The EMBO Journal Vol. 22 No. 15 pp. 3803–3815, 2003

Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors

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Traditional treatment of infectious diseases is based on compounds that kill or inhibit growth of bacteria. A major concern with this approach is the frequent development of resistance to antibiotics. The discovery of communication systems (quorum sensing systems) regulating bacterial virulence has afforded a novel opportunity to control infectious bacteria without interfering with growth. Compounds that can override communication signals have been found in the marine environment. Using *Pseudomonas aeruginosa* PAO1 as an example of an opportunistic human pathogen, we show that a synthetic derivate of natural furanone compounds can act as a potent antagonist of bacterial quorum sensing. We employed GeneChip® microarray technology to identify furanone target genes and to map the quorum sensing regulon. The transcriptome analysis showed that the furanone drug specifically targeted quorum sensing systems and inhibited virulence factor expression. Application of the drug to *P.aeruginosa* biofilms increased bacterial susceptibility to tobramycin and SDS. In a mouse pulmonary infection model, the drug inhibited quorum sensing of the infecting bacteria and promoted their clearance by the mouse immune response.

Keywords: antagonists/biofilm/furanone/GeneChip/microarray/quorum sensing

Introduction

As the 21st century commences, it is becoming increasingly apparent that the 20th century, which opened with the promise of the eradication of most infectious diseases, closed with the specter of re-emergence of many deadly infectious diseases. It has become clear that bacteria can adapt by mutation to the selective pressure imposed by antibiotics. Today, a global concern has emerged that we are entering a post-antibiotic era with a reduced capability to combat microbes (Geddes, 2000).

A large number of opportunistic pathogenic Gram-negative bacteria employ *N*-acylhomoserine lactone (AHL) as their command language to coordinate population behavior during invasion and colonization of higher organisms (Hentzer *et al*., 2002a). The communication systems share common regulatory features: AHL signal molecules are synthesized from precursors by a synthase protein I and interact with transcriptional activator proteins R to regulate expression of target genes. AHL signaling is often referred to as quorum sensing (QS) because the system enables a given bacterial species to sense when a critical (i.e. quorate) population density has been reached in the host and in response activate expression of target genes required for succession (Fuqua *et al*., 1994).

QS-controlled genes often encode virulence factors and gene products required for bacteria–host interactions (Pirhonen *et al*., 1993; Parsek and Greenberg, 2000; Pearson *et al*., 2000). More importantly, there is growing evidence that QS influences more complex behavioral processes such as the ability to form surface-associated, structured and cooperative consortia referred to as biofilms (Davies *et al*., 1998; Eberl *et al*., 1999; Huber *et al*., 2001). Biofilm formation plays an important role in bacterial pathogenesis and is a common cause of persistent infections (Costerton *et al*., 1999; Høiby *et al*., 2001; Middleton *et al*., 2002; Singh *et al*., 2002). Biofilm bacteria are resistant to disinfectants, antibiotics and the action of host immune defenses (Koch and Høiby, 1993; Costerton *et al*., 1999).

Pseudomonas aeruginosa is an increasingly prevalent opportunistic human pathogen and is the most common Gram-negative bacterium found in nosocomial and life-threatening infections of immunocompromised patients (Van Delden and Iglewski, 1998). Patients with cystic fibrosis are especially disposed to *P.aeruginosa* infections, and for these persons the bacterium is responsible for high rates of morbidity and mortality (Høiby and Frederiksen, 2000; Lyczak *et al*., 2002).

Pseudomonas aeruginosa possesses two QS systems: the LasR–LasI and the RhlR–RhlI, with the cognate signal molecules *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OdDHL) and *N*-buturyl-L-homoserine lactone (BHL),

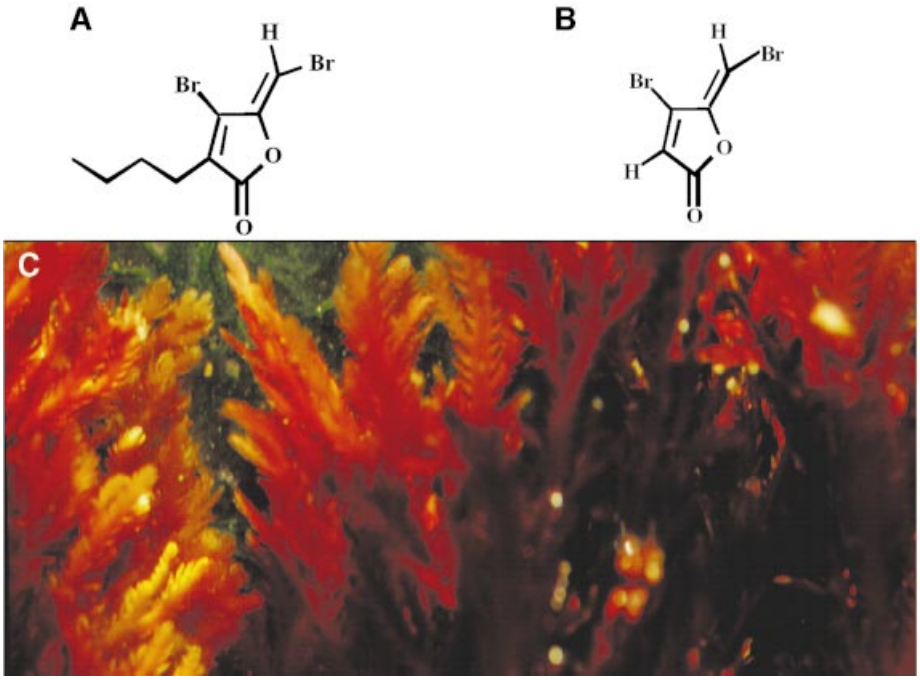

Fig. 1. From algal metabolite to *Pseudomonas* drug. (**A**) Compound 2, a natural furanone compound isolated from (**C**) *D.pulchra.* (**B**) compound C-30, a synthetic furanone with enhanced QSI activity.

respectively. The two QS circuits orchestrate a symphony of virulence factors such as exoproteases, siderophores, exotoxins and several secondary metabolites (Passador *et al*., 1993; Winson *et al*., 1995). *In vitro* immunoassays on human leukocytes have shown that OdDHL possesses immunomodulatory properties, for example, inhibition of lymphocyte proliferation and downregulation of tumor necrosis factor-α production and IL-12 production (Telford *et al*., 1998). In addition, OdDHL has been demonstrated to activate T cells *in vivo* to produce inflammatory cytokine γ-interferon (Smith *et al*., 2001) and thereby potentially promote a Th2-dominated response leading to increased tissue damage and inflammation.

We have attempted to attenuate bacterial pathogenesis by interfering with bacterial QS systems. Our approach is based on natural signal antagonists isolated from a marine environment. Seaweeds are devoid of advanced immune systems but some have evolved to rely, at least in part, on secondary metabolite chemistry for protection against colonizing organisms. In particular, the Australian red macro-alga (seaweed) *Delisea pulchra* is largely unfouled in nature due to the production of biologically active halogenated furanones (de Nys *et al*., 1993). These secondary metabolites are released at the surface of the plant at concentrations that inhibit colonization by both prokaryotes and eukaryotes (de Nys *et al*., 1995, 2002; Maximilien *et al*., 1998; Dworjanyn *et al*., 1999). We subsequently discovered that these compounds are QS inhibitors (QSIs), resulting in inhibition of colonization traits in a number of bacteria (Givskov *et al*., 1996; Gram *et al*., 1996; Maximilien *et al*., 1998; Hentzer *et al*., 2002b). The present article demonstrates that the *P.aeruginosa* communication systems can be blocked by a novel halogenated furanone compound. This is a highly specific and effective approach to attenuating bacterial virulence and controlling bacterial infections.

Results

Development of furanone compounds

Our laboratories have previously reported on the generation of synthetic furanone compounds and their QSI activities (Manefield *et al*., 2002). In this work we have applied a novel substance, termed furanone C-30 (Figure 1). This compound displays an enhanced antagonistic activity against *P.aeruginosa* QS systems.

Inhibition of virulence factor production

To test the efficacy of furanone C-30 to inhibit *P.aeruginosa* QS-controlled phenotypes, we investigated the effect on production of some QS-controlled extracellular virulence factors, namely protease, pyoverdin and chitinase. The production of these virulence factors was partially or completely suppressed in *P.aeruginosa* cultures grown in the presence of 1 or 10 μM (~2.5 μg/ml) furanone C-30 (Figure 2). Importantly, the furanone did not affect growth of the planktonic cultures (Figure 2A). QS-deficient mutants of *P.aeruginosa* PAO1 show similar growth rates to the parental wild-type strain (Glessner *et al*., 1999).

