Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover

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***N*-acyl-L-homoserine lactones (AHLs) are co-regulatory ligands required for control of the expression of genes encoding virulence traits in many Gram-negative bacterial species. Recent studies have indicated that AHLs modulate the cellular concentrations of LuxR-type regulatory proteins by binding and fortifying these proteins against proteolytic degradation (Zhu & Winans, 2001). Halogenated furanones produced by the macroalga *Delisea pulchra* inhibit AHL-dependent gene expression. This study assayed for an *in vivo* interaction between a tritiated halogenated furanone and the LuxR protein of *Vibrio fischeri* overproduced in *Escherichia coli*. Whilst a stable interaction between the algal metabolite and the bacterial protein was not found, it was noted by Western analysis that the half-life of the protein is reduced up to 100-fold in the presence of halogenated furanones. This suggests that halogenated furanones modulate LuxR activity but act to destabilize, rather than protect, the AHL-dependent transcriptional activator. The furanone-dependent reduction in the cellular concentration of the LuxR protein was associated with a reduction in expression of a plasmid encoded P_{luxI} -*gfp*(ASV) fusion suggesting that the reduction in LuxR concentration is the mechanism by which furanones control expression of AHL-dependent phenotypes. The mode of action by which halogenated furanones reduce cellular concentrations of the LuxR protein remains to be characterized.**

Keywords: *Delisea pulchra*, homoserine lactone, intercellular signal

INTRODUCTION

Many Gram-negative bacterial species employ *N*-acyl-L-homoserine lactones (AHLs) to control the synthesis of products that facilitate interactions with the surrounding environment, including interactions with eukaryotic host species (reviewed by Eberl, 1999; Kievit & Iglewski, 2000). AHL dependent gene expression has been suggested to constitute a mechanism by which bacteria can alter their behaviour in response to cell density and is thus commonly referred to as quorum sensing (reviewed by Swift *et al.*, 1999).

The diverse range of AHL regulated phenotypes includes the production of degradative extracellular enzymes by

Pseudomonas aeruginosa and *Erwinia carotovora* (Jones *et al.*, 1993), bioluminescence in *Vibrio fischeri* (Sitnikov *et al.*, 1995) and *Vibrio harveyi* (Bassler *et al.*, 1993), plasmid transfer in *Agrobacterium tumefaciens* (Piper *et al.*, 1993), antibiotic production in *E. carotovora* (Bainton *et al.*, 1992), and more complex phenotypes such as surface motility in *Serratia liquefaciens* (Eberl *et al.*, 1999) and development of biofilm architecture in *P. aeruginosa* (Davies *et al.*, 1998).

AHLs are synthesized by homologues from either the AinS or LuxI family of AHL synthases and mediate transcription of various target genes through an interaction with, in most cases, a homologue of the LuxR protein of *V. fischeri* (reviewed by Fuqua *et al.*, 1996). AHLs show variation in the length, degree of saturation and adjoining substitutions of the acyl chain (reviewed by Fuqua & Eberhard, 1999). These structural variations account for the different responses elicited by different

Abbreviations: AHL, *N*-acyl-L-homoserine lactone; 3-oxo-C6-HSL, *N*-3-oxo-hexanoyl-homoserine lactone; RFU, relative fluorescence unit.

AHLs in quorum sensing assays (McClean *et al.*, 1997; Zhu *et al.*, 1998). The molecular mechanism by which AHLs trigger the transcriptional activation of target promoters via an interaction with LuxR homologues remains to be fully elucidated (see Discussion), but appears to involve AHL binding to and induction of conformational changes in the regulatory protein which lead to multimerization and DNA binding (Choi & Greenberg, 1992; Qin *et al.*, 2000; Welch *et al.*, 2000). Other cellular components are involved in the expression of AHL-regulated genes including the cAMP receptor protein (Nealson *et al.*, 1972), the H-NS protein (Ulitzur *et al.*, 1997), and the molecular chaperones GroES and GroEL (Adar *et al.*, 1992).

The discovery of quorum sensing has afforded a novel opportunity to control unwanted microbial activity without the use of growth inhibitory agents such as antibiotics, preservatives and disinfectants that select for resistant organisms. A means of interfering with AHL-mediated gene expression not only has potential in a number of applied contexts, including the treatment of lung infections in cystic fibrosis patients, but would also constitute an evolutionary advantage for plant and animal species under selective pressure from quorum sensing pathogens.

