed to provide a red-fluorescent tag on the biofilm bacteria. A mini-Tn5 transposon with the \textit{dsred} gene under the control of the strong constitutive \textit{lac} promoter was inserted into the chromosome of PAO1 containing the \textit{lasB} reporter system.

The dual-labelled PAO1 strain was inoculated and grown in flow cells in the absence and presence of 5 µg furanone 56 ml⁻¹. The flow cells were inspected daily for 10 days and scanning confocal photomicrographs were captured (Figs 6 and 7). For clarity, the green and red-fluorescent signals from the same area of the biofilm are shown separately. Because the cells constitutively express Rfp, the red colour correlates with cell mass, whereas the green fluorescence indicates active transcription of the lasB-gfp(ASV) reporter gene in response to on-going bacterial communication. We observed that the \textit{lasB} reporter in \textit{P. aeruginosa} PAO1 was activated in a cell-density-dependent manner as small microcolonies did not fluoresce green in contrast to larger microcolonies, which fluoresced bright-green (Fig. 6).

As evident from Fig. 7, early biofilm formation (day 1) is not or is only slightly affected by the furanone, though bacterial signalling appeared to be greatly reduced. By day 7, the untreated biofilm had grown to a mean thickness of 61 ± 6 µm and bright green fluorescence was emitted by the cells. In contrast, the furanone-treated biofilm was 23 ± 4 µm thick and cells were far less green-fluorescent. Complete inhibition of the \textit{lasB-gfp(ASV)} reporter in all biofilm bacteria by addition of furanone in concentrations which had no effect growth (<10 µg furanone 56 ml⁻¹), was not achievable.

**Repression of LuxR-activated QSC gene transcription**

We speculated that the AHL-antagonistic properties of furanone 56 were specific to the \textit{P. aeruginosa} las quorum sensing system. To test this, a previously published quorum sensing reporter based on the \textit{Vibrio Fischeri luxR} gene and \textit{Phuxl-gfp(ASV)} (Andersen et al., 2001; Wu et al., 2000) was transferred to PAO-JP2. Biofilms of PAO-JP2, grown as described above, were exposed to 250 nM OHHL [N-(3-oxo-hexanoyl)l- homoserine lactone] and green fluorescence developed within 1 h. Green fluorescence decreased significantly within 2 h and completely disappeared after 7 h when
**Fig. 7.** Effect of furanone 56 on wild-type *P. aeruginosa* quorum sensing and biofilm formation. *P. aeruginosa* PAO1 carrying the lasB-based reporter and a dsred expression cassette on mini-Tn5 transposons was cultivated in flow cells in the absence or presence of 5 µg furanone 56 ml\(^{-1}\). In the simulated fluorescence projections generated by SCLM, green fluorescence indicates active transcription of the QSC lasB promoter. Red fluorescence arises from constitutive expression of the dsred gene and, therefore, correlates to bacterial biomass accumulation at the substratum. Single cells may emit both green and red fluorescence but, for clarity, the colours are shown in separate images. The lower images provide sagittal views to visualize biofilm structure and thickness (day 7). Bars, 20 µm. The medium was ABt containing 0–3 mM glucose.

15 µg furanone 56 ml\(^{-1}\) was supplied in the medium flow (Fig. 8).

**DISCUSSION**

QSC gene expression, i.e. cell-density-dependent gene regulation, has been shown to be a common phenomenon in many Gram-negative bacteria (Fuqua & Greenberg, 1999; Greenberg, 1997; Parsek & Greenberg, 2000). In most known cases, quorum sensing systems control expression of virulence factors and hydrolytic enzymes (for recent reviews see Eberl, 1999; de Kievit & Iglewski, 2000). More complex phenotypes are also known to be QSC, including swarming motility of *S. liquefaciens* which is a specialized, flagella-driven movement by which a bacterial community can, in the presence of extracellular biosurfactant, spread as a biofilm over a surface (Eberl et al., 1996, 1999; Givskov et al., 1997, 1998; Rasmussen et al., 2000). Evidence is accumulating that the ability to form surface-associated, structured and co-operative consortia (referred to as biofilms) in many organisms may involve quorum sensing regulation (Costerton et al., 1999; Davies et al., 1998; Eberl et al., 1999). *P. aeruginosa* has become one of the important model organisms for research in this field. This opportunistic pathogen produces a battery of extracellular virulence factors. The quorum sensing circuits of *P. aeruginosa* have been demonstrated to exert positive transcriptional control on the majority of genes encoding virulence factors, e.g. lasB (elastase), lasA (staphyloprotease), toxA (exotoxin A) and aprA (alkaline protease) (Brint & Ohman, 1995; Gambello et al., 1993; Gambello & Iglewski, 1991; Ochsner & Reiser, 1995; Pearson et al., 1995; Seed et al., 1995; Toder et al., 1991). Recent studies estimated that 1–4% of *P. aeruginosa* genes are subject to quorum sensing control (Whiteley et al., 1999) and, thereby, support the view that quorum sensors are involved in global control of gene expression.