Identification of target genes of furanone C-30 action

QSI-screening assays and repression of *P.aeruginosa* virulence factor production suggest that QS circuits are targeted by the furanones. However, these observations do not exclude other targets of the furanone. DNA microarray technology offers the ability to overview the bacterial transcriptome and hence to reveal furanone target specificity by monitoring changes in transcript accumulation. We have used an Affymetrix GeneChip® *P.aeruginosa* Genome Array covering all the 5570 predicted

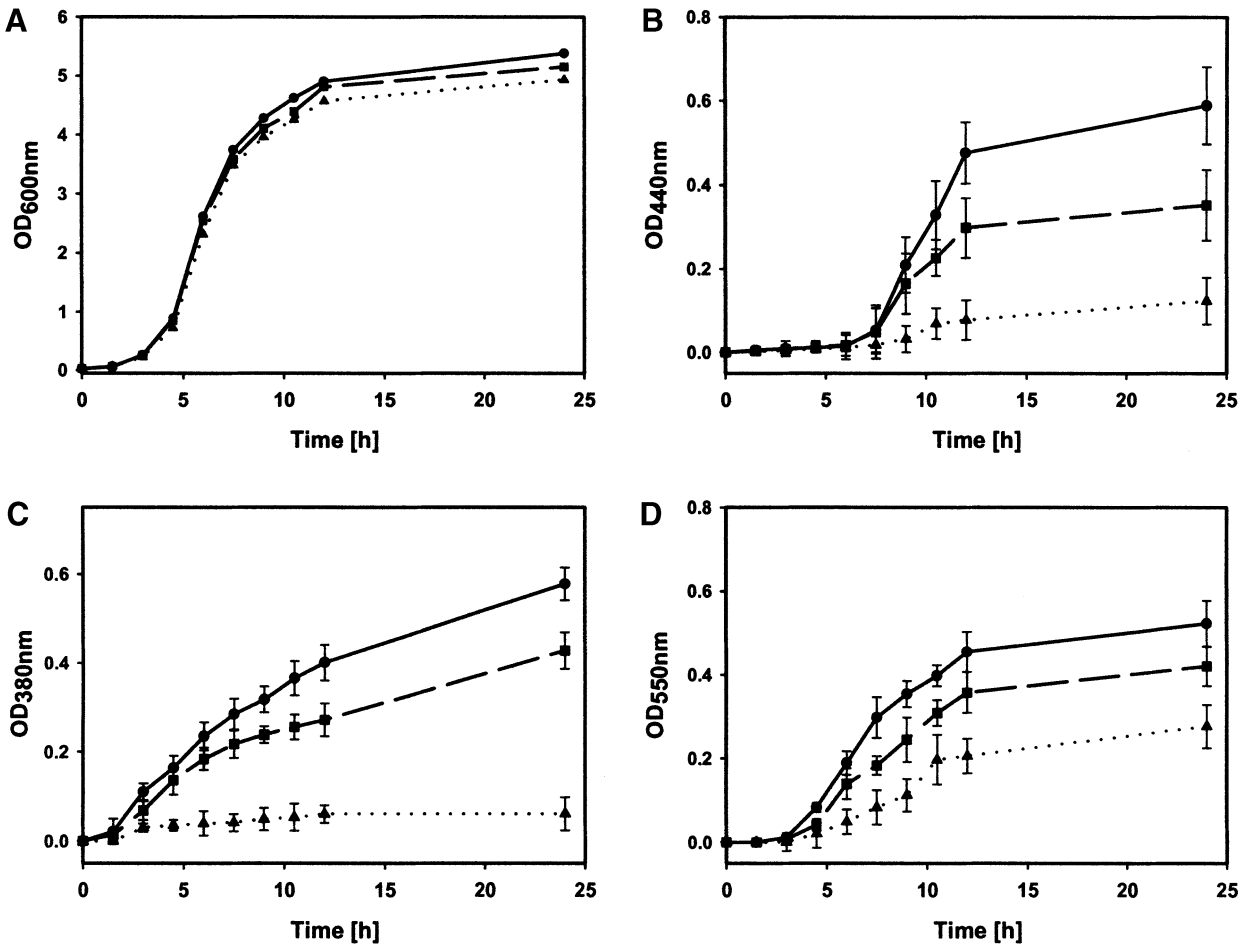

Fig. 2. Influence of furanone C-30 on growth and expression of virulence factors of *P.aeruginosa* PAO1. Cultures were grown in the absence (circles) or presence of 1 μM (squares) and 10 μM (triangles) furanone C-30. (**A**) Growth rate; (**B**) exoprotease activity; (**C**) pyoverdin activity; (**D**) chitinase activity. The data represent mean values of three independent experiments. Error bars represent the standard errors of the means.

P.aeruginosa PAO1 genes to study the effect of furanone C-30 on global gene expression.

Planktonic cultures of *P.aeruginosa* PAO1 were grown with or without addition of furanone C-30. Analysis of microarray hybridization signals showed that 93 genes (1.7% of all genes) were >5-fold affected by the furanone compound (Figure 3). In all, 85 genes (1.5%) were repressed and eight genes (0.1%) were activated in response to C-30. About 43% of the C-30-regulated genes encode hypothetical proteins of unknown function. Currently, 44% of the *P.aeruginosa* genes are classified as encoding such hypothetical proteins (Whiteley *et al.*, 2001). Among the 85 furanone-repressed genes, 30% have previously been reported as QS-controlled major *P.aeruginosa* virulence factors. These genes include the *lasB* gene encoding elastase, *lasA* encoding LasA protease, the *rhlAB* operon for rhamnolipid production, the *phzA-G* operon encoding phenazine biosynthesis, the *hcnABC* operon for hydrogen cyanide production and the *chiC* gene encoding chitinase activity.

Transcription of the *lasRI* and *rlhRI* genes was not significantly affected by C-30 (<2-fold repression). However, we observed that expression of two QS-associated genes, *fabH1* and *fabH2*, was drastically repressed by C-30. These genes encode 3-oxo-acyl carrier protein (ACP) synthase III. Furthermore, two ACP-encoding genes (PA3334 and PA1869) were significantly down-regulated by C-30. Acyl-ACPs have been proposed to be the acyl donors for synthesis of AHLs (Schaefer *et al.*, 1996; Parsek *et al.*, 1999).

Transcription of the *phnAB* operon encoding anthranilate synthase was repressed by C-30. Recently, the *phnAB* operon was suggested to encode the biosynthetic function for the *Pseudomonas* quinolone signal PQS (Gallagher *et al.*, 2002). PQS signaling is, in concert with the AHL-based QS systems, involved in regulation of virulence factor production, in particular phenazine, pyocyanin and hydrogen cyanide, and in autolysis of *P.aeruginosa* colonies (D'Argenio *et al.*, 2002), and hence potentially also in biofilms.

Among the C-30-activated genes we observed the *mexEF* genes encoding a multidrug efflux transporter and the *mexR* gene encoding the multidrug resistance operon repressor. Other C-30-activated genes included oxidoreductases, ABC transporters and MFS transporters.

To obtain an independent validation of our microarray data, we have studied expression of reporter fusions using the green fluorescent protein (GFP) as the reporter. We observed that reporter gene expression correlated well with microarray data. For example, GFP expression from a

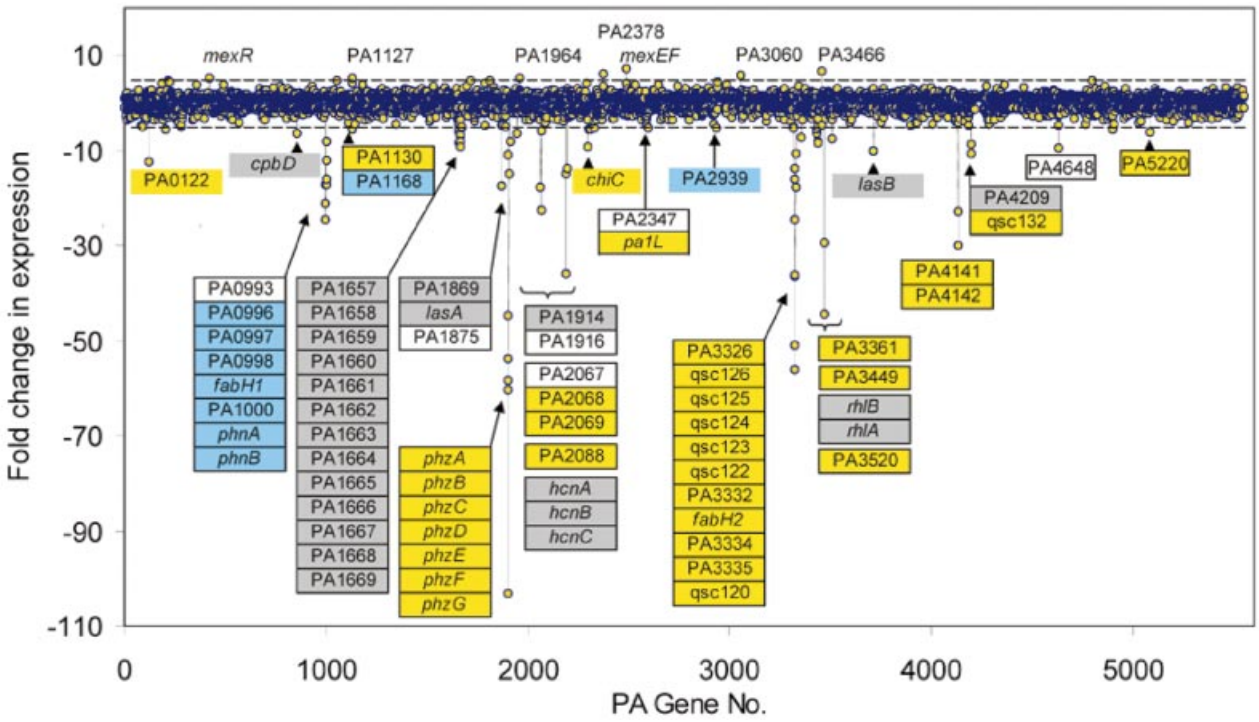

Fig. 3. Effect of furanone C-30 on genome-wide gene expression profile of *P.aeruginosa*. Differential gene expression in planktonic cultures of *P.aeruginosa* PAO1 in response to 10 μM C-30 analyzed by microarrays. Positive values represent C-30-induced genes. Negative values indicate C-30-repressed genes. The dashed lines indicate 5-fold induction or repression. Genes significantly affected by C-30 are indicated. The color coding of the individual genes indicates which of the two QS systems are required for induction: blue, LasR-controlled genes (activated by addition of only OdDHL); gray, both LasR and RhlR are required for full expression (activated by OdDHL, and further induced by addition of BHL); yellow, RhlR-controlled genes (genes induced only by simultaneous addition of OdDHL and BHL). C-30-regulated genes recorded as non-QS-controlled are not colored. Data represent samples retrieved at an OD_{600} of 2.0.

lasB promoter fusion was 7-fold repressed in the presence of 10 μM C-30 (data not shown). In comparison, microarray analysis showed that a similar C-30 treatment caused *lasB* mRNA accumulation to fall ~10-fold. Virulence factor measurements (Figure 2) showed that exoprotease was reduced 9-fold in the presence of 10 μM C-30.