Gram-negative bacteria engage in AHL-dependent phytopathogenic (Barras *et al.*, 1994; Zhang *et al.*, 1993) and phytosymbiotic (Rodelas *et al.*, 1999) relationships with terrestrial plants. Whilst there are no known examples of such relationships in the marine environment, marine plants are at once rich in secondary metabolite chemistry and, in the absence of more advanced immune systems, prone to disease (Correa, 1996; Fenical, 1997). For these reasons marine plants are likely candidates for the evolution of AHL antagonist activity (Kjelleberg & Steinberg, 2001). The marine macroalga *Delisea pulchra* produces a range of lactones, known more specifically as halogenated furanones, which inhibit quorum sensing (Givskov *et al.*, 1996; Manefield *et al.*, 1999; Rasmussen *et al.*, 2000). It has previously been proposed that the production of the halogenated furanones in specialized cells, which migrate to the surface of the alga to release the compounds (Dworjanyn *et al.*, 1999), is likely to have evolved in response to the negative impacts of AHL-dependent colonization of its surfaces by marine bacterial species (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997; Kjelleberg & Steinberg, 2001).

It has been demonstrated that halogenated furanones have inhibitory effects in a variety of biological assays designed to measure AHL-mediated gene expression (Givskov *et al.*, 1996). Such inhibition was found to be partially relieved by increasing AHL concentrations in the bioassays, indicative of competition for a regulatory function (Manefield *et al.*, 1999). Furthermore, halogenated furanones were found to have activity in an *in vivo* ligand-binding assay employed to monitor the displacement of AHLs from the LuxR protein (Manefield *et al.*, 1999). These results have been in accordance

with a model in which halogenated furanones compete with AHLs for a common binding site on LuxR and LuxR homologues. In this study we tested directly for an interaction between a halogenated furanone and the LuxR protein, and discovered that the furanones inhibit AHL-mediated gene expression through accelerated degradation of the transcriptional activator.

METHODS

Strains, plasmids and culture conditions. The LuxR overproduction strain *Escherichia coli* XL-1 Blue(pHK724) (pGroESL) and the control strain *E. coli* XL-1 Blue(pGroESL) were cultured and washed during assays in Luria-Bertani broth (containing 10 g NaCl l⁻¹). Plasmid pHK724 carries a *P*_{lac}-*luxR* fusion and encodes resistance to 50 µg ampicillin ml⁻¹ (Hanzelka & Greenberg, 1995). Plasmid pGroESL carries *P*_{lac}-*groES* and *P*_{lac}-*groEL* fusions and encodes resistance to 25 µg chloramphenicol ml⁻¹ (Goloubinoff *et al.*, 1989). The LuxR-based green fluorescent AHL monitor strain *E. coli* MT102(pJBA89) was cultured and diluted during assays in minimal ABT medium [(NH₄)₂SO₄, 15.1 mM; Na₂HPO₄·2H₂O, 33.7 mM; KH₂PO₄, 22.0 mM; NaCl, 51.0 mM; MgCl₂·6H₂O, 1.0 mM; CaCl₂, 0.1 mM; FeCl₃, 10 µM; thiamin, 7.4 µM] (Clark & Maaløe, 1967) supplemented with 0.5% glucose and 0.5% Casamino acids. Plasmid pJBA89 carries *luxR* and *P*_{luxR}-*gfp*(ASV) fusion and encodes resistance to 50 µg ampicillin ml⁻¹ (Andersen *et al.*, 2001). The *clp lon* double mutant *E. coli* KY2347 [MG1655 Δ(*clpPX-lon*) 1196::cat] (Zhu & Winans, 2001) and the isogenic parent *E. coli* MG1655 (wild-type *E. coli* K-12 *ilvG rph-1*) (Guyer *et al.*, 1981), both harbouring pJBA89, were cultured in LB diluted 20 times in ABT medium. Unless otherwise stated, the relevant antibiotics at the above-mentioned concentrations were included in cultures to ensure plasmid maintenance.

