Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein

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Acylated homoserine lactone (AHL)-mediated gene expression controls phenotypes involved in colonization, often specifically of higher organisms, in both marine and terrestrial environments. The marine red alga *Delisea pulchra* produces halogenated furanones which resemble AHLs structurally and show inhibitory activity at ecologically realistic concentrations in AHL bioassays. Evidence is presented that halogenated furanones displace tritiated OHHL [*N*-3-(oxohexanoyl)-L-homoserine lactone] from *Escherichia coli* cells overproducing LuxR with potencies corresponding to their respective inhibitory activities in an AHL-regulated bioluminescence assay, indicating that this is the mechanism by which furanones inhibit AHL-dependent phenotypes. Alternative mechanisms for this phenomenon are also addressed. General metabolic disruption was assessed with two-dimensional PAGE, revealing limited non-AHL-related effects. A direct chemical interaction between the algal compounds and AHLs, as monitored by ¹H NMR spectroscopy, was shown not to occur *in vitro*. These results support the contention that furanones, at the concentrations produced by the alga, can control bacterial colonization of surfaces by specifically interfering with AHL-mediated gene expression at the level of the LuxR protein.

Keywords: quorum sensing, AHL, acylated homoserine lactone, *Delisea pulchra*, halogenated furanones

INTRODUCTION

Acylated homoserine lactone (AHL)-mediated gene transcription controls a variety of bacterial phenotypes (for reviews see Fuqua *et al.*, 1996; and Swift *et al.*, 1996). The expression of bioluminescence in *Vibrio fischeri* is the most extensively studied example (Sitnikov *et al.*, 1995). Two genes constitute the primary level of regulatory control involved in the expression of this phenotype. One encodes an AHL synthase protein, known as a LuxI homologue. The other encodes a regulatory protein which alters transcriptional promoter activity of nominated genes in the presence of the AHL

signal molecule and is known as a LuxR homologue. For example, in *V. fischeri* LuxI synthesizes *N*-3-(oxohexanoyl)-L-homoserine lactone (OHHL) which binds to LuxR, activating transcription of genes encoding the luminescent phenotype (*luxCDABEG*). AHLs comprise the homoserine lactone ring with any one of a variety of modified acyl chains. At a secondary level of regulation other factors, including GroESL (Adar & Ulitzur, 1993), cAMP and cAMP receptor protein (Dunlap, 1989), the *traM* gene product (Fuqua *et al.*, 1995), opines (Fuqua & Winans, 1994), RpoS and HNS (Ulitzur *et al.*, 1997) influence expression of AHL-dependent phenotypes.

AHL-regulated phenotypes often facilitate bacterial colonization of eukaryotes. Examples include elastase production by *Pseudomonas aeruginosa* in lung

Abbreviations: 2-D, two-dimensional; AHL, acylated homoserine lactone; OHHL, *N*-3-(oxohexanoyl)-L-homoserine lactone.

infections of cystic fibrosis patients (Pearson *et al.*, 1997) and exoenzyme production by *Erwinia carotovora* in the maceration of economically important crops (Pirhonen *et al.*, 1993). For instances where these bacterial activities are deleterious to the host, it is likely that higher organisms may have evolved a means of defending themselves from AHL-mediated colonization. Such an evolutionary response has been suggested to have occurred in the red marine alga *Delisea pulchra* (Kjelleberg *et al.*, 1997).

D. pulchra, endemic to the south-eastern coast of Australia, produces a range of halogenated enones and furanones, some of which have strong inhibitory activity against fouling organisms and herbivores (de Nys *et al.*, 1995; Steinberg & van Altena, 1992; R. de Nys and others, unpublished). Additionally, furanones have specific effects on colonization phenotypes of marine bacteria at concentrations found on the surface of the alga (Maximilien *et al.*, 1998). Swarming motility of marine isolates known to produce metabolites active in AHL bioassays (Maximilien, 1996) is one such phenotype. Swarming has been shown to be an AHL-dependent behaviour in *Serratia liquefaciens* (Eberl *et al.*, 1996). The above-mentioned algal metabolites structurally resemble AHLs, and thus we hypothesized that *D. pulchra* defends itself against extensive bacterial colonization by using halogenated furanones as antagonists of AHL-mediated gene expression. This hypothesis was supported by the inhibition of two AHL-dependent phenotypes (swarming and bioluminescence) by two furanones from *D. pulchra*, with no effect on non-AHL-regulated phenotypes such as swimming motility, production of flagella, constitutively expressed bioluminescence and growth (Givskov *et al.*, 1996). Furthermore, because furanones inhibited artificially stimulated AHL-dependent luminescence in the absence of LuxI, we hypothesized that the site of action was on the LuxR protein.