Mapping of the P.aeruginosa QS regulon

Among the 1.7% of *P.aeruginosa* genes that were significantly affected by C-30, one-third have previously been reported as QS controlled, many of which encode major *P.aeruginosa* virulence factors. Until recently, QS target genes have only been identified by genetic analysis, not by transcriptome analysis. As a consequence, a larger fraction of our C-30-repressed genes might be as yet unidentified QS target genes. We have performed an in-depth mapping of genes responsive to exogenous OdDHL and BHL using a *lasI rhlI* double mutant constructed from the sequenced PAO1 strain (Stover *et al*., 2000).

We identified 163 genes activated in response to addition of AHLs, corresponding to 2.9% of the PAO1 genome (Figure 4). The QS-induced genes are scattered throughout the genome, supporting the view that the QS systems function as global regulatory systems. Many of the identified QS-controlled genes are organized in putative operons. A simple estimate predicts 34 such operons. Sequence analysis showed that 20% of the QS-regulated genes and operons contained *las* box-like sequences in their corresponding promoter regions (Figure 4). Whiteley *et al*. (1999) categorized QS-controlled genes into four classes depending on early or late induction either by OdDHL or by OdDHL and BHL in combination. In our analysis we observed similar expression profiles notwithstanding different experimental conditions.

The induction profiles of AHL-controlled genes in the *lasI rhlI* double mutant are generally in good agreement with the profiles recorded in PAO1 (Figure 4). We observed that some QS-controlled genes were activated slightly later in the wild type than in the signal-complemented double mutant (e.g. the putative operon PA3327–PA3336), whereas other genes (e.g. PA2331) were activated earlier in the wild type than in the AHL-complemented mutant. The absolute expression level of quorum-induced genes is generally not lower in the wild-type strain despite the fact that AHL signals are produced and accumulate as a consequence of bacterial growth and are not present in high concentrations throughout the growth cycle as was the case with the double-mutant cultures. These observations indicate that the timing of QS-induced gene expression is only slightly altered in a signal-complemented *lasI rhlI* mutant.

Many genes show temporal induction patterns where they are not only activated in response to increasing cell density but also repressed in stationary phase, as illustrated by the expression profile of the putative operon covering PA4129–PA4130. Such observations suggest that additional regulators of QS are involved, but they remain to be characterized.

Comparative analysis of the C-30 target genes and the QS regulon shows that 80% of the furanone-repressed genes are also QS controlled, using a 5-fold cut-off limit for furanone repression and QS induction. Likewise, 46% of AHL-induced genes were >5-fold repressed by C-30, and another 39% were 2- to 5-fold repressed (Figure 4). In general, there is a strong correlation between genes strongly induced and repressed by AHLs and C-30, respectively. Quorum-induced genes that are <5-fold repressed by C-30 are generally 5-fold (or less) induced by exogenous AHLs. Importantly, C-30-repressed genes are found among all four classes of QS-controlled genes. A few QS-controlled genes fail to be efficiently repressed by furanone despite a strong AHL induction. In particular, *rsaL*, which encodes a putative regulator of QS, is highly (>1000-fold) expressed upon addition of AHLs to the *lasI rhlI* mutant culture, but *rsaL* transcription is not repressed by the addition of C-30.

In order to investigate which cellular processes are controlled by QS, we have categorized the genes into functional groups according to the annotation by PseudoCAP (Supplementary figure S2, available at *The EMBO Journal* Online). This analysis shows that *P.aeruginosa* QS-controlled genes are slightly overrepresented in functional groups related to virulence and survival during infection, for example, adaptation/protection, antibiotic resistance and susceptibility, central intermediary metabolism, fatty acid and phospholipid metabolism, and protein secretion/export apparatus. In particular, many of the secreted factors (21%, corresponding to 18 genes) produced by *P.aeruginosa* are under QS control. All genes involved in quinolone signal response are under QS control; however, this functional group consists of only seven genes organized in three transcriptional units. The functional classification of C-30-repressed genes is strikingly similar to the grouping of QS-controlled genes, which indicates that the furanone interferes with QS-controlled gene expression in an unbiased fashion.

We hypothesized that the constitution of the QS regulon might depend on experimental conditions. For instance, the biofilm environment might provide an ideal environment for QS signaling because bacteria are present in a very high local concentration. Additionally, the bacteria are believed to exhibit a biofilm-specific physiology radically different from that of bacteria in a planktonic mode of growth (Sauer *et al*., 2002). To test our hypothesis, we grew biofilms of *P.aeruginosa* PAO1 and the *lasI rhlI* mutant in a silicone tube biofilm reactor as described in the Supplementary data. Our analysis showed that 254 genes were AHL induced in *P.aeruginosa* biofilms. Since biofilms are heterogeneous populations consisting of cells in many different growth stages, we compared gene induction ratios in biofilm samples with the planktonic data averaged over all five cell-density sample points. Among the planktonic QS genes showing an average induction ratio >5-fold, 86% of them were also >5-fold induced in the biofilm samples (Supplementary table S1).

Effect on biofilm stress tolerance

Many commonly used traditional antibiotics have been demonstrated to be ineffective on biofilm cells compared with planktonic cells (Anwar and Costerton, 1990). We wanted to test whether C-30 shows a similar inadequacy to cope with biofilm bacteria.

For *P.aeruginosa*, it has been demonstrated that the ability to form the characteristic mushroom-structured and SDS-resistant biofilms is affected by QS (Davies *et al*., 1998). We observed that biofilms grown in the presence of C-30 were, in contrast to a non-furanone-treated control, efficiently dissolved by an overnight treatment with 0.1% SDS (see Supplementary figure S1). The sensitivity to tobramycin, an aminoglycoside antibiotic routinely used in cystic fibrosis clinics (Høiby *et al*., 2000), was also assessed. Bacterial viability staining showed that biofilms grown in the presence of C-30 were significantly more susceptible to this antibiotic (Figure 5). The antibiotics efficiently penetrated and killed the furanone-treated biofilm cells, leaving 5–10% of cells alive (mainly present at the substratum). In the non-furanone-treated control, only the cells at the surface of the biofilm were killed by the tobramycin treatment. C-30-treated planktonic *P.aeruginosa* cells were two to three orders of magnitude more sensitive to tobramycin (data not shown).

QS inhibition in vivo

Cell-to-cell communication between infecting bacteria in the lungs of infected mice can be visualized by use of GFP reporter technology (Wu *et al*., 2000; Riedel *et al*., 2001). An *Escherichia coli*-based dual-labeled AHL sensor was introduced intratracheally to 4×10^7 colony-forming units (CFU) per lung. The sensor bacteria constitutively express the red fluorescent protein (RFP) for easy detection and localization in the lung tissue, and additionally express GFP in response to the presence of AHL signals. Introduction of OHHL into the mouse blood circulation caused activation of the LuxR-controlled *PluxI-gfp*(ASV) fusion (Figure 6A and B). This demonstrates that OHHL is transported by the blood, penetrates the lung tissue and induces QS-controlled gene expression in the infecting bacteria.

We used this model system to evaluate the efficacy of C-30 *in vivo*. Furanone C-30 [~2 μg/g body weight (BW)] co-administered intravenously with OHHL caused repression of LuxR-controlled activation of the dual-labeled AHL sensor (Figure 6C). C-30 inhibition was reversed by increasing dosages of OHHL (Figure 6D, E and F). This shows that the furanone compound can be transported by the blood circulation to the lungs, penetrate the lung tissues, enter the bacteria and, in turn, repress QS-controlled gene expression.

The ability to suppress *P.aeruginosa* QS *in vivo* was further tested by infecting mouse lungs with 2×10^7 CFU/lung of *P.aeruginosa* harboring a dual-labeled PA quorum sensor (Hentzer *et al*., 2002b). The infection was allowed to establish for 2 days before C-30 (~1.7 μg/g BW) was introduced through the tail vein. Over a time span of 4–6 h after administration of C-30, the GFP signal from the dual-labeled quorum sensor was significantly reduced (Figure 7). After 8 h, the GFP signal reappeared, indicating that the furanone had cleared from the mouse blood circulation and hence *de novo* GFP synthesis recommenced (not shown). The experiments reveal important information about the mode of action of C-30. First, it significantly represses QS-regulated gene expression