*P. aeruginosa* has been shown to form organized, surface-attached microbial communities, called bio-
films. This trait has been linked to pathogenicity of the organism in relation to pulmonary infections in cystic fibrosis (Hoiby & Koch, 1990; Koch & Hoiby, 1993; Pedersen et al., 1992). The biofilm mode of growth seems to provide the ideal scenario for AHL-mediated quorum sensing. In contrast to the planktonic mode of growth, where signal molecules are likely to become diluted in the medium and carried away by flow, biofilms offer a diffusion-limited environment which may allow the signal compounds to reach the critical threshold concentration (Charlton et al., 2000). A recent study linked quorum sensing and biofilm development by demonstrating that a lasI mutant is incapable of forming a highly structured wild-type-like biofilm (Davies et al., 1998). This observation emphasizes the need for studying quorum sensing in P. aeruginosa at the community level and investigating the interplay between bacterial communication, biofilm mode of growth and pathogenesis.

Clinical studies have shown that the development of resistance to antibiotics in P. aeruginosa is a serious side-effect of current anti-pseudomonal treatment (Ciofu et al., 1994). This has encouraged us to engage in the development of novel non-antibiotic, anti-bacterial therapies based on QSI compounds that specifically block bacterial signalling systems. In contrast to traditional anti-microbial agents, QSI compounds work at concentrations that are well below the minimal inhibitory concentration. This concept is attractive, since such compounds will not create a selection pressure for development of resistance. Furthermore, bacteria that are insensitive to the QSI compounds because of mutations in the LuxR-type receptor proteins are expected to be unable to signal each other and therefore unable to coordinate their effort. Finally, since the selected QSIs are non-toxic for bacteria at the concentrations used, they are not expected to exhibit adverse effects on beneficial bacterial consortia present in the host (for example the gut flora).

In this study we have developed novel molecular tools which allow in situ detection of AHL-mediated quorum sensing and quorum sensing inhibition in P. aeruginosa biofilms. Our monitor system relies on a reporter gene fusion to a QSC promoter from P. aeruginosa. We have chosen the well characterized lasB promoter (Bever & Iglewski, 1988; Fukushima et al., 1997; Gambello & Iglewski, 1991; Rust et al., 1996; Todner et al., 1994) and used a translational reporter fusion that retains the 5′ untranslated region of the lasB transcript, the native RBS and splicing to the translational start. This might be important as the 5′ untranslated lasB mRNA is involved in post-transcriptional iron control of elastase expression (Brumlik & Storey, 1992, 1998). An unstable variant of Gfp (Andersen et al., 1998, 2001) has been used as reporter. This protein is an optimal bacterial reporter for non-invasive, real-time studies of gene expression at the single-cell level because no exogenous substrates and cofactors are required, except for trace amounts of oxygen for maturation, and Gfp normally does not interfere with growth of the host (Chalfie et al., 1994). Notably, the unstable Gfp variant allows detection of transient bacterial communication.

The present quorum sensing reporter is highly sensitive, even when present as a single chromosomal copy, and detects OdDHL at concentrations as low as 20 nM (data not shown). In agreement with the study of Passador et al. (1996), we found that OdDHL was most efficient in stimulating lasB promoter activity, whereas ODHL and OOH were less efficient (Fig. 2). None of the other AHL compounds tested resulted in detectable expression of the reporter gene fusion. The concentration of OdDHL needed for half-maximal activation of the lasB promoter was \( \approx 250 \text{nM} \), i.e. about 1/20 of that found in stationary-phase culture fluids of PAO1. Pearson et al. (1995) reported that 1 \( \mu \text{M} \) OdDHL was required for half-maximal activation of a similar construct. However, our estimate is based on a reporter system in a single chromosomal copy in PAO-JP2, whereas the former study used a plasmid-based reporter (pKDT17) in a P. aeruginosa rhlR mutant (PAO-R1). The differences in copy number and strain background could account for the different estimates.

