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*-acyl 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*-acyl 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*-acyl-homoserine lactone; BHL, *N*-butanoyl-L-homoserine lactone; Gfp, green-fluorescent protein; OdDHL, *N*-[3-oxo-dodecanoyl]-L-homoserine lactone; OHHL, *N*-[3-oxo-hexanoyl]-L-homoserine lactone; QSC, quorum-sensing-controlled; QSI, 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, pyoverdine), toxins (exotoxin A) and *lasI* itself. In the *rhl* system, the *rhlI* 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 *lasI* 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

Strain/plasmid/ primer	Relevant genotype and characteristics	Source or reference
<i>E. coli</i>		
MT102	F ⁻ <i>thi araD139 ara-leu</i> Δ7679 Δ(<i>lacIOPZY</i>) <i>galU gal'K r⁻ m⁺ Sm^R</i>	T. Hansen, Novo Nordisk A/S
CC118 λpir	Δ(<i>ara-leu</i>) <i>araD ΔlacX74 galE galK phoA20 thi-1 rps-1 rpoB argE</i> (Amp) <i>recA thi pro hsdRM⁺ RP4-2-Tc::Mu-Km::Tn7 λpir</i>	Herrero <i>et al.</i> (1990)
<i>P. aeruginosa</i>		
PAO1	Wild-type <i>P. aeruginosa</i>	Holloway (1955)
PAO-JP2	<i>lasI rhII</i> derivative of PAO1, Hg ^R Tc ^R	Pearson <i>et al.</i> (1997)
Plasmids		
pJBA25	Ap ^R ; pUC18 <i>NotI</i> RBSII- <i>gfp</i> (ASV)-T ₀ -T ₁	J. B. Andersen
pJBA27	Ap ^R ; pUC18 <i>NotI</i> P _{A1/O4/O3} -RBSII- <i>gfp</i> mut3*-T ₀ -T ₁	Andersen <i>et al.</i> (1998)
pJBA132Gm	Tc ^R Gm ^R ; <i>luxR PluxI-gfp</i> (ASV)	Andersen <i>et al.</i> (2001)
pUCP22Not	Ap ^R Gm ^R ; <i>Pseudomonas</i> shuttle and cloning vector, <i>ori</i> pRO1614	Herrero <i>et al.</i> (1990)
pKDT17	Ap ^R ; <i>lasB-lacZ Plac-lasR</i>	Pearson <i>et al.</i> (1994)
pUTTc	Ap ^R Tc ^R ; Tn5-based delivery plasmid	de Lorenzo <i>et al.</i> (1990)
pTn5-Gm	Ap ^R Gm ^R ; Tn5-based delivery plasmid	Whiteley <i>et al.</i> (2000)
pDsRed	Ap ^R ; DsRed expression vector	Clontech Laboratories
pMH210	Ap ^R Gm ^R ; <i>Plac-dsred</i> fragment of pDsRed cloned into pUCP22Not	This study
pTn5-Red	Ap ^R Tc ^R ; pUTTc carrying <i>Plac-dsred</i> -T ₀ -T ₁	This study
pMH306	Ap ^R Gm ^R ; pUCP22Not carrying P _{A1/O4/O3} -RBSII- <i>gfp</i> mut3*-T ₀ -T ₁	This study
pMH391	Ap ^R Gm ^R ; <i>Pseudomonas</i> shuttle and <i>gfp</i> (ASV) fusion vector with RBSII- <i>gfp</i> (ASV)-T ₀ -T ₁	This study
pMHLB	Ap ^R Gm ^R ; pMH391 carrying <i>PlasB-gfp</i> (ASV)	This study
pMHLAS	Ap ^R Gm ^R ; pMH391 carrying <i>PlasB-gfp</i> (ASV) <i>Plac-lasR</i>	This study
pTn5-LAS	Ap ^R Gm ^R ; pTn5-Gm carrying <i>PlasB-gfp</i> (ASV) <i>Plac-lasR</i>	This study
pBAD18	Ap ^R ; <i>araC</i> P _{BAD} promoter	Guzman <i>et al.</i> (1995)
pBADGfp	Ap ^R Gm ^R ; <i>Pseudomonas</i> shuttle vector with <i>araC</i> P _{BAD} - <i>gfp</i> (ASV)	This study
pTn5-BADGfp	Ap ^R Gm ^R ; pTn5-Gm carrying <i>araC</i> P _{BAD} - <i>gfp</i> (ASV)	This study
pRK600	Cm ^R ; <i>ori</i> ColE1 RK2-Mob ⁺ RK2-Tra ⁺ ; helper plasmid in triparental conjugations	Kessler <i>et al.</i> (1992)
Primers		
lasB fwd	5'-GCTCTAGAGCGGCCAGGAAAGCGTGCAA-3'	
lasB rev	5'-GCTGCTGCATGCTTGTTTCAGTTCTCCTGGT-3'	
lasR fwd	5'-CGGGATCCGGCACGACAGGTTTCCCGAC-3'	
lasR rev	5'-GCCGGCCAGTGCCAAGCTTGC-3'	
araCP fwd	5'-GGGTACGTCGACTGATCACCTATGCTACTCCGTCAAGCCG-3'	
araCP rev	5'-GCGCTCGTCGACTGATCAGTTCAAATCCGCTCCCGGCGG-3'	