PA gene[1]	Gene	Description[2]	AHL	Induction in *lasI rhlI* mutant[3]										Induction in *P. aeruginosa* PAO1[3]				
				OdDHL					OdDHL + BHL					no furanone[4]				Furanone
			OD_{620}	0.5	1.3	1.6	2.0	2.7	0.5	1.3	1.6	2.0	2.7	1.3	1.6	2.0	2.7	Repression[5]
PA0007		hypothetical protein		-1	-2	2	1	5	2	2	2	2	3	-4	-2	1	-1	<2
PA0026		hypothetical protein*		3	2	4	5	4	2	3	3	4	4	1	2	3	2	2-5
PA0027		hypothetical protein		1	2	4	4	4	1	2	4	3	6	6	7	7	1	2-5
PA0052		hypothetical protein*		-2	2	3	11	3	-1	2	3	12	3	2	3	3	15	2-5
PA0059	*osmC*	osmotically inducible protein OsmC*		-1	2	1	2	-2	-2	1	1	5	4	-2	-1	-1	7	2-5
PA0122		conserved hypothetical protein*		-1	2	2	4	3	2	5	10	23	21	1	6	19	38	>5
PA0143	*nuh*	nonspecific ribonucleoside hydrolase		4	3	3	3	4	4	3	5	3	4	1	2	2	-1	2-5
PA0144		hypothetical protein*		-2	6	2	2	6	1	6	6	1	5	-4	-3	-1	-2	<2
PA0158		probable RND efflux transporter*		1	1	2	5	-1	2	1	3	5	2	-1	1	2	-1	2-5
PA0178		probable two-component sensor		-1	3	1	2	5	-1	2	1	2	3	-2	-1	-2	2	>5
PA0179		probable two-component response regulator		-1	3	2	2	6	-2	2	1	2	4	-1	1	1	7	2-5
PA0567		conserved hypothetical protein		4	2	2	39	4	4	1	3	79	6	-2	1	11	136	>5
PA0572		hypothetical protein		2	20	26	18	16	2	24	21	17	20	3	4	8	2	2-5
PA0582	*folB*	dihydroneopterin aldolase		-1	1	1	1	12	1	-1	-1	-1	10	-2	-1	-2	-1	2-5
PA0583		hypothetical protein		-1	-2	-1	2	2	-1	-1	1	6	3	3	2	2	8	2-5
PA0844	*plcH*	hemolytic phospholipase C precursor		-1	2	3	4	1	-2	5	3	2	7	-1	-1	2	1	2-5
PA0850		hypothetical protein		1	-1	5	1	9	-1	-1	8	2	6	2	-1	2	2	<2
PA0852	*cpbD*	chitin-binding protein CbpD precursor, qsc129*		2	6	6	6	13	2	10	14	16	37	2	5	15	10	>5
PA0981		hypothetical protein		2	5	2	5	-4	-1	-1	5	3	1	-3	1	-4	-2	>5
PA0996		probable coenzyme A ligase, *pqsA*		-2	21	26	14	5	-1	16	17	9	3	2	12	16	5	>5
PA0997		hypothetical protein, *pqsB*		-1	21	32	21	4	-2	16	20	14	2	2	27	31	6	>5
PA0998		hypothetical protein, *pqsC*		2	32	37	18	7	2	23	23	12	3	8	164	141	23	>5
PA0999	*fabH1*	3-oxoacyl-[acyl-carrier-protein] synthase III, *pqsD*		4	17	30	17	6	3	11	18	11	3	3	30	33	7	>5
PA1000		hypothetical protein, *pqsE*		2	14	34	20	10	3	10	23	14	3	5	41	64	14	>5
PA1001	*phnA*	anthranilate synthase component I		1	10	26	16	7	1	8	17	9	3	2	24	33	8	>5
PA1002	*phnB*	anthranilate synthase component II		4	13	17	23	8	3	9	12	12	3	2	35	33	18	>5
PA1003	*mvfR*	probable transcriptional regulator, *pqsR*		6	2	2	1	-1	6	2	2	1	-1	1	2	3	4	<2
PA1130	*rhlC*	rhamnosyl transferase 2		-2	2	1	2	2	-1	3	4	5	7	3	6	8	8	>5
PA1131		probable MFS transporter*		-4	2	1	-1	2	-2	2	3	2	6	-2	1	-1	2	2-5
PA1152		hypothetical protein		-1	1	6	1	6	-2	-1	9	-1	14	2	1	2	-2	<2
PA1168		hypothetical protein		-1	3	7	12	8	1	11	9	13	18	2	7	2	8	>5
PA1196		probable transcriptional regulator		-1	-1	6	4	4	1	-1	1	2	2	-2	-2	-1	-2	<2
PA1246	*aprD*	alkaline protease secretion protein AprD		2	2	3	3	6	2	2	4	3	5	10	11	18	4	2-5
PA1247	*aprE*	alkaline protease secretion protein AprE		-1	2	2	2	5	-2	2	3	2	5	1	2	1	1	2-5
PA1248	*aprF*	alkaline protease secretion protein AprF		2	2	2	2	5	1	2	3	2	3	2	1	3	1	2-5
PA1249	*aprA*	alkaline metalloproteinase precursor		2	3	3	3	5	-2	4	3	3	2	18	45	34	64	2-5
PA1250	*aprI*	alkaline proteinase inhibitor AprI		3	4	6	6	6	3	5	9	6	5	1	3	4	1	2-5
PA1323		hypothetical protein		1	1	2	4	-2	-1	2	3	9	3	-3	-2	1	21	2-5
PA1324		hypothetical protein		-1	2	2	4	-2	2	1	2	7	3	-2	-2	2	34	<2
PA1386		probable ATP-binding component of ABC transporter		-1	4	6	3	-3	1	4	6	4	2	2	2	2	4	>5
PA1431	*rsaL*	regulatory protein RsaL		239	223	588	2048	181	223	181	446	2195	169	2	2	3	5	<2
PA1432	*lasI*	autoinducer synthesis protein LasI*		-1	8	4	5	5	4	2	5	5	6	-1	-2	-2	1	<2
PA1656		hypothetical protein		2	3	2	1	-1	2	2	5	3	1	-1	1	1	-1	2-5
PA1657		conserved hypothetical protein		2	6	6	5	4	3	12	14	17	7	-2	2	3	-2	>5
PA1658		conserved hypothetical protein		2	3	2	2	-1	2	4	5	6	1	-2	2	3	-1	>5
PA1659		hypothetical protein		1	3	2	1	1	1	3	5	3	1	-2	2	2	-1	>5
PA1660		hypothetical protein		-1	3	1	2	14	1	5	3	7	4	1	1	2	-4	>5
PA1664		hypothetical protein		1	2	3	1	2	1	3	5	4	3	-2	1	1	-2	>5
PA1665		hypothetical protein		7	3	9	2	-2	5	4	11	5	1	2	5	8	1	>5
PA1666		hypothetical protein		1	3	2	2	4	-1	3	2	4	8	-1	-1	1	-4	>5
PA1667		hypothetical protein		7	3	2	2	2	11	3	4	3	2	-3	2	3	-2	>5
PA1669		hypothetical protein		1	1	3	7	-2	1	2	5	6	1	-3	-3	1	-4	>5
PA1784		hypothetical protein*		1	4	3	5	5	-1	3	2	5	5	-1	-1	2	17	2-5
PA1869		probable acyl carrier protein, qsc117*		8	3	2	9	6	17	16	49	104	97	1	3	19	4	>5
PA1871	*lasA*	LasA protease precursor*		-1	4	5	12	23	-1	8	17	45	84	1	4	8	18	>5
PA1875		hypothetical protein		-1	5	2	5	3	-3	2	8	5	3	1	1	3	7	>5
PA1877		probable secretion protein		-1	20	1	1	1	-2	2	1	3	2	-4	-3	-2	2	2-5
PA1891		hypothetical protein		4	11	2	5	-1	1	4	2	1	1	-2	-1	-1	-2	>5
PA4210	*phzA*	probable phenazine biosynthesis protein		-2	-1	-2	-2	-2	-2	3	9	20	26	-2	-1	11	2	>5
PA4211	*phzB*	probable phenazine biosynthesis protein		-1	1	1	1	-1	-2	20	28	34	69	-1	5	26	7	>5
PA1901	*phzC*	phenazine biosynthesis protein PhzC, qsc131		1	-2	-2	1	1	-1	4	6	16	9	-1	3	9	4	>5
PA1902	*phzD*	phenazine biosynthesis protein PhzD		-1	-2	3	2	-1	2	4	14	45	11	-1	9	29	12	>5
PA1903	*PhzE*	phenazine biosynthesis protein PhzE		1	1	-1	1	-1	-1	4	4	23	15	-1	2	10	2	>5
PA1904		probable phenazine biosynthesis protein		2	2	-1	4	2	-1	9	6	147	30	-3	4	40	6	>5
PA1905		probable pyridoxamine 5 -phosphate oxidase		-2	2	-1	1	1	-2	4	4	13	13	-2	2	13	3	>5
PA1914		conserved hypothetical protein		-2	2	4	12	23	-2	1	4	20	49	-1	-1	8	55	>5
PA2030		hypothetical protein		2	2	2	2	9	2	3	3	3	9	-1	2	4	18	2-5
PA2031		hypothetical protein		2	2	2	2	6	1	3	4	4	7	1	2	4	33	2-5
PA2055		probable MFS transporter		-2	2	-1	2	2	-2	-2	9	5	-1	-1	-4	-4	1	<2
PA2068		probable MFS transporter		-2	1	3	3	-1	1	3	14	4	13	-1	2	10	3	>5
PA2069		probable carbamoyl transferase*		1	1	1	1	9	-1	2	6	7	194	-3	4	18	7	>5
PA2070		hypothetical protein		2	-3	4	1	4	-1	-2	4	14	2	-1	2	3	-1	<2
PA2076		probable transcriptional regulator		9	3	1	3	7	6	2	1	-1	5	2	2	3	3	>5
PA2079		probable amino acid permease		-1	2	6	-1	1	1	7	6	1	2	3	-1	3	12	>5
PA2080		hypothetical protein		4	3	5	2	2	3	3	3	2	3	2	2	2	2	2-5
PA2082		probable transcriptional regulator*		2	-4	-1	6	1	1	1	1	6	1	3	1	4	7	2-5
PA2088		hypothetical protein		-2	1	1	2	1	-2	1	5	4	-2	-1	4	5	4	>5
PA2193	*hcnA*	hydrogen cyanide synthase HcnA*		7	23	6	37	4	20	181	39	169	60	-1	2	4	2	>5
PA2194	*hcnB*	hydrogen cyanide synthase HcnB, qsc128		7	16	8	12	-1	20	60	49	52	49	-2	1	2	-1	>5
PA2195	*hcnC*	hydrogen cyanide synthase HcnC		2	6	2	2	3	5	49	11	15	39	-4	1	2	1	>5

Fig. 4. Quorum-induced genes and C-30-repressed genes in *P.aeruginosa*. Quorum-induced genes identified in *lasI rhlI* mutant grown with or without added exogenous AHL signals. The transcription profile of the quorum-induced genes in *P.aeruginosa* PAO1 is shown together with the maximal repression caused by C-30. [1]The gene number and name are from the *Pseudomonas* Genome Project (www.pseudomonas.com). Genes previously reported as QS regulated are shown in bold type.], genes organized in operon. The criteria for organization into putative operons were as follows: (i) genes are transcribed in the same orientation; (ii) intergenic regions are <200 bp; (iii) genes exhibit similar transcription profiles. [2]Description from the *Pseudomonas* genome project. *qsc* gene names refer to the study by Whiteley *et al*. (1999); *pqs* gene names refer to the study by Gallagher *et al*. (2002).