However, direct evidence that halogenated furanones interfere with the interaction between AHLs and the putative binding site of a LuxR homologue remains to be obtained. In this study we used [³H]OHHL and a LuxR overexpression vector to confirm that halogenated furanones displace the bacterial signal from the LuxR-binding site with potencies corresponding to their respective inhibitory activities in an AHL-regulated bioluminescence assay. Components of the *V. fischeri* AHL system were considered most appropriate for use in this investigation given that their function is well characterized and that they are of marine origin (Hanzelka & Greenberg, 1995). Two-dimensional (2-D) PAGE was also employed to assess whether the effect of halogenated furanones on AHL-mediated swarming and bioluminescence can be explained by a subtle metabolic disruption of bacterial cell function. Additionally, the possibility that the algal compounds act via a direct chemical interaction with the bacterial AHL signal was addressed by ¹H NMR spectroscopy and TLC. Our results substantiate the hypothesis that halogenated furanones produced by the eukaryote *D. pulchra* act on

bacteria by displacing AHLs from their cognate LuxR-like regulatory proteins.

METHODS

Bacterial strains and plasmids. The bioluminescent monitor system consisted of *Escherichia coli* MT102 harbouring pSB403. pSB403 consists of the *V. fischeri luxR* and the *luxI* promoter region cloned from pSB237 (Bainton *et al.*, 1992) transcriptionally fused to a promoterless *luxCDABE* cassette derived from *Xenorhabdus luminescens* (Winson *et al.*, 1998). It also encodes tetracycline resistance (20 µg ml⁻¹). When present in *E. coli* MT102, pSB403 cannot produce OHHL but can respond to its exogenous addition with a resultant bioluminescent phenotype. The LuxR overexpression system consists of *E. coli* XL-1 Blue harbouring pHK724 and pGroESL (Hanzelka & Greenberg, 1995). pHK724 encodes the *ptac*-controlled *luxR* gene from *V. fischeri* and ampicillin resistance (50 µg ml⁻¹). pGroESL encodes *plac*-controlled *groESL* genes and chloramphenicol resistance (25 µg ml⁻¹). All strains were propagated on Luria-Bertani (LB) media (Maniatis *et al.*, 1982) with appropriate antibiotics for plasmid maintenance, unless otherwise specified.

Extraction of natural furanones. Furanones were extracted from *D. pulchra* according to protocols established by de Nys *et al.* (1993). Algal tissue was frozen, freeze-dried, extracted with dichloromethane and reduced *in vacuo*. Pure compounds were isolated from this crude extract by vacuum liquid chromatography and HPLC, and identified by ¹H and ¹³C NMR spectroscopy (de Nys *et al.*, 1993). Furanones were dissolved in ethanol for use in assays.

Synthesis of OHHL, [³H]OHHL and compound 8. Synthetic OHHL was prepared according to the method of Eberhard *et al.* (1986). OHHL was dissolved in ethyl acetate for use in assays. [³H]OHHL was prepared by an improved synthesis protocol (W. Lao, M. A. Long, N. Kumar & R. W. Read, unpublished) based on the method of Kaplan *et al.* (1985). Compound 8 was prepared according to protocols established by Manny *et al.* (1997).

Bioluminescence assay and growth. Bioluminescence was quantified as relative light units on a Wallac 1450 Microbeta Plus liquid scintillation counter. OHHL and furanones were added to dilute 10 ml cultures (OD₆₀₀ < 0.005) and the luminescence response of 100 µl samples in triplicate was measured after 4 h incubation at 30 °C. Concentration ranges between 10 nM and 100 nM OHHL and up to 100 µM of different furanones (compounds 1, 2, 3, 4, 5 and 8) were employed. Appropriate concentrations of the ethanol solvent were incorporated into control treatments.

Growth was monitored by following the OD₆₀₀ of 10 ml cultures in appropriate media from lag-, through exponential- into stationary-phase growth at 30 °C. Growth measurements were performed in triplicate.