Media. The basic medium was either modified Luria–Bertani (LB) medium (Bertani, 1951) containing 4 g NaCl l⁻¹ or ABt minimal medium [AB minimal medium (Clark & Maaløe, 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 with 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 thermocycler 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 pMH391 was constructed by inserting the 1765 bp *NotI* fragment containing the RBSII-*gfp*(ASV)-T₀-T₁ cassette of pJBA25, as described by Andersen *et al.* (1998), into *NotI*-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

Fig. 1. Schematic drawings of *lasB* reporter fusions (not to scale). (a) *lasB*-*gfp*(ASV) translational fusion vector pMHLB. (b) pMHLAS with *lasB* fusion and *lasR* expressed from the *lac* promoter. (c) *gfp* expression cassette of pMH306. (d) L-Arabinose-controlled *gfp*(ASV) expression cassette of pBADGfp. The indicated *NotI* fragments are maintained on a *Pseudomonas* shuttle vector of the pUCP series and on the mini-Tn5 delivery vector, pTn5-Gm. The genetic components are: P_{lasB} , elastase (*LasB*) promoter fragment; *gfp*(ASV), gene encoding the unstable Gfp(ASV); T_0 , transcriptional terminator from phage λ ; T_1 , transcriptional terminator from *rrnB* operon of *E. coli*; $P_{A1/04/03}$, a strong, synthetic LacI-repressible promoter; *araC* P_{BAD} , the promoter of the *E. coli* *araBAD* operon and the gene encoding the positive and negative regulator of this promoter, *araC*.

codon is preserved and, therefore, that the activity of the reporter gene fusion closely reflects the expression of the *lasB* gene. The quorum sensing reporter system, pMHLAS, was constructed by a two-step cloning procedure. The *PlasB*-*gfp*(ASV) translational fusion was made by amplifying a 348 bp PCR product starting 345 bp upstream of the *lasB* initiation codon, using the primers *lasB* fwd and *lasB* rev (Table 1) and chromosomal DNA of *P. aeruginosa* PAO1 as template. The PCR fragment was subsequently digested with *XbaI* and *SphI* and inserted into the corresponding site of pMH391. This gave rise to the plasmid pMHLB (Fig. 1a) which carries the translational *PlasB*-*gfp*(ASV) followed by translational stop codons in all three reading frames and two strong transcriptional terminators (Andersen *et al.*, 1998).

To enhance the sensitivity of the quorum sensing monitor, the *lasR* gene under control of the *lac* promoter was inserted upstream of *PlasB*-*gfp*(ASV). The *lac* promoter was chosen to drive *lasR* expression since previous studies have demonstrated that *lasR* under its own promoter was insufficient to activate the *lasB* promoter in the presence of OddHL in *E. coli* (Pearson *et al.*, 1995). The presence of *lasR* on the monitor plasmid allows use of very sensitive *E. coli*-based monitor strains harbouring the construct in high copy-numbers. A 1002 bp *Bam*HI fragment containing the *Plac*-*lasR* expression cassette was generated by PCR amplification with the primer set *lasR* fwd and *lasR* rev (Table 1) and with pKDT17 as template. The fragment was inserted into the unique *Bam*HI site of pMHLB. The resulting plasmid, pMHLAS, contained divergent transcribed *Plac*-*lasR* and *PlasB*-*gfp*(ASV) fusions

on a 3126 bp fragment flanked by *NotI* restriction sites (Fig. 1b).

The *NotI* cassette was excised from pMHLAS and inserted into the unique *NotI* site of the pTn5-Gm vector to create pTn5-LAS.

A vector for constitutive Gfp expression in *P. aeruginosa* was constructed by cloning the 2.0 kb *NotI* fragment of pJBA27 (Andersen *et al.*, 1998) containing the $P_{A1/04/03}$ -RBSII-*gfp*mut3* T_0 - T_1 cassette into *NotI*-digested pUCP22Not. The resulting plasmid was termed pMH306 (Fig. 1c).