PA gene[1]	Gene	Description[2]	Induction in *lasI rhlI* mutant[3]										Induction in *P. aeruginosa* PAO1[3]				
		AHL	OdDHL					OdDHL + BHL					no furanone[4]				Furanone
		OD_{600}	0.5	1.3	1.6	2.0	2.7	0.5	1.3	1.6	2.0	2.7	1.3	1.6	2.0	2.7	Repression[5]
PA2300	*chiC*	chitinase*	1	2	-1	2	4	2	2	3	7	52	-1	1	3	3	>5
PA2302		probable non-ribosomal peptide synthetase, qsc100	2	12	11	10	10	1	16	9	12	12	2	7	6	2	2-5
PA2303		hypothetical protein, qsc107	2	79	37	21	9	2	52	32	34	11	1	4	4	1	2-5
PA2304		hypothetical protein	10	15	10	17	11	3	13	15	18	11	3	8	7	4	2-5
PA2305		probable non-ribosomal peptide synthetase	3	16	10	6	14	2	20	14	6	12	2	3	3	2	<2
PA2327		probable permease of ABC transporter	1	-1	1	2	6	1	2	1	3	3	6	4	2	2	2-5
PA2331		hypothetical protein	1	-1	2	2	2	-1	2	-1	6	2	53	18	8	2	>5
PA2414		L-sorbosone dehydrogenase	-2	2	-2	3	2	-1	-1	-1	4	1	2	-1	-1	13	2-5
PA2415		hypothetical protein*	-1	2	1	3	1	-2	1	2	4	1	-1	-3	-2	3	2-5
PA2423		hypothetical protein	4	4	5	6	5	5	3	6	6	5	5	6	7	13	2-5
PA2433		hypothetical protein*	-2	2	1	4	-2	-3	2	1	9	3	-2	-4	-1	35	2-5
PA2564		hypothetical protein	-3	5	-1	9	2	-1	3	1	5	5	-3	-2	-3	1	>5
PA2566		conserved hypothetical protein*	-4	1	2	3	4	-4	1	5	6	17	-2	3	3	28	2-5
PA2570	*pa1L*	PA-I galactophilic lectin*	-2	2	1	-1	2	-2	2	4	5	20	5	3	9	135	>5
PA2587		probable FAD-dependent monooxygenase, qsc105, *pqsH**	10	5	24	13	11	6	4	24	16	13	3	8	3	4	<2
PA2588		probable transcriptional regulator	1	3	3	2	8	-2	2	3	3	17	-2	1	-1	3	2-5
PA2591		probable transcriptional regulator*	10	14	3	6	20	7	12	5	6	13	2	3	3	4	<2
PA2592		probable periplasmic spermidine/putrescine-binding protein, qsc104*	2	2	4	6	4	2	4	8	10	9	2	4	10	5	2-5
PA2593		hypothetical protein	-2	1	-1	-1	-2	2	-1	5	5	5	-1	2	2	1	2-5
PA2594		conserved hypothetical protein	-3	-2	1	1	11	-1	-1	1	1	9	4	1	2	-2	2-5
PA2629	*purB*	adenylosuccinate lyase	-1	-1	-1	-1	2	-1	-1	2	-1	5	-1	-1	-1	-4	<2
PA2747		hypothetical protein	5	1	2	3	-3	6	1	3	5	-3	-1	3	3	41	2-5
PA2753		hypothetical protein	5	2	1	6	2	4	-1	-2	2	-4	-8	-2	-2	-3	<2
PA2939		probable aminopeptidase	-1	1	6	17	20	-2	1	8	15	26	-3	3	15	57	>5
PA3274		hypothetical protein*	3	-3	2	3	-2	1	-1	4	6	9	-2	5	3	27	2-5
PA3326		probable Clp-family ATP-dependent protease*	-2	2	2	3	3	1	12	15	11	14	4	17	13	9	>5
PA3327		probable non-ribosomal peptide synthetase, qsc126	-1	2	-1	-1	-1	-1	10	7	7	2	2	6	3	1	>5
PA3328		probable FAD-dependent monooxygenase, qsc125	9	1	3	-1	-1	3	23	32	12	4	2	15	13	2	>5
PA3329		hypothetical protein, qsc124	-1	2	3	2	-1	1	724	97	194	23	11	115	127	-1	>5
PA3330		probable short chain dehydrogenase, qsc123	-2	2	7	4	6	-2	446	97	137	69	7	90	91	2	>5
PA3331		cytochrome P450, qsc122	4	3	2	2	1	5	49	24	45	5	2	24	31	3	>5
PA3332		conserved hypothetical protein	-2	2	2	-1	1	-2	52	26	17	4	3	35	37	2	>5
PA3333	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III, qsc121	-1	4	2	2	3	3	84	26	28	11	2	15	15	1	>5
PA3334		probable acyl carrier protein	2	3	2	6	2	2	45	39	56	9	6	63	72	4	>5
PA3335		hypothetical protein	2	6	2	1	1	1	64	15	28	15	-1	4	5	1	>5
PA3336		probable MFS transporter, qsc120	-1	2	-1	1	-1	-2	15	15	14	1	-1	13	10	2	>5
PA3361	*pa2L*	hypothetical protein*	-1	2	-2	1	-1	-2	3	3	9	16	-2	1	3	6	>5
PA3371		hypothetical protein	-1	1	1	3	6	-1	1	2	5	6	1	-1	2	12	2-5
PA3441		probable molybdopterin-binding protein	3	1	2	2	3	2	2	3	3	5	21	10	17	2	>5
PA3449		conserved hypothetical protein	1	-2	3	3	1	-3	-1	8	5	-2	5	3	3	1	>5
PA3477	*rhlR*	transcriptional regulator*	2	2	2	2	3	3	2	2	2	3	3	3	5	4	<2
PA3478	*rhlB*	rhamnosyltransferase chain B	-1	2	2	5	3	1	9	26	37	45	2	8	19	16	>5
PA3479	*rhlA*	rhamnosyltransferase chain A*, qsc119	6	2	2	8	7	2	15	37	84	64	6	29	96	87	>5
PA3520		hypothetical protein*	-1	3	3	2	2	-1	3	8	7	10	-2	-2	4	8	>5
PA3691		hypothetical protein	-1	1	2	4	-2	-1	1	2	6	-2	-2	-2	2	26	2-5
PA3692		probable outer membrane protein	-1	2	2	3	-2	-1	2	2	6	3	-3	-2	1	24	2-5
PA3724	*lasB*	elastase LasB*	1	13	30	21	64	-1	37	158	52	119	11	43	116	377	>5
PA3890		probable permease of ABC transporter	3	3	-1	6	-3	1	1	1	12	4	-1	-2	1	5	2-5
PA3904		hypothetical protein	16	17	34	49	10	17	24	69	56	23	3	2	4	-1	<2
PA3905		hypothetical protein	8	3	5	6	6	6	13	16	11	7	4	5	6	2	2-5
PA3906		hypothetical protein	12	13	9	4	2	9	14	18	5	3	3	3	3	-3	2-5
PA3907		hypothetical protein, qsc103	5	6	4	4	2	5	6	11	4	2	3	5	5	-1	2-5
PA3908		hypothetical protein	16	6	4	3	5	10	8	11	3	4	2	4	3	-2	2-5
PA3939		hypothetical protein*	2	-2	2	2	1	-2	-3	7	9	-4	2	2	-1	-2	2-5
PA3991		hypothetical protein	-2	6	-2	3	2	-2	10	4	9	3	-4	2	-3	2	>5
PA4085	*cupB2*	chaperone CupB2	-3	4	3	2	-2	-3	6	1	5	2	-2	3	-2	2	>5
PA4129		hypothetical protein	1	6	7	7	3	-2	15	14	17	2	3	3	4	2	2-5
PA4130		probable sulfite or nitrite reductase	1	7	8	6	2	-2	20	17	16	2	5	7	10	8	2-5
PA4131		probable iron-sulfur protein	2	7	3	5	1	2	13	7	9	1	3	2	2	2	<2
PA4132		conserved hypothetical protein	1	3	2	2	-1	-1	3	5	4	-2	2	2	1	-3	2-5
PA4133		cytochrome c oxidase subunit (cbb3-type)	2	8	7	7	-1	1	26	28	28	2	5	6	7	3	2-5
PA4134		hypothetical protein	2	2	3	-2	5	2	6	6	7	2	4	2	4	1	<2
PA4139		hypothetical protein	1	2	1	3	3	2	3	2	6	4	7	5	19	31	2-5
PA4141		hypothetical protein	-1	2	3	6	4	1	20	28	42	42	2	27	65	77	>5
PA4142		probable secretion protein	-1	4	-1	1	2	-3	5	10	6	18	2	3	11	4	>5
PA4175	*prpL*	Pvds-regulated endoprotease	2	5	6	17	23	1	6	8	20	21	3	8	19	15	2-5
PA4209	*phzM*	probable phenazine-specific methyltransferase	4	-1	-3	-1	7	2	3	2	6	42	3	4	9	17	>5
PA4217	*phzS*	flavin-containing monooxygenase, qsc132	-1	1	-1	-1	-1	-1	4	3	10	11	-1	1	6	2	>5
PA4306		hypothetical protein	3	2	3	5	3	1	-1	4	5	4	1	4	17	127	>5
PA4590	*pra*	protein activator	2	3	3	3	3	2	3	2	4	8	-2	2	2	16	2-5
PA4677		hypothetical protein	1	8	21	10	20	1	8	20	7	15	2	2	3	5	<2
PA4738		conserved hypothetical protein	-1	3	1	3	-2	-1	2	3	6	-3	-1	-4	3	40	<2
PA4739		conserved hypothetical protein	-1	3	3	7	-2	-1	3	5	12	-3	1	2	6	52	<2
PA4778		probable transcriptional regulator	2	2	3	3	3	2	2	3	3	5	2	2	3	1	2-5
PA5058	*phaC2*	poly(3-hydroxyalkanoic acid) synthase 2*	-1	2	3	4	4	-1	2	4	4	5	1	1	3	3	2-5
PA5059		probable transcriptional regulator	-1	2	5	3	15	-2	3	12	3	24	1	5	14	2	2-5
PA5220		hypothetical protein, qsc138*	2	1	-2	1	-1	1	3	3	7	8	-2	2	2	3	>5
PA5230		probable permease of ABC transporter	2	2	1	2	10	1	1	1	2	5	-2	-3	-3	-2	>5
PA5231		probable ATP-binding/permease fusion ABC transporter	2	2	2	3	12	1	1	2	2	9	-3	-3	-3	-3	2-5
PA5232		conserved hypothetical protein	1	1	2	2	6	1	1	2	2	5	-3	-3	-3	-2	2-5
PA5480		hypothetical protein	-3	2	3	7	7	-1	-1	4	2	2	-2	-2	-1	-1	2-5
PA5481		hypothetical protein	-2	1	5	5	5	-2	1	6	7	5	-2	4	9	57	2-5
PA5482		hypothetical protein	-1	1	2	6	4	-2	2	4	10	4	3	3	16	163	2-5