We have cultivated P. aeruginosa strains harbouring the
quorum sensing reporter in laboratory-based flow cells. Using SCLM, we were able to monitor quorum sensing in situ at the single-cell level in biofilms. In this study we did not perform a detailed study on the induction of the reporter system in wild-type biofilms in relation to microcolony quorum size or threshold OdDHL concentration. However, we did observe that the size of the microcolonies did correlate with induction of the lasB reporter fusion as would be expected (Fig. 6). In PAO-JP2 biofilms, lasB promoter activity could be induced by OdDHL at concentrations as low as 20 nM.

Furanone compounds produced by D. pulchra have previously been demonstrated to specifically interfere with several AHL-regulated bacterial processes without any effect on bacterial growth or general protein synthesis capability (Givskov et al., 1996; Manefield et al., 2000). The current hypothesis is that the furanone compounds antagonize AHLs by competition for the binding site on the receptor protein. Recently, Manefield et al. (1999) showed that halogenated furanones, at the concentrations produced by the algae, are capable of displacing OHHL molecules from the cognate LuxR receptor protein.

In this study we have employed a novel synthetic furanone which displays enhanced AHL-antagonistic properties and has no or little effect on growth of P. aeruginosa. Quantitative data from planktonic cultures showed that furanone 56 caused a significant reduction in OdDHL-activated expression of a lasB-gfp(ASV) reporter in P. aeruginosa. The interference by furanone 56 occurs in a competitive fashion, although the stochiometric furanone/OdDHL ratio is approximately 400:1. This ratio is in good agreement with the study by Kline et al. (1999) using structural analogues of OdDHL as possible agonists and antagonists of OdDHL. The disproportionate ratio probably reflects the well-documented high affinity of LasR for OdDHL (Gray et al., 1994; Passador et al., 1996). This might also explain our failure to achieve complete inhibition of lasB expression by the addition of non-toxic concentrations of furanone 56. The compound repressed lasB promoter activity in a heterologous E. coli background devoid of an AHL-mediated quorum sensing system. This supports the model that the algal metabolite specifically interferes with AHL-dependent gene transcription at the level of the LasR regulatory protein.

The P. aeruginosa las and rhl quorum sensing circuits are subject to additional levels of regulation. Transcription of lasR was shown to be positively regulated by the virulence factor regulator Vfr (Albus et al., 1997) and to be subject to negative regulation by the product of the rslA gene, recently identified downstream of lasR (de Kievit et al., 1999). Production of BHL was shown to be induced in a P. aeruginosa gacA mutant and a model has been proposed that places GacA upstream of LasR and RhlR (Reimmann et al., 1997). Moreover, recent results suggest that the rhl system is controlled by RpoS, a sigma factor required for the general stress response of P. aeruginosa (Whiteley et al., 2000). It might be speculated that the furanone interferes with one or more of these higher level regulatory circuits. To exclude this possibility, we investigated if the furanone affects a heterologous quorum sensing system hosted by P. aeruginosa. The Vibrio fischeri lux quorum sensing system represents a distinct cell-to-cell communication system not amenable to endogenous P. aeruginosa regulators and might be regarded as a ‘clean’ system in P. aeruginosa. In the present study, furanone 56 was observed to interfere with OHHL-LuxR activated expression of a luxR-luxI-gfp(ASV) fusion. This strengthens the hypothesis that the furanone antagonizes AHLs by interaction with LuxR-type receptors. Second, the effect on the luxR-luxI-gfp(ASV) reporter indicates that furanone 56 has a broad activity in interaction with LuxR-type receptor proteins, i.e. the particular furanone is not limited only to being an antagonist of OdDHL-LasR complex formation in P. aeruginosa, but might also be used to interfere with AHL-mediated cell-to-cell communication in other Gram-negative bacteria.