[³H]OHHL binding and displacement. Binding and displacement protocols closely follow that of Hanzelka & Greenberg (1995). *E. coli* XL-1 Blue(pHK724, pGroESL) and *E. coli* XL-1 Blue(pGroESL) cultures were grown from OD₆₀₀ 0.01 to 0.30 with shaking at 30 °C in the presence of 1 mM IPTG to stimulate the *plac* and *ptac* promoters. At OD₆₀₀ 0.3, 0.5 µM [³H]OHHL with or without 10–100 µM furanone (compound 2, 4 or 8) or 0.5–5.0 µM OHHL was added to 1 ml aliquots of both cultures in triplicate. Cultures were incubated at room temperature with shaking for a further 10 min. Cells were centrifuged at 16000 g for 4 min at 4 °C. The cell pellets were

washed twice with 500 µl LB medium and finally resuspended in 100 µl scintillation fluid. Radioactivity of samples was quantified on a Wallac 1450 Microbeta Plus liquid scintillation counter.

2-D PAGE. *E. coli* MT102(pSB403) was incubated in Difco minimal salts medium (without dextrose) plus 0.2% glucose and 0.1% Casamino acids with shaking at 30 °C. At OD₆₀₀ 0.03 the culture was divided into two vessels, one of which was treated with 0.5 µM OHHL, the other with ethyl acetate as a control, and incubation continued. At OD₆₀₀ 0.2 the OHHL-treated culture was again divided into two, one half of which was treated with 50 µM compound 4 and the other half with ethanol as a control. Incubation was continued for 30 min. Three 0.5 ml aliquots of each culture were transferred to 25 ml glass flasks and treated with [³⁵S]methionine for 10 min with shaking at room temperature. Incorporation was stopped by chasing with 50 mM unlabelled methionine for 4 min. Cultures were centrifuged at 20000 *g* for 20 min at 4 °C and pellets frozen at -75 °C. Proteins were extracted from cell pellets and resolved according to their charge and mass as described by O'Farrell (1975). The IEF dimension contained 4.1% (w/v) polyacrylamide and 6.25% ampholytes (pH 4–8, Millipore). The second dimension was an 11.5% SDS-polyacrylamide gel. All conditions were according to Millipore specifications. Autoradiograms were prepared by 10 d exposure to Fuji RX film. Analysis was performed with the Bioimage 2-D analyser. Triplicate samples were resolved for each treatment on at least two separate occasions. Putative identity of spots was found by manually assessing the gene-protein database of *E. coli* K-12 (VanBogelen *et al.*, 1996).

In vitro reaction of OHHL and compound 4. Solutions of OHHL (9.3 mg, 4.4×10^{-5} mol) and 4-bromo-5-(bromo-methylene)-3-(1-hydroxybutyl)-2(5H)-furanone (compound 4) (14.2 mg, 4.4×10^{-5} mol) in 0.5 ml CDCl₃ (non-polar medium) or 0.5 ml d₆-DMSO (polar medium) were kept at room temperature, in the presence or absence of 1 ml stationary-phase *E. coli* MT102(pSB403) culture (from which the compounds were extracted with ethyl acetate) and monitored by 300 Hz ¹H NMR spectroscopy and normal-phase silica gel TLC (eluent employed were either dichloromethane or 1% methanol in dichloromethane) over a 48 h period.

RESULTS

Selecting a halogenated furanone for investigation

D. pulchra produces more than 20 halogenated furanones (de Nys *et al.*, 1993). To identify which furanone would constitute the most effective antagonist of the AHL system for use in the following investigation, we compared the activity of these naturally produced compounds and a number of synthetic analogues against the bioluminescent monitor strain *E. coli* MT102(pSB403). Of the molecules tested, compounds 3, 5 and 4 (Fig. 1) exhibited the greatest activity and were thus employed in this study. Compound 8 (Fig. 1), a synthetic halogenated furanone, was included as an example of a molecule with relatively weak activity. Compounds 1 and 2, which have been employed predominantly in previous studies, were also included (Givskov *et al.*, 1996; Gram *et al.*, 1996). Compounds 1–5 are the most abundant furanones produced by the alga (de Nys *et al.*, 1993).