AHL and furanone preparation. Synthetic N-oxohexanoyl-L-homoserine lactone was prepared according to the method of Eberhard *et al.* (1981) and stored in ethyl acetate at 20 mg ml⁻¹ (approx. 100 µM, depending on the compound) at -20 °C in a non-frost free freezer. The halogenated furanones compound 2, compound 8 and compound 56 were synthesized as previously described by Manny *et al.* (1997). Compound 4 was extracted from *D. pulchra* and purified by HPLC according to protocols established by de Nys *et al.* (1993). Compound 30 was synthesized according to protocols reported by Wells (1963). Tritiated compound 4 was prepared by reducing a ketone group at the C(1') position on the alkyl chain of a compound 4 precursor with tritiated borane producing an enantiomeric mixture of the S and R forms of compound 4 with a tritium atom replacing the hydrogen atom at the C(1') position. The furanones were stored in ethanol at 20 mg ml⁻¹ at -20 °C in a non-frost free freezer.

Binding analysis. A variation on the method of Hanzelka & Greenberg (1995) was employed to assay for an *in vivo* affinity between the LuxR protein and ³H-labelled compound 4. LuxR, GroES and GroEL overproduction was induced in exponential phase *E. coli* XL-1 Blue(pHK724)(pGroESL) and *E. coli* XL-1 Blue(pGroESL) cultures (OD₆₀₀ 0.3) by incubation with 100 µM IPTG for 2 h. Various concentrations of the labelled halogenated furanone were then added to 1 ml aliquots of the induced cultures. After 10 min incubation, the cells were washed, resuspended in scintillation fluid and monitored for tritium as previously described for ³H-3-oxo-C6-HSL (Manefield *et al.*, 1999; Hanzelka & Greenberg, 1995). A standard curve describing the relationship between

the quantity of the labelled furanone and its relative activity in c.p.m., obtained by diluting known amounts of ^3H -labelled compound 4 in 100 μl scintillation fluid, was used to convert the raw data obtained from c.p.m. to nmol.

Determination of GFP expression. The effect of halogenated furanones on expression of a 3-oxo-C6-HSL stimulated, LuxR controlled $P_{\text{luxI}}\text{-gfp(ASV)}$ fusion [encoding an unstable Gfp protein with half life ranging from 40 min to 110 min in exponential and stationary phase cells, respectively (Andersen *et al.*, 1998, 2001)] was determined as follows. Aliquots (200 μl) of exponential phase *E. coli* MT102(pJBA89) cultures were distributed to the wells of microtitre dishes in which 3-oxo-C6-HSL and furanone compounds in the required concentrations were already present. After 2 h incubation at 37 °C the relative fluorescence units (RFU) of each sample were captured with a Wallac Victor2, 1420 Multilabel Counter using a 485 nm excitation filter and a 535 nm emission filter. The OD_{450} was also determined for each sample to monitor growth of the bacterial strain.

LuxR Western analysis. *E. coli* XL-1 Blue(pHK724)(pGroESL) was grown in the presence of 100 μM IPTG to OD_{600} 0.3. Cells were harvested by centrifugation at 10000 g, 4 °C for 5 min and resuspended in an equal volume of fresh media. The washed culture was split into 5 ml aliquots and treated with various concentrations of halogenated furanones and/or 3-oxo-C6-HSL (*N*-3-oxo-hexanoyl-homoserine lactone). Samples were taken over time for determination of optical density and for Western analysis. Samples taken for Western analysis were frozen immediately and kept at -20 °C. Prior to SDS-gel electrophoresis, the samples were adjusted to the same absorbance. Western analysis of the separated proteins was performed using an anti-LuxR antibody from Quorum Sciences as the primary antibody and an anti-rabbit horse-radish peroxidase conjugated antibody from Amersham Pharmacia Biotech as the secondary antibody, according to the manufacturers recommendations. Localization of the secondary antibody was visualized using chemiluminescent detection reagents from Amersham Pharmacia Biotech and a Hamamatsu C2400-47 double intensified CCD camera (Hamamatsu). Colour images were saved in 16-bit format (the scale has a resolution of 16 colours) using ARGUS-50 software (Hamamatsu). The relative amounts of LuxR protein in each sample were estimated from a standard curve relating colour to relative LuxR content constructed from a two-fold dilution series of fully IPTG induced cells. Prior to SDS-gel electrophoresis, the samples were adjusted to the same absorbance.

To assess whether the translation of persistent *luxR* mRNA during the above described treatment contributed to the cellular pool of LuxR measured in the Western procedure, 100 μg chloramphenicol ml^{-1} was administered after the removal of IPTG, just prior to furanone treatment, to disrupt the ribosomal activity required for LuxR production. Cells treated thus were assessed by Western blotting as above.