The furanone did not have any significant effect on bacterial growth rates at concentrations below 10 μg ml⁻¹. In addition, we observed no negative, non-AHL related effects on bacterial protein synthesis when Gfp expression under the control of the araBAD promoter was induced by suboptimal levels of L-arabinose. The data are in agreement with previous two-dimensional PAGE analysis, demonstrating that furanones have no gross effect on bacterial protein synthesis (Manefield et al., 1999).

The lasB transcription data were complemented by measurements of the production of two QSC virulence factors, elastase and chitinase. In PAO-JP2, OdDHL, added alone or in combination with BHL, clearly stimulated protease and chitinase expression. Addition of furanone 56 reduced the activities to near the uninduced level. OdDHL and BHL synergistically induced protease activity as reported by Pearson et al. (1995). Importantly, the furanone compound was capable of repressing protease activity in the presence of OdDHL and BHL. This suggests that the furanone is also active against the rhl quorum sensing system.

We have developed a novel dual-labelling methodology to study quorum sensing in wild-type P. aeruginosa biofilms. P. aeruginosa PAO1 was manipulated to contain the lasB-gfp(ASV) fusion as a green-fluorescent reporter of quorum sensing. Additionally, the strain was equipped with a chromosomally integrated Rfp expression cassette to provide a constitutive red-fluorescent colour tag on biofilm bacteria. To our knowledge, this is the first report on the utilization of Rfp in P. aeruginosa. Inhibition of AHL-mediated signalling in the wild-type strain presents additional challenges: the AHL concentration cannot be controlled and the reporter system is subject to additional regulation by the rhl quorum sensing system, which works in conjunction with the las circuit to maximize lasB expression (Pearson et al., 1995). Furthermore, the reporter system in the wild-type responds to endogenous
and exogenous OdDHL, whereas the PAO-JP2-based reporter strain responds solely to incoming signal molecules. Considering the potential involvement of efflux pumps in transport of furanone compounds, this might be an important difference. Transcription of the lasB promoter was reduced approximately twofold in planktonic cultures of PAO1. In biofilms, the reporter system was partially shut down in the presence of 5 μg furanone 56 ml⁻¹. It is uncertain if the relatively weak reduction of lasB expression would be sufficient to render the wild-type strain significantly less virulent. However, keeping in mind that lasB belongs to the top of the quorum sensing cascade (Latifi et al., 1996; Seed et al., 1995), it is likely that qsc genes located at lower levels in the regulatory hierarchy might be more severely affected as these genes require higher OdDHL concentrations for activation. The observations by Davies et al. (1998) indicate the existence of qsc genes involved in late P. aeruginosa biofilm maturation. Our study shows that early biofilm formation, i.e. attachment to the surface, is not affected by the furanone. However, we observed that the wild-type biofilm, when grown in the presence of furanone, failed to mature and showed an architecture that strongly resembled that of the PAO1 lasI mutant observed by Davies et al. (1998). This leads to the hypothesis that the furanone may inhibit expression of the yet unidentified qsc gene(s) responsible for biofilm maturation.

In the present study we have demonstrated the use of a furanone compound as a QSI compound. Furanone 56 interferes with quorum-sensing-regulated transcription of a lasB-gfp(ASV) reporter fusion and reduces extracellular elastase and chitinase activity. The furanone has no or little effect on bacterial growth and protein synthesis. In our study we have focussed on the effect the furanone exerts on the las quorum sensing system, because the las system is located at the top of the quorum sensing hierarchy and controls the activity of the rhl system. However, our data suggest that the furanone is active against the rhl system. Furthermore, we have demonstrated that the furanone is capable of penetrating the P. aeruginosa biofilm matrix where it interferes with QSC gene expression and, as a consequence, with biofilm maturation. Our study raises a number of interesting questions. What is the involvement of the efflux pumps in QSI sensitivity? Can furanone-mediated displacement of OdDHL from LasR be demonstrated? Does the QSI compound in turn affect the biocide sensitivity of PAO1 biofilms? Can the QSI properties be optimized by altered furanone structures?

What is the effect of furanones on P. aeruginosa quorum sensing and pathogenicity in a mouse lung model? Several of these questions are currently being addressed in our laboratories by means of the molecular tools presented in this report. Furanone compounds seem to hold promise as AHL-antagonists and for development of novel non-antibiotic, anti-pathogenic agents, which interfere with bacterial cell-to-cell communication and render bacteria less virulent and more sensitive to biocide treatment.

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