	R1	R2	R3	R4
compound 1	H	Br	Br	Br
compound 2	H	Br	H	Br
compound 3	OAc	Br	H	Br
compound 4	OH	Br	H	Br
compound 5	OAc	Br	H	I
compound 8	H	H	Br	Br

Fig. 1. Structural variations of five halogenated furanones produced by the benthic marine macroalga *D. pulchra* (compounds 1–5). Compound 8 was produced synthetically.

Fig. 2. Inhibitory effect of six halogenated furanones (compounds 1, 2, 3, 4, 5 and 8) at a concentration of 100 µM (50 µM for compound 4) on bioluminescence from OHHL-stimulated *E. coli*(pSB403) (spotted bars). Partial relief of inhibition is shown as a result of increasing the concentration of OHHL from 10 nM (spotted bars) to 100 nM (white bars). Luminescence output from cultures treated with either 10 nM or 100 nM OHHL alone was identical (black bar). The experiment was performed in triplicate and error bars represent standard deviation.

Fig. 2 shows inhibition of the bioluminescent phenotype by compounds 1, 2, 3, 4, 5 and 8. None of the compounds had an effect on growth of *E. coli*(pSB403) at the concentrations tested (data not shown). Each furanone displayed a different potency, with compound 4 eliciting the largest response followed by compound 5, com-

Fig. 3. *In vivo* displacement of [³H]OHHL (0.5 μM) from *E. coli*(pHK724, pGroESL) cells overproducing LuxR. Bars represent radioactive signal bound in *E. coli*(pHK724, pGroESL) cultures treated with: (1) 0.5 μM [³H]OHHL alone; or (3–9) 0.5 μM [³H]OHHL plus (3) 0.5 μM OHHL, (4) 5 μM OHHL, (5) 10 μM compound 4, (6) 50 μM compound 4, (7) 100 μM compound 4, (8) 100 μM compound 2, or (9) 100 μM compound 8. Bar 2 represents *E. coli*(pGroESL) treated with 0.5 μM [³H]OHHL alone. All treatments were performed in triplicate and error bars represent standard deviation.

pound 3 and compounds 1, 2 and 8 in that order. Additionally, the relief of inhibition produced by adding more OHHL to the culture medium for each compound is shown. Competitive relief of inhibition was partial, with the effect of the strongest inhibitors being the most difficult to antagonize with OHHL. Based on potency, compound 4 was selected as the most appropriate halogenated furanone with which to perform the studies reported in this paper.

Displacement of [³H]OHHL from LuxR by furanones

It has previously been shown that [³H]OHHL binds to *E. coli* cells overproducing LuxR (Adar & Ulitzur, 1993; Hanzelka & Greenberg, 1995). This is the strongest evidence supporting an interaction between OHHL and LuxR. The current model of inhibition by furanones suggests that the algal metabolites act by disrupting this interaction (Givskov *et al.*, 1996). To test this directly we first added [³H]OHHL to a final concentration of 0.5 μM to cultures of *E. coli* XL-1 Blue(pGroESL) and *E. coli* XL-1 Blue(pHK724, pGroESL). pGroESL encodes chaperone proteins which assist in the folding of a functional LuxR (Hanzelka & Greenberg, 1995). In preliminary binding experiments three different concentrations of [³H]OHHL (0.04, 0.2 and 2 μM) were tested. In support of previously published results, significantly more [³H]OHHL bound to *E. coli* cells overproducing LuxR than to wild-type cells (Fig. 3). At 0.2 μM [³H]OHHL the cells bound only 16% of what was bound when 2 μM was used (data not shown). This suggested that 0.2 μM [³H]OHHL did not saturate the LuxR overproduction system. Based on this, 0.5 μM

[³H]OHHL was considered a close to saturating concentration to employ in the study. Compound 4, the most inhibitory furanone in the luminescent assay, was then tested at different concentrations (10, 50 and 100 μM) for its ability to displace [³H]OHHL from *E. coli*(pHK724, pGroESL) cultures. Fig. 3 shows that compound 4 displaces [³H]OHHL from *E. coli* cells overproducing LuxR in a concentration-dependent manner. Similarly, compound 2, which has been used predominantly in previous studies (Givskov *et al.*, 1996), was tested at 100 μM for its effect on displacement. Compound 2 also reduced binding of [³H]OHHL to *E. coli*(pHK724, pGroESL) cultures, but to a lesser extent than compound 4, corresponding to its lower activity in the luminescent assay. Finally we tested the ability of compound 8, which is much less active in the bioluminescent phenotypic assay than compound 2 or compound 4 (Fig. 2), to displace [³H]OHHL from *E. coli*(pHK724, pGroESL) cultures. Fig. 3 shows that the effect of 100 μM compound 8 on the OHHL-LuxR interaction is much less than for the other furanones.