The *araC*- P_{BAD} -controlled *gfp*(ASV) expression vector was constructed by PCR amplification of a 1658 bp fragment containing the *araC*- P_{BAD} region using the primers *araCP* fwd and *araCP* rev (Table 1) and pBAD18 as template. The *araC*- P_{BAD} fragment was digested with *BclI* and *XbaI* and then ligated into the *Bam*HI-*XbaI* site of pMH391, giving rise to pBADGfp (Fig. 1d). The *araC*- P_{BAD} -*gfp*(ASV) 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 *PvuII*. A 916 bp blunt-ended fragment containing the *dsred* gene under the *lac* promoter was isolated and inserted into the blunt-ended *EcoRI*-*HindIII* site of pMH391. This resulted in pMH210 with *Plac*-*dsred* followed by translational stop codons in all three reading frames and two strong transcriptional terminators. The *dsred* expression cassette was excised as a 1916 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* PAO1 and PAO-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 ABt medium supplemented with 0.5% glucose at 30 °C, shaking at 250 r.p.m. At an OD₆₀₀ of approximately 0.8, the cultures were diluted and split into subcultures in glass culture flasks. AHLs 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) set 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 OD₆₀₀.

***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 ABt 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.

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 OD_{600} . 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], OHL [*N*-octanoyl-L-homoserine lactone], OHHL, HHL [*N*-hexanoyl-L-homoserine lactone] and BHL. The results are mean values $\pm$ SEM of three independent experiments. (b) OdDHL-mediated induction of the *PlasB-gfp*(ASV) *Plac-lasR* reporter cassette on a mini-Tn5 transposon integrated into the chromosome of PAO-JP2. The results are mean values $\pm$ 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).

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 ABt with 10% LB. The cultures were grown at 30 °C for 18 h before they were diluted to an OD_{600} 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 OD_{600} of 1.0. The culture was divided into subcultures to which was added OdDHL (1 μ M) and BHL (3 μ M), individually or in combination, and 0, 3 or 5 μ g 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 μ l, 2%; Sigma) in 50 mM Tris/HCl and sterile-filtered (0.2 μ m pore size) supernatant (150 μ l) 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 10000 r.p.m., NaOH (1.4 ml, 1 M) was added to the supernatant. The relative protease activity was measured as A_{440} of the supernatant divided by the OD_{600} of the culture.

The chitinase activity assay was performed as described by the

manufacturer (Loewe Biochemica). Supernatant (560 μ l) of cultures prepared as described for the elastase assay was mixed with carboxymethyl/chitin/remazol brilliant violet (200 μ l) and sodium phosphate buffer (40 μ l, 1 M, pH 7.5). The reaction mixture was incubated for 18 h at 40 °C in a waterbath. The reaction was stopped by the addition of HCl (200 μ l, 2 M) and kept for 15 min on ice. After centrifugation (10 min at 15000 r.p.m.), the A_{550} of the supernatant was measured. Relative chitinase activity was calculated as A_{550}/OD_{600} 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 $\times$ /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 on-line experiment, the microscope was programmed to track single randomly selected microcolonies; the sensitivity of photo multipliers and the laser intensity were adjusted and 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.

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.*, 1997; 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

Fig. 3. Inhibition of quorum sensing by furanone 56. (a) Molecular structure of furanone 56 (MW 175 g mol⁻¹). The asterisk indicates position 3 on the furanone ring. (b) Response of PAO-JP2 mini-Tn5-*PlasB-gfp*(ASV) *Plac-lasR* to OdDHL and furanone 56. The fluorescence signal has been normalized to 100% for 100 nM OdDHL and 0 µg furanone 56 ml⁻¹. (c) Induction of the mini-Tn5-based *PlasB-gfp*(ASV) reporter in wild-type *P. aeruginosa* PAO1 in the presence of 0 (●), 5 (▽) or 10 µg furanone 56 ml⁻¹ (■); ◇, PAO1 with the pMH391 vector control. (d) Growth of *P. aeruginosa* PAO1 in the presence of furanone 56 [symbols as for (c)].

Fig. 4. *P. aeruginosa* PAO-JP2 virulence factor production in the presence of OdDHL and furanone 56. (a) Elastase activity; (b) chitinase activity.

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*(ASV) fusion was 8 nM (data not shown). When the *PlasB-gfp*(ASV) 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 *D. 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 *Serratia 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*(ASV) 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*(ASV) 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*(ASV) expression is stimulated by OdDHL, while furanone 56 antagonizes this activation.