An asterisk indicates a putative *las* box-like sequence in the promoter. *las* box-like sequences were identified using the Patser tool at the Regulatory Sequence Analysis Tools (RSAT) website (http://bioinformatics.bmc.uu.se/~jvanheld/rsa-tools/). The *las* box consensus matrix was derived from the sequences of Whiteley *et al*. (1999) using the consensus pattern discovery tool at the RSAT-site. [3]Induction is given as the fold change in GeneChip hybridization signal. Black shading indicates genes at least 5-fold repressed or induced. [4]The reference for the induction ratio in PAO1 is a sample taken at an optical density of 0.5. [5]For every cell density sample point, the furanone repression of gene expression is calculated relative to a sample taken at the same optical density from a non-treated parallel control culture.

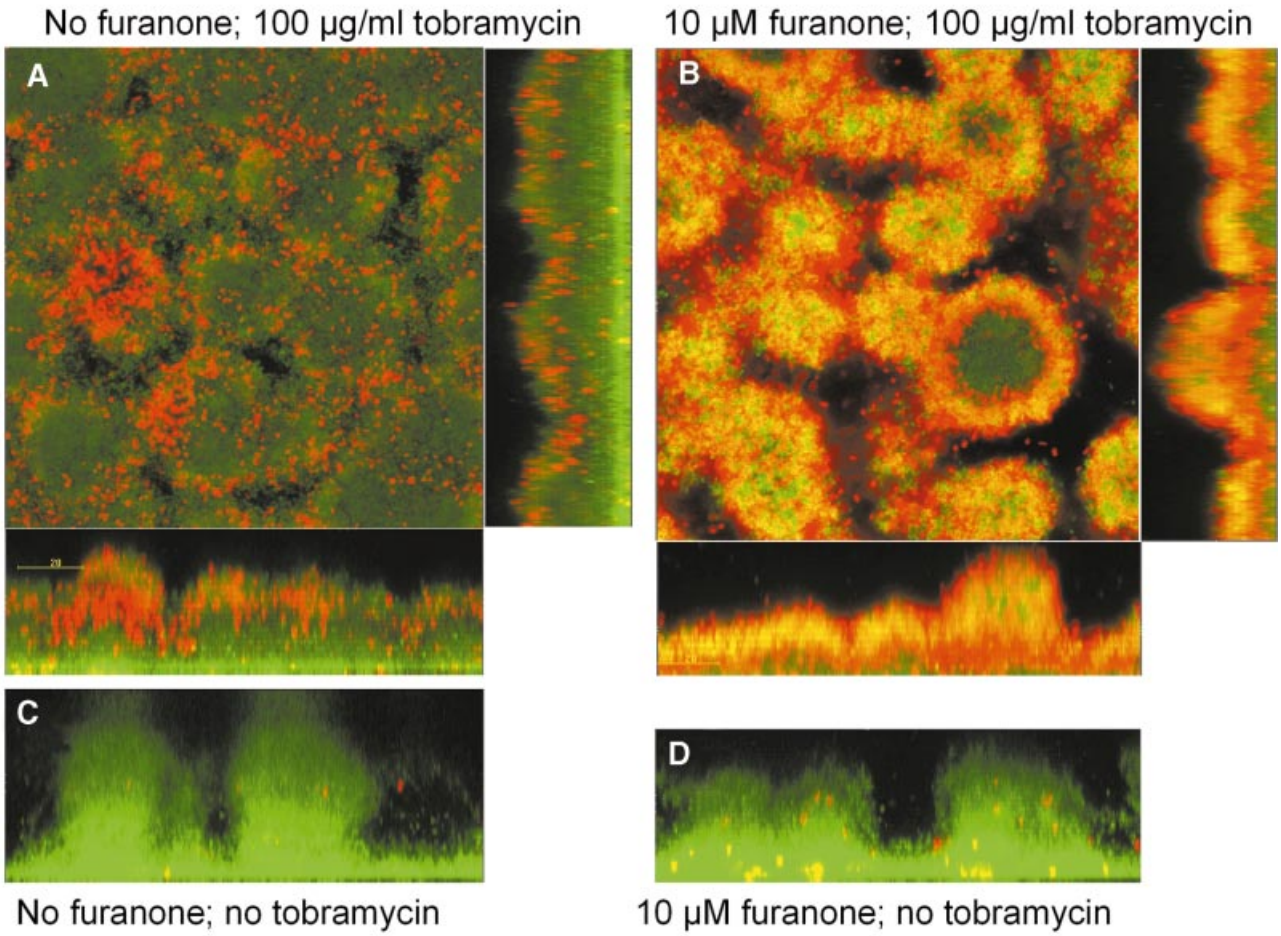

Fig. 5. Sensitivity of furanone C-30-treated *P.aeruginosa* biofilms to tobramycin. Scanning confocal laser microscopy (SCLM) photomicrographs of *P.aeruginosa* PAO1 biofilms grown in the absence (left panel) or presence (right panel) of 10 µM C-30. After 3 days, the biofilms were exposed to 100 µg/ml tobramycin for 24 h. Bacterial viability was assayed by staining using the LIVE/DEAD *Bac*Light Bacterial Viability Kit: red areas are dead bacteria, and green areas are live bacteria. The biofilms were exposed to (**A**) no furanone and 100 µg/ml tobramycin, (**B**) 10 µM C-30 and 100 µg/ml tobramycin, (**C**) non-treated control and (**D**) 10 µM C-30 and no tobramycin.

in vivo; secondly, the effect is concentration dependent; and thirdly, the compound is turned over within the duration of the experiment. This tells us that the QSI effect of a single C-30 injection lasts for ~6 h in the present animal model.

Clearance of the infecting bacteria

Having established the conditions required for *in vivo* QS inhibition, the infection model was used to study the effect of C-30 on bacterial persistence in the lung. Twenty mice were infected with *P.aeruginosa* PAO1 at day 0. Immediately after this, the mice were split into two groups (10 each), which received subcutaneous injections of either C-30 (~0.7 µg/g BW) or placebo with 8 h intervals for the following 3 days. Seven days post-infection, lungs were removed, homogenized and plated for CFU determination. The C-30-treated groups of animals displayed on average three orders of magnitude lower bacterial content than placebo groups (Figure 8). The efficiency of bacterial clearing was positively correlated with the concentration of C-30. In a similar experimental set-up, treatment with ~0.4 µg/g BW resulted in a 10-fold lower CFU compared with the placebo group, whereas treatments with ~0.2 µg/g BW had no detectable effect.

Discussion

In this article we show that a synthetic halogenated furanone inhibits expression of QS-controlled behavior in *P.aeruginosa*, in particular the production of extracellular virulence factors, inhibits the development of antibiotic-resistant biofilms and reduces the persistence of infecting bacteria in a pulmonary mouse model. We have employed comprehensive molecular, genetic and biochemical techniques, as well as biological model systems, to support our conclusions.