The ability of unlabelled OHHL to displace [³H]OHHL from LuxR-overproducing cells is also shown in Fig. 3. This allows for a comparison of the displacing efficacy between OHHL and furanones. The results suggest that compound 4 is approximately 25 times less efficient than OHHL at displacing [³H]OHHL from LuxR-overproducing *E. coli* cells.

Furanones have limited impact on global protein production: 2-D PAGE analysis

2-D PAGE was employed to find out if compound 4 has any significant non-target effects on protein synthesis in the bioluminescent monitor strain *E. coli*(pSB403). We regarded *E. coli*, which has no AHL signalling system of its own and a comprehensive proteome database, as the most appropriate organism in which to assess non-AHL-related effects of furanones. Three samples of cells labelled with [³⁵S]methionine were prepared and analysed: (i) *E. coli*(pSB403) untreated (data not shown); (ii) *E. coli*(pSB403) plus 0.5 μM OHHL (Fig. 4a); (iii) *E. coli*(pSB403) plus 0.5 μM OHHL and 50 μM compound 4 (Fig. 4b). It was possible to compare approximately 400 protein spots based on whether they could be matched positionally and did not vary in intensity by more than 20% of the mean value on triplicate gels. A greater than twofold change in spot density was considered as a difference in protein expression.

The addition of OHHL to *E. coli*(pSB403) culture medium upregulated only three of the proteins resolved in the analysis (Fig. 4). According to their apparent pI and *M_r*, these three proteins are likely to be LuxA, LuxB and LuxD (labelled 1, 2 and 3, respectively, in Fig. 4) from the *X. luminescens* lux cassette encoded on pSB403 (Johnston *et al.*, 1990; Cochrum *et al.*, 1991). Addition of compound 4 to the culture medium downregulated the expression of these three proteins (Fig. 4). There were an additional nine changes (three downregulated

Fig. 4. 2-D PAGE gel of *E. coli*(pSB403) treated with 0.5 μ M OHHL (a) and 0.5 μ M OHHL plus 50 μ M compound 4 (b) prior to [35 S]methionine pulse-labelling. Six proteins (1–6) are downregulated by 50 μ M compound 4 (black arrows), including those three (1–3) upregulated by 0.5 μ M OHHL. An additional six proteins (A–F) are upregulated by 50 μ M compound 4 (white arrows). Spots with a twofold density shift were considered to have altered expression.

Table 1. Putative identities of proteins with altered expression in the presence of compound 4

Spot	Coordinates*	A-N†	Protein	Gene
Downregulated				
1	Not found	–	LuxA	<i>luxA</i>
2	Not found	–	LuxB	<i>luxB</i>
3	Not found	–	LuxD	<i>luxD</i>
4	106, 108	B036.0	OmpF	<i>ompF</i>
5	97, 146.5	B066.0	DnaK/Hsp70	<i>dnaK</i>
6	76.5, 134.5	D050.0	Glutamate–ammonia ligase	<i>glnA</i>
Upregulated				
A	Not found	–	–	–
B	55.5, 130.5	F048.8	Glucose-6-phosphate dehydrogenase	<i>zwf</i>
C	79.5, 149.5	C075	Not known	–
D	83.5, 106.5	C037.5	Not known	–
E	40, 82.5	G025.9	Not known	–
F	85.5, 68.5	B020.9	Alkylhydroperoxide reductase	<i>ahpC</i>

* x, y coordinates in *E. coli* K-12 gene-protein database.