RESULTS

Assaying LuxR affinity for a halogenated furanone

To further explore the hypothesis that halogenated furanones have affinity for the LuxR protein, it was of interest to test directly for an interaction between the protein and the algal metabolites and, if possible, to ascertain whether a correlation exists between binding activity and biological activity in bioassays. Because the LuxR protein has never been isolated with AHL-binding

Fig. 1. Dose response relationship for ^3H -labelled compound 4 binding to XL-1 Blue(pHK724)(pGroESL) (●) and XL-1 Blue(pGroESL) (■). ^3H -labelled compound 4 (0–800 nmol) was added to 1 ml aliquots of each culture and incubated for 10 min. The cultures were then washed several times before assaying for the tritium signal using a scintillation counter. Radioactivity was related to ^3H -labelled compound 4 concentration by a standard curve. These data are representative of two separate experiments in which treatments were performed in duplicate.

activity we employed the *in vivo* approach used to demonstrate 3-oxo-C6-HSL binding activity in the LuxR protein overproduced in *E. coli* (Hanzelka & Greenberg, 1995). To this end tritium-labelled compound 4 was synthesized (see Methods) and incorporated at various concentrations (0–800 nmol) into 1 ml aliquots of XL-1 Blue(pHK724)(pGroESL) and XL-1 Blue(pGroESL) cultures overproducing LuxR and GroESL or GroESL alone, respectively. The concurrent overproduction of the molecular chaperones GroES and GroEL with LuxR enhances the affinity of the native AHL ligand for the overproducing cells (Hanzelka & Greenberg, 1995). After a short incubation, cells were washed and assayed for the quantity of ^3H -labelled compound 4 retained.

Fig. 1 describes the dose response relationships obtained for the two strains. The *E. coli* culture overproducing the LuxR protein retained between 0.4% and 1.5% of the tritiated furanone added, depending on the concentration added. By comparison, the control culture overproducing the chaperone proteins alone retained from 0.4% to 1.1% of the labelled compound added. Whilst this small difference may represent an association between the algal metabolite and the LuxR protein, these results suggest that any LuxR–compound 4 complex formed is unstable in nature.

Halogenated furanones induce instability of the LuxR protein

To resolve the apparent paradox in the observation that halogenated furanones do not form a stable complex with the LuxR protein but can displace 3-oxo-C6-HSL

Fig. 2. Western blots following the decay of the LuxR protein over time in the absence and presence of compound 30. (a) An *E. coli* XL-1 Blue(pHK724)(pGroESL) culture was stimulated to produce LuxR with 100 μM IPTG and washed with fresh media. The culture was sampled after 0, 45, 90 and 135 min and analysed by Western blotting employing a LuxR antibody. (b) Cultures were stimulated to produce LuxR, washed as in (a) and split into eight subcultures which were treated with various compound 30 concentrations (0.1–100 μM). Samples were removed after 0, 0.5, 1, 5, 10 and 30 min, and analysed as in (a). These blots are representative of three separate experiments. The colours reflect intensity of the band, which correlates with protein quantity. The highest band intensity is represented by white pixels with red, green, yellow and blue pixels representing decreasing intensity, respectively.

from LuxR (Manefield *et al.*, 1999), we hypothesized that binding of halogenated furanones to LuxR accelerates the proteolytic degradation of the protein. Since AHL-regulated phenotypes are not expressed in the absence of LuxR-type regulatory proteins, the data presented here could be reconciled with that published previously if the presence of halogenated furanones affected the steady-state cellular concentration of functional LuxR protein.

To address this hypothesis we examined the amounts of LuxR protein in cells treated with furanones. To assess the *in vivo* stability of LuxR, XL-1 Blue(pHK724)(pGroESL) cells were induced with 100 μM IPTG, washed free of the inducer and sampled at different time points after the wash. The amounts of LuxR protein in the different samples were then visualized by an SDS-PAGE-Western blot procedure using LuxR antibody and a chemiluminescence-based detection system. A CCD camera reported chemiluminescence as colour digital images with increasingly lighter colours relating to increases in band intensity (Fig. 2a).