Using microarray technology, we demonstrated that mRNA accumulation from 1.7% of the *P.aeruginosa* PAO1 genes is significantly affected by C-30. Among these, one-third have previously been reported as QS controlled, many of which encode major *P.aeruginosa* virulence factors. Among the C-30-repressed genes, we found many that have not previously been described as QS regulated. We decided to use transcriptome analysis to identify the QS regulon under the same conditions used to

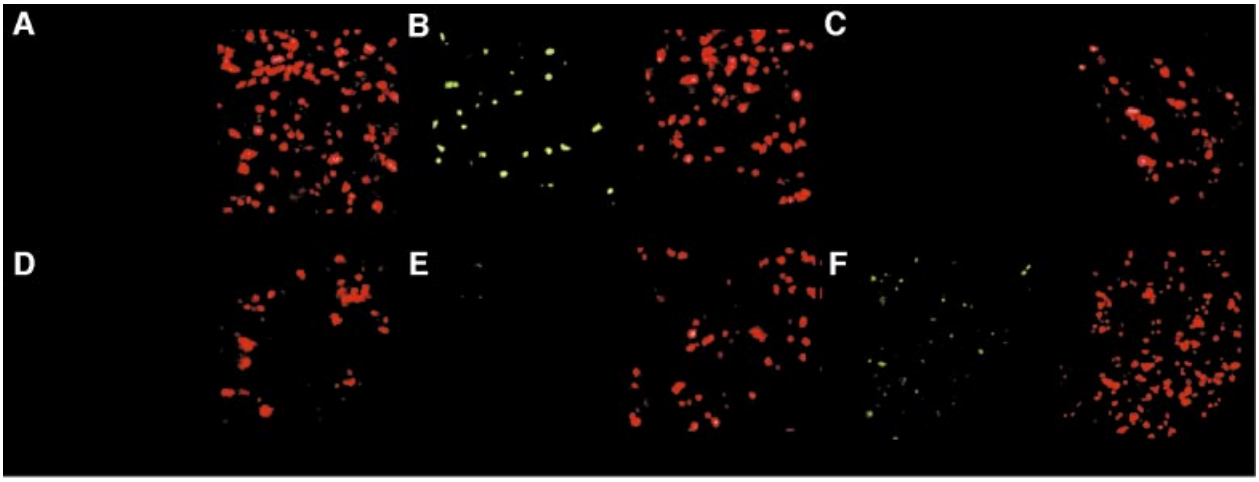

Fig. 6. Inhibition of QS *in vivo*. Photomicrographs of mouse lung tissue infected with an *E.coli*-based dual-labeled AHL sensor. The strain expresses GFP in response to the presence of exogenous AHL signals and carries a *dsred* expression cassette to provide a red fluorescent tag on the sensor bacteria for simple identification in tissue samples. Mice carrying the sensor bacteria in the lungs were administered OHHL and furanone C-30 via intravenous injection. (**A**) No injection; (**B**) 200 μM OHHL; (**C**) 200 μM OHHL + 2 μg/g BW C-30 (corresponds to ~10 μM); (**D**) 400 μM OHHL + 2 μg/g BW C-30; (**E**) 800 μM OHHL + 2 μg/g BW C-30; (**F**) 1200 μM OHHL + 2 μg/g BW C-30.

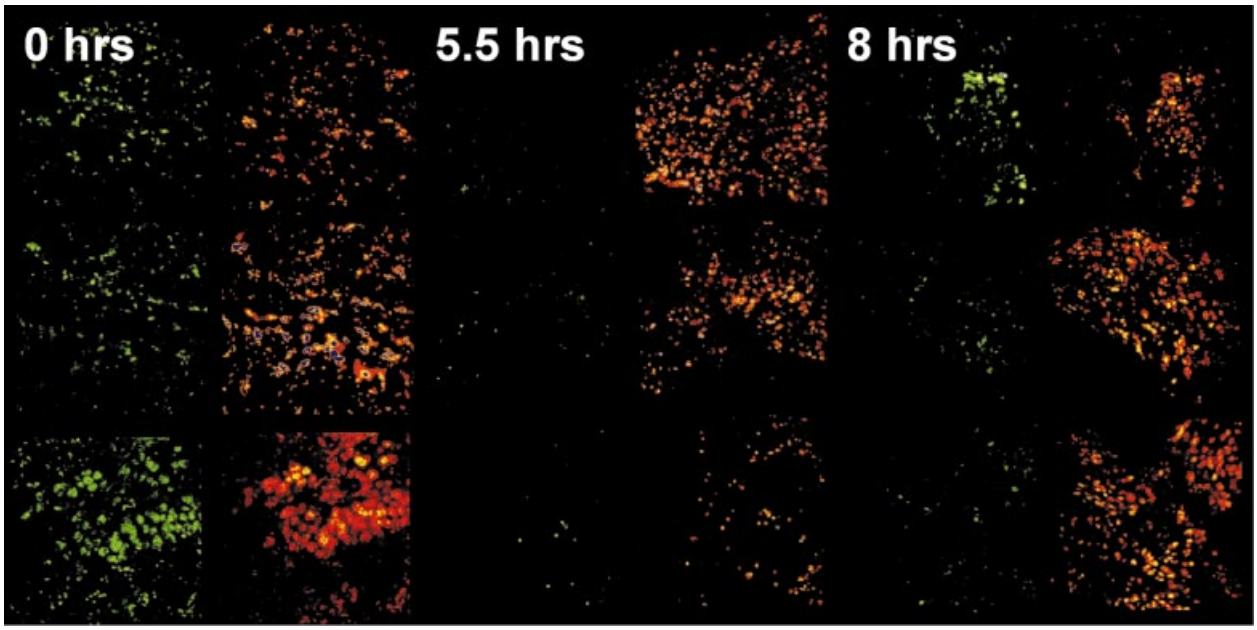

Fig. 7. Inhibition of *P.aeruginosa* QS in mouse lungs. Photomicrographs of mouse lung tissue infected with *P.aeruginosa* carrying the dual-labeled PA quorum sensor for detection of QS signaling and a red fluorescent tag for simple identification in tissue samples. Mice were administered C-30 via intravenous injection at time zero. Infected animals were killed in groups of three at the time points indicated and the lung tissue samples were examined by SCLM.

map the furanone target genes. We constructed a signaling-deficient *lasI rhlI* mutant from the sequenced *P.aeruginosa* PAO1 strain to identify furanone target genes and AHL-activated genes. Using a 5-fold cut-off, we identified 163 genes activated by quorum signals (Figure 4). Among these genes, most previously reported QS genes were present, though some, like *toxA*, *sodA* and *kat*, were absent. We examined the expression profiles of these genes in the wild type, but found that they were expressed below the detection limit. This shows, not surprisingly, that the constitution of the QS regulon depends on the experimental conditions.

We also compared the expression profiles of the AHL-induced genes in the signaling-deficient mutant with wild-type expression patterns. The expression profiles were similar despite differences in the experimental scenarios (mutant grown in the presence of saturating concentrations of AHLs). This observation is in agreement with those of other researchers, but nonetheless surprising in the light of the paradigm which states that QS-controlled induction

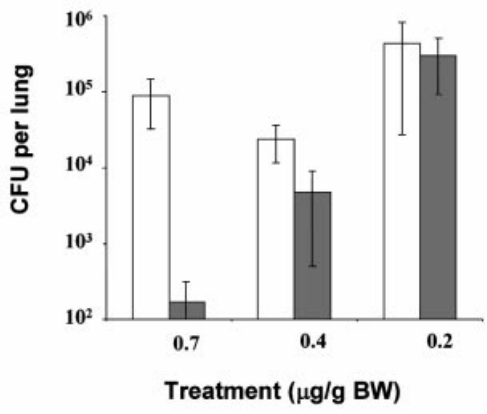

Fig. 8. Lung bacteriology. Healthy CBA/J mice (10 in each group) were infected with PAO1 as described in the text. Furanone C-30 (solid bars; concentration as indicated below the *x*-axis) or saline (open bars) were given three times a day (every 7 h) for 3 days. The mice were killed on day 7 after the bacterial challenge and the bacterial content of the infected lungs was determined as described in the text. Average values were plotted and the standard deviation is shown by error bars. The values were tested by means of an *F*-test (analysis of variance), and the *p* values for the 0.7, 0.4 and the 0.2 μM C-30 concentrations were 0.0007, 0.004 and 0.5, respectively.

occurs in response to build-up of extracellular signal molecules. The fact that gene expression cannot be induced prematurely at low cell densities in response to the addition of high doses of exogenous signals indicates that the onset of induction is not simply dictated by the signal concentration. Several other studies have pointed to regulatory factors involved in timing the onset of quorum induction; such factors include MvaT, QscR and RsmA (Chugani *et al*., 2001; Pessi *et al*., 2001; Diggle *et al*., 2002).

A comparative analysis of the QS regulon and C-30 target genes shows that 80% of the furanone-repressed genes are in fact controlled by QS. Furanone-repressed genes are not restricted to genes regulated by either the *las* or the *rhl* encoded systems, but are found throughout the continuum of QS-induced genes. Among the QS-induced genes, the majority were repressed by C-30. Importantly, the remaining non-repressed genes did not exhibit an obvious cell density-dependent expression profile in the wild-type strain. This is likely attributed to the different experimental conditions: QS-controlled genes were identified in a *lasI rhlI* mutant background grown with saturating levels of exogenous AHLs, whereas the furanone target genes were identified in the wild-type strain producing AHLs with increasing cell density. In essence, our analysis shows a clear overlap between strongly QS-induced genes and efficiently furanone-repressed genes. These genes include major virulence factors like *lasA*, *lasB*, *hcnAB*, *rhlAB*, *chiC*, *phnAB* and *phzABCDEFG* (see Figure 4). Intriguingly, expression of the *lasI lasR* and *rhlI rhlR* gene clusters, which encode the central components of the *P.aeruginosa* QS system, were not notably affected by the furanone treatment. This observation suggests that C-30 does not interfere with some of the regulatory systems controlling transcription of the *lasRI* and *rhlRI* genes, but rather that the furanone acts on these QS regulators at the post-transcriptional level.

Recently, two independent studies reported on identification of *P.aeruginosa* QS-controlled genes by microarray analysis (Schuster *et al*., 2003; Wagner *et al*., 2003). We have compared the results from the three studies in order to investigate whether the constitution of the QS regulon is affected by experimental factors. Our comparison shows a major overlap between the independent studies, but also points to several subsets of genes that appear to be QS controlled only under certain experimental conditions (Supplementary figure S3). We have termed the common group of QS-controlled genes the 'general QS regulon' of *P.aeruginosa* (Supplementary table S2). This designation relies entirely on data extracted from experiments using Affymetrix GeneChip® *P.aeruginosa* Genome Arrays and is sensitive to the significance cut-off value applied. Interestingly, the general QS regulon (except PA0144) was also induced by cells during biofilm growth (Supplementary table S1).