† Alpha-numeric ID of protein spots in *E. coli* K-12 gene-protein database.

and six upregulated) in protein expression in response to compound 4. The identity of some of these proteins, as predicted by comparison with the *E. coli* K-12 database (VanBogelen *et al.*, 1996) are presented in Table 1. One

spot (A) could not be associated with any proteins in the database and the identities of three of the remaining eight proteins are currently not known. Compound 4 (50 μ M) downregulated production of the outer-mem-

Fig. 5. ^1H NMR spectra of (a) compound 4, (b) OHHL and (c) a mixture of compound 4 and OHHL in CDCl_3 incubated at room temperature for 24 h. Characteristic spectra for both compounds (a, b) observed individually are present in the mixture (c), indicating no reaction between the two compounds.

brane porin protein OmpF, the chaperone DnaK and the nitrogen-limitation-induced glutamate–ammonia ligase. The same treatment upregulated production of glucose-6-phosphate dehydrogenase (*zwf* gene product), which acts in the oxidative branch of the pentose-phosphate pathway, and alkylhydroperoxide reductase (*ahpC* gene product), which responds to oxidative stress. The significance of these particular changes is considered in the Discussion. These minor changes in non-AHL-related proteome expression and the significant changes in expression of the putative LuxA, LuxB and LuxD proteins indicated that halogenated furanones do not disrupt normal metabolic activity and that the effect on the expression of the target genes is specific.

Furanones and OHHL do not react *in vitro*

The inhibition of AHL-regulated phenotypes by furanones could potentially be explained by a covalent interaction between the two molecules. To test this hypothesis compound 4 was mixed *in vitro* with OHHL in a polar (DMSO) or non-polar (chloroform) medium in the presence or absence of an *E. coli* culture. The polarity of the solvent was varied to see if this was an important factor in a hypothetical interaction. The polarity of DMSO is similar to that of water and is thus more ecologically relevant. The *E. coli* culture was

included to ascertain whether its presence facilitated a reaction between the compounds. After 2, 8, 24 or 48 h no changes in the proton chemical shifts for either OHHL or compound 4, as determined by ^1H NMR spectroscopy, were observed in any of the four treatments, indicating no reaction between the two compounds. Fig. 5 shows the ^1H NMR spectra for compound 4 and OHHL individually and mixed in chloroform after 24 h. Similarly, migration of the mixture on TLC plates indicated the presence of OHHL and compound 4 only (data not shown).

DISCUSSION

The attention currently being paid to chemical signalling in bacterial ecology is unprecedented. Signals are employed by and against a wide cross-section of the microbial community (Kleerebezem *et al.*, 1997; Fuqua *et al.*, 1996; Dunny *et al.*, 1995; Guangyong *et al.*, 1997). Out of the diverse range of signalling systems, AHL-mediated gene expression is unparalleled in its predominant regulation of colonization phenotypes, often of higher organisms. Given this and the diversity of biologically active secondary metabolites in marine and terrestrial eukaryotes, it would be expected that these higher organisms have evolved chemical inhibitors of AHL-mediated gene expression. We have hypothesized that furanones from the red alga *D. pulchra* function in just this fashion.

To date, evidence for this hypothesis, besides the structural similarities between furanones and AHLs, stems from the furanone-imposed inhibition of colonization phenotypes of marine bacteria (Maximilien *et al.*, 1998) and AHL-dependent phenotypes of assay strains (Givskov *et al.*, 1996). These inhibitory effects occur at ecologically realistic concentrations of the algal metabolites, as based on epifluorescence microscopy (furanones occur in specialized gland cells at the plant surface) and direct measurement of surface concentrations of furanones (de Nys *et al.*, 1998). The ecological consequences of furanones at the surface of *D. pulchra* are lower levels of bacteria on parts of the plant that have high levels of furanones and lower levels of bacteria on *D. pulchra* in comparison to other algae in the same environment (Maximilien *et al.*, 1998).

To confirm our hypothesis that *D. pulchra* inhibits bacteria by interfering with AHL-mediated gene expression, we need to demonstrate (i) the mechanism of action of furanones in AHL systems and (ii) that furanones inhibit AHL systems in bacteria that co-occur with *D. pulchra*. Genetic characterization of AHL systems in these marine bacteria is in progress (unpublished data). Here we focus on identifying the mechanism of action of furanones in AHL systems. The *V. fischeri* AHL system was preferred for this study owing to its thorough characterization and the marine origin of the organism.

To test directly whether halogenated furanones displace AHL from the putative binding site on the regulatory

protein we employed the LuxR overproducer *E. coli*(pHK724, pGroESL) and [^3H]OHHL. Compound 4 was shown to be capable of such displacement, with compound 2 and compound 8 showing decreasing activity. The different activities in the displacement assay correspond to the inhibitory strengths that the furanones exhibited in the luminescence assay. These findings, in conjunction with the partial relief of furanone-imposed inhibition of bioluminescence by OHHL (Fig. 2), support a model in which furanones bind or disfigure the OHHL-binding site of LuxR.