The *PlasB-gfp*(ASV) reporter was inserted into the chromosome of wild-type *P. aeruginosa*. Expression of *lasB-gfp*(ASV) expression was followed along the growth curve in the presence of furanone 56. Fig. 3(c) shows that *lasB-gfp*(ASV) expression was induced in a cell-density-dependent manner. The quorum size for *lasB-gfp*(ASV) induction corresponded to a 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*(ASV) 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 the furanone in concentrations used for this study had no or only little effect on growth. A similar assay with *P. aeruginosa* PAO-JP2 showed no effect on growth rate (data not shown). Furthermore, we tested whether the furanone affected *P. aeruginosa* protein synthesis. The wild-type *P. aeruginosa* PAO1 strain containing the constitutive Gfp expression vector pMH306 was grown in the presence of furanone 56 at concentrations from 0 to 10 μg ml⁻¹. 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

Fig. 5. Inhibition of OdDHL-mediated signalling in *P. aeruginosa* biofilm. Twenty-four-hour-old biofilms of *P. aeruginosa* PAO-JP2 carrying the mini-Tn5-based *PlasB-gfp*(ASV) *Plac-lasR* reporter were established in flow cells. ABt-LB medium was switched to contain 40 nM OdDHL (I), 40 nM OdDHL and 2 μg furanone 56 ml^{-1} (II) and 80 nM OdDHL and 2 μg furanone 56 ml^{-1} (III). Prior to the switch (0 h), the microscope was programmed to track selected microcolonies. Reflection and epifluorescence images were recorded by SCLM during the 8 h on-line experiment. Bar, 20 μm .

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 *araC-P_{BAD}-gfp*(ASV) cassette induced by suboptimal levels of L-arabinose (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 *P. aeruginosa* biofilms

Wild-type *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 *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 *dsred* gene under the control of the strong constitutive *lac* promoter was inserted into the chromosome of PAO1 containing the *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 d 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 *lasB* reporter in *P. aeruginosa* PAO1 was activated in a cell-density-dependent manner as small microcolonies

Fig. 6. Cell-density-dependent activation of the *PlasB-gfp*(ASV) reporter in *P. aeruginosa* PAO1 biofilm. Green fluorescence indicates active transcription of the QSC *lasB* gene. Red fluorescence arises from constitutive expression of the *dsred* gene and, therefore, correlates to bacterial biomass accumulation at the substratum (right panels). Simulated fluorescence projections generated by SCLM after 12 (I) and 48 h (II) post-inoculation. Bar, 20 μm . The medium was ABt minimal medium containing 0.3 mM glucose.

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 *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 *P. aeruginosa* *las* quorum sensing system. To test this, a previously published quorum sensing reporter based on the *Vibrio fischeri luxR* gene and *PluxI-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* (staphylolytic protease), *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-

Fig. 8. Effect of furanone 56 on the *V. fischeri lux* quorum sensing system in a *P. aeruginosa* background. Plasmid pJBA132Gm carrying the *luxR luxI-gfp*(ASV) reporter (Andersen *et al.*, 2001) was transferred to PAO-JP2. The resulting strain, PAO-JP2(pJBA312Gm), was grown in ABT-LB medium in flow cells and studied by SCLM. A 24-h-old, non-fluorescent biofilm (a) was exposed to 250 nM OHHL. Within 1 h the biofilm bacteria became green-fluorescent (b). The medium was then further modified to contain 250 nM OHHL and 15 µg furanone 56 ml⁻¹. After an additional 2 h, biofilm bacteria were significantly less green-fluorescent (c). Six hours following the introduction of furanone 56, green fluorescence had almost completely disappeared (d). Bar, 20 µm.

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; Toder *et al.*, 1994) and used a translational reporter fusion that retains the 5' untranslated region of the *lasB* transcript, the native RBS and spacing 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 OOHL 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 ≈ 250 nM, i.e. about 1/20 of that found in stationary-phase culture fluids of PAO1. Pearson *et al.* (1995) reported that 1 µM 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 alga, 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 stoichiometric 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 *rsaL* gene, recently identified downstream of *lasR* (de Kievit *et al.*, 1999). Production of BHL was shown to be reduced in a *P. aeruginosa gacA* mutant and a model has been proposed that places GacA upstream of LasR and RhIR (Reimann *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-PluxI-gfp*(ASV) fusion. This strengthens the hypothesis that the furanone antagonizes AHLs by interaction with LuxR-type receptors. Second, the effect on the *luxR-PluxI-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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