In a previous study, 39 QS-controlled genes were identified in *P.aeruginosa* by *lacZ*-based promoter probing (Whiteley *et al*., 1999). We have analyzed this data set as a control for our *P.aeruginosa* microarray data. The comparison shows good overall agreement between the independent data sets, both in terms of signal responses and signal specificities (Supplementary table S3).

Among the furanone target genes, we have identified the *phnAB* operon (covering PA0996–PA1002) (Cao *et al*., 2001). Similar data (Schuster *et al*., 2003; Wagner *et al*., 2003) show that this operon is subject to QS regulation. Previously, this operon was reported to be controlled by MvfR, a LysR-like transcriptional regulator required for maximum *P.aeruginosa* PA14 virulence in a plant leaf and the burned mouse model (Rahme *et al*., 2000) as well as in nematodes (Mahajan-Miklos *et al*., 1999). Recently, the *phnAB* operon was shown to encode enzymes responsible for biosynthesis of the *Pseudomonas* quinolone signal (PQS), thus giving rise to the alternative *pqs* operon designation (Gallagher *et al*., 2002). The PQS signaling system is hierarchically placed between the LasR and RhlR controlled circuits. Our array data confirm the proposed model in which *pqsH* is controlled by the *las* system (Gallagher *et al*., 2002), but in addition our analysis shows that the entire *pqs* operon is controlled by the *las* system. Interestingly, our data indicate that a *las*-dependent upregulation of *mvfR* expression precedes AHL-induced expression of the *pqs* operon.

The microarray analysis demonstrates that C-30 does not affect basal life processes. The furanones exhibit a high degree of specificity for the *las* and *rhl* quorum sensors. Furthermore, it is notable that C-30, in concentrations that significantly lower quorum-induced gene expression in planktonic cells, is equally active on biofilm bacteria despite the profoundly different lifestyles. In contrast, classical antibiotics used for the treatment of *P.aeruginosa* infections, such as tobramycin and piperacillin, are required in 100- to 1000-fold higher concentrations to eradicate biofilm bacteria compared with their planktonic counterparts (Anwar and Costerton, 1990). We propose that this difference is inherent to the furanone mode of action. Unlike classic antibiotics, which often target intracellular proteins involved in basal life processes, furanones might comprise a natural chemical defense system developed through the course of evolution

to target and inactivate receptors of bacterial communication systems which, in turn, control virulence factor production, surface colonization and biofilm formation. Taken together, the effects on *P.aeruginosa* virulence factor production and biofilm sensitivity probably account for the rapid clearing of the bacteria in the pulmonary mouse model. Most importantly, the results demonstrate that bacterial virulence can be controlled by means of substances that specifically block cell-to-cell communication. Given the large number of bacteria that employ QS systems (Eberl, 1999), QSI compounds may find applications in many different settings, such as medicine, agriculture and food technology. Chemical attenuation of bacterial virulence, rather than bactericidal or bacteristatic strategies, is a highly attractive concept because such antipathogenic agents are less likely to pose a selective pressure for development of resistant mutants.

In the context of the present study, we envision that the concept of direct targeting virulence is promising as an early prophylactic treatment of individuals with *P.aeruginosa* infections. QSI drugs might prevent the formation of detrimental biofilms in the lung, on implants or in wounds. For cystic fibrosis patients, this might suffice to alter the delicate host–pathogen balance in favor of the host clearance mechanisms and thereby reduce the severity of infection.

Materials and methods

Bacterial strains

The *P.aeruginosa* PAO1 was obtained from the *Pseudomonas* Genetic Stock Center (www.pseudomonas.med.ecu.edu, strain PAO0001). This PAO1 isolate has served as the DNA source for the *Pseudomonas* Genome Project (www.pseudomonas.com) and subsequently as the template for the design of the *P.aeruginosa* GeneChip® (Affymetrix Inc., Santa Clara, CA). The *lasI rhlI* mutant was constructed using previously described knockout systems (Beatson *et al*., 2002). The knockout mutant was verified by genetic analysis and by screening for AHL production.

Detection of QS signaling

Detection of AHL signaling in planktonic cultures, biofilms and mouse lungs was achieved using an *E.coli* JM105 strain carrying the monitor plasmid pJBA132Gm, which encodes an unstable GFP reporter [*luxR-PluxI-gfp(ASV)*] for AHL detection (Wu *et al*., 2000; Andersen *et al*., 2001). For the present study, a *dsred* expression cassette was inserted into the *Eco*RV restriction site of this plasmid. This construct was referred to as a 'dual-labeled AHL sensor'. OdDHL and BHL signaling were monitored using the *P.aeruginosa* PAO1 strain carrying a translational *lasB-gfp*(ASV) reporter fusion pMHLAS and a *dsred* expression cassette on a mini-Tn*5* transposon element as described previously (Hentzer *et al*., 2002b). This is referred to as a 'dual-labeled PA quorum sensor'.

Measurement of virulence factors

Strains were grown in Luria broth medium in the absence and presence of C-30 (1 and 10 μM) and harvested as described previously (Hentzer *et al*., 2002b). Assays for proteolytic activity and chitinase activity were performed as described elsewhere (Hentzer *et al*., 2002b). Pyoverdin production was monitored by 380 nm absorbance as described elsewhere (Prince *et al*., 1993).

Microarray analysis

Batch cultures of *P.aeruginosa* PAO1 were grown in ABt minimal medium with 0.5% casamino acids at 37°C and 200 r.p.m. A culture was inoculated with exponentially growing cells to an OD_{600} of 0.05. A reference sample for QS-controlled gene expression was retrieved at low cell density (OD_{600} = 0.5). The culture was split into two at a density of 0.7, and 10 μM furanone C-30 was added to one of the cultures. Further samples were retrieved at cell densities of 1.3, 1.6, 2.0 and 2.7. Samples for RNA isolation were immediately transferred to 2 vols of RNAlater (Ambion Inc., Austin, TX) and stored at –80°C. Microarray analysis of the QS regulon was performed by growing a culture of the *lasI rhlI* mutant to an OD_{600} of 0.3. The culture was split into three and the following additions were made: (i) no AHLs; (ii) 2 μM OdDHL; (iii) 2 μM OdDHL and 5 μM BHL. Samples were retrieved at the OD_{600} values given above. RNA purification and further processing for microarray analysis were performed according to the guidelines provided by the manufacturer of the GeneChip® *P.aeruginosa* Genome Array (Affymetrix Inc.). Microarray data analysis was performed using Affymetrix Microarray Suite 5.0 and Data Mining Tool 3.0 software. Average microarray hybridization signal intensity was scaled to 2500. For data analysis, the minimum and maximum signal thresholds were set to 200 and 100 000, respectively. The data represent the results of at least two independent experiments. A Mann–Whitney test was used to identify genes showing differential expression ($p \leqslant 0.05$). In this article, we have only shown genes >5-fold repressed or induced and with an absolute difference in hybridization signal of >600. In our experimental settings, these criteria corresponded to a p value $\leqslant 0.03$.

Biofilm experiments

Biofilms were cultivated in continuous-culture once-through flow chambers perfused with sterile ABtrace minimal medium containing 0.3 mM glucose as described previously (Hentzer *et al*., 2002b). Furanone C-30 was added to the ABtrace medium where appropriate. Biofilm development was examined by SCLM using a Zeiss LSM 510 system (Carl Zeiss GmbH, Jena, Germany) equipped with an argon laser and a helium–neon laser for excitation of fluorophores. Tolerance of biofilms to tobramycin and SDS was assessed by introducing 100 μg/ml tobramycin or 0.1% SDS, respectively, to the influent medium to 3-day-old biofilms. After 24 h, biofilms were examined by SCLM. Bacterial viability in planktonic and biofilm cultures was assessed using the LIVE/DEAD *Bac*Light bacterial viability staining kit (Molecular Probes Inc., Eugene, OR) as described elsewhere (Hentzer *et al*., 2001).

Pulmonary mouse model

Healthy CBA/J mouse strains aged 11 weeks (average body weight 30 g) were used in animal studies. Immobilization of *P.aeruginosa* (5×10^8 CFU/ml) and *E.coli* (1×10^9 CFU/ml) in seaweed alginate beads and the surgical introduction of bacteria into mouse lungs (40 μl injected per lung) were performed as described previously (Wu *et al*., 2000). The mice were given OHHL by intravenous injection. The mouse total body volume was estimated to be 30 ml, which for the calculation of concentrations is ~30 g. Furanone C-30 was administered by either intravenous or subcutaneous injection. AHL signal molecules and C-30 were prepared as stock solutions in 50% ethanol, which were diluted 20-fold in 0.9% NaCl prior to injection. Intravenous injection in the tail vein was applied to ensure that drugs were transported directly to the lung before passing the liver. Subcutaneous injections were applied where slow drug release was required. Preparation of lung tissue samples, subsequent SCLM examination and determination of lung bacteriology were performed as described elsewhere (Wu *et al*., 2000).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

This work was supported by grants from the Danish Technical Research Council, the Danish Medical Research Council, the Plasmid Foundation and the Villum Kann-Rasmussen Foundation to M.G., and from the Cystic Fibrosis Foundation, Therapeutics Inc. and the German Mukoviszidose e.V. to M.G. and L.E. M.A.S. was supported by a Skou stipend. The technical assistance of Linda Stabell was greatly appreciated.

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Received December 11, 2002; revised May 8, 2003;
accepted June 2, 2003