It may be questioned whether halogenated furanones exert their effect on non-essential phenotypes such as swarming and bioluminescence by a non-specific disruption of primary metabolic function. Monitoring the optical density of cultures as a measure of growth in the presence of the compounds suggests this is not the case (Givskov *et al.*, 1996); however it can be argued that the disruption may be too subtle to affect cell division and thus may not be detected. Analysis of protein production by 2-D PAGE provides definitive evidence that furanones, at the concentrations employed, have limited non-AHL-related effects. Of approximately 400 proteins resolved only 12 were affected by the addition of 50 μM compound 4. Of these 12, three are upregulated by OHHL, suggesting they are *lux* proteins and are thus expected to show shifts in expression. The predicted pI and M_r of these protein spots are in accordance with this.

Attempts to identify the remaining changes by comparison with the *E. coli* K-12 gene-protein database revealed the upregulation of glucose-6-phosphate dehydrogenase (*zwf* gene product) and alkylhydroperoxide reductase (*ahpC* gene product). These gene products belong to two separate regulons (SoxRS and OxyR, respectively) which are active in the presence of oxidative stress. Each operon contains approximately 40 proteins. Analysis of the data presented in Fig. 4 revealed that no changes in expression of the remaining proteins in these regulons occurred. This suggests that the upregulation of *zwf* and *ahpC* does not represent an induction of oxidative stress responses in the presence of 50 μM compound 4. The significance of the downregulation of the *dnaK* and *glnA* gene products is not currently understood. The downregulation of OmpF, in light of the enhanced synthesis of two of the oxidative stress gene products, might in principle be explained by increased expression of the SoxR-dependent *micF* gene, the product of which inhibits synthesis of OmpF. However, the lack of induction of all but two of the visualized protein spots in the oxidative stress regulons makes this explanation less plausible. Upregulation of the unidentified proteins designated with alpha-numeric IDs C075, C037.5 and G025.9 is not associated with the oxidative stress regulons (VanBogelen *et al.*, 1996).

The limited effect of OHHL on expression of proteins in *E. coli*(pSB403) is a reminder of the non-native background in which the bioluminescence assay is performed. OHHL does not perform the function of

signalling molecules contained in *E. coli* (Garcia-Lara *et al.*, 1996).

Given the similarities in their structures, the mode of action by which furanones interfere with OHHL-driven bioluminescence is most likely to involve the AHL-binding site. However, it is possible that the observed effects of the halogenated furanones on AHL systems could be explained by a direct chemical interaction between the algal metabolites and the bacterial signals which disables the AHL's ability to activate a LuxR homologue. For example, the hydroxyl group of the most inhibitory furanone (compound 4) might have undergone a transacylation reaction with the keto or amide function of OHHL. Assessment of a mixture of compound 4 and OHHL by ^1H NMR and TLC revealed the presence of the individual compounds only, indicating that there is no *in vitro* reaction between the two. This was observed in both a non-polar and polar medium, and in both the presence and absence of an *E. coli* culture, suggesting that no reaction would take place *in situ* in the natural aqueous environment.

The use of six unique furanone structures in the bioluminescence assay provided the opportunity to assign functional importance to particular moieties. Specifically, the hydroxy group at the C1' position (see compound 4 in Fig. 1) confers a higher inhibitory activity than the acetoxy group (see compound 3), which in turn confers higher activity than a hydrogen atom (see compound 2) at this position. Additionally, this hydroxy group confers an irreversibility of inhibition to the furanone that the acetoxy group does not. Finally, the presence of a bromine atom extending from the carbon 4 position on the furan ring (compare C1 to C8) increases the biological activity of the structure. Such clear differences in activity associated with these minor changes in chemical structure have prompted a more comprehensive structure/function investigation which will be the focus of future studies.

This study adds crucial supportive evidence to the contention that the eukaryote *D. pulchra* controls bacterial colonization of its surface by producing metabolites which specifically interfere with AHL-dependent gene transcription at the level of the LuxR-like regulatory protein. In an evolutionary context, the discovery of naturally produced AHL antagonists by a benthic marine macroalga suggests that other organisms, also subject to AHL-mediated colonization by Gram-negative bacteria, may also possess mechanisms for interfering with bacterial communication.

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