A correlation between the coloured intensity scale and the relative amount of LuxR protein was established with a twofold serial dilution of a LuxR preparation (data not shown), revealing that the synthesized LuxR protein decayed with a half-life of approximately 65 min (Fig. 2a). Indistinguishable results were obtained in the presence and absence of 100 μg chloramphenicol ml⁻¹ indicating that translation of the *luxR* transcript does not contribute to the cellular pool of LuxR protein after removal of the P_{tac} inducer. LuxR had a comparable, perhaps marginally longer half-life in the presence of 2 μM 3-oxo-C6-HSL (data not shown). When IPTG induced XL-1 Blue(pHK724)(pGroESL) cells were washed free of IPTG and then exposed to a synthetic halogenated furanone with strong inhibitory activity in a LuxR-dependent GFP-based AHL bioassay (compound 30), it was obvious from the Western analysis that LuxR levels decreased much faster. This indicated that the halogenated furanone promoted turnover of the LuxR protein. The half-life of LuxR was found to decrease with increasing concentrations of compound 30 (Fig. 2b).

A correlation between furanone activity, cellular LuxR concentration and expression of the *luxI* promoter

To determine whether furanone-dependent decay of the LuxR protein was the mechanism by which halogenated furanones inhibit expression from the promoter of the *luxI* gene (P_{luxI}) we assessed the relationship between furanone activity, cellular LuxR concentration and P_{luxI} expression. We tested a series of compounds (Table 1) for their ability to inhibit 3-oxo-C6-HSL-induced LuxR-dependent expression of fluorescence from a P_{luxI} -*gfp* (ASV) fusion in the AHL monitor strain *E. coli* MT102(pJBA89). The effect of two natural and three synthetic halogenated furanones (Table 1) on 3-oxo-C6-HSL stimulated *LuxR* P_{luxI} -*gfp*(ASV) expression was

Table 1. The structures and inhibition indexes of various halogenated furanones

Compound no.	Structure	Inhibition index*
2		0.47
4		0.26
8		0.76
30		0.16
56		0.48

* A low inhibition index indicates strong biological activity (see text for details).

determined at eight different furanone concentrations (ranging from 0 to 40 μ M) and three different 3-oxo-C6-HSL concentrations (ranging from 25 to 100 nM). Incubation with 100 nM 3-oxo-C6-HSL and no furanone addition was defined as having 100 % RFU. A total of 24 RFU values obtained for each furanone compound were used to calculate an inhibition index expressing the quantity of furanone (μ mol) per quantity of 3-oxo-C6-HSL (nmol) required to inhibit P_{luxI} - gfp (ASV) expression to an arbitrary level (40 %). This is termed the ID_{40} value. The three values obtained, one for each 3-oxo-C6-HSL concentration, were plotted as a function of 3-oxo-C6-HSL concentration (illustrated for compound 30 in Fig. 3) and the gradient of the best straight line passing through the origin was taken as the inhibition index (IIX_{40}). The IIX_{40} expresses the number of moles furanone per mmole 3-oxo-C6-HSL required to inhibit expression of fluorescence to 40 % of the untreated

Fig. 3. Determination of IIX_{40} for compound 30. 3-oxo-C6-HSL-stimulated $LuxR$ P_{luxI} - gfp (ASV) fluorescence (y axis) was determined at eight different furanone (x axis) and three different 3-oxo-C6-HSL concentrations (z axis). Incubation with 100 nM 3-oxo-C6-HSL and no furanone addition was defined as having 100 % RFU. ID_{40} values (one for each 3-oxo-C6-HSL concentration) were determined as described in the text.

sample. A low IIX_{40} value therefore indicates that a compound is an efficient quorum-sensing inhibitor. Using this method, it was found that the most active compounds tested are compounds 30 (synthetic) and 4 (natural). Compounds 2 (natural) and 56 (synthetic) have medium activity whereas compound 8 (synthetic) has poor activity (Table 1).

The range of inhibitory activities displayed provided an opportunity to relate this activity to the degradation of $LuxR$. We therefore assessed the effect of each compound on the stability of $LuxR$. IPTG-induced XL-1 Blue(pHK724)(pGroESL) cells were washed free of IPTG, treated with 50 μ M compound 2, 4, 8, 30 or 56 and sampled for Western analysis. As shown in Fig. 4, treatment with compound 8 (inhibition index of 0.76) did not decrease the $LuxR$ half-life compared to the untreated samples. Samples treated with compound 2 (inhibition index of 0.47), compound 4 (inhibition index of 0.26) and compound 56 (inhibition index of 0.48) accelerated the $LuxR$ turnover when compared to the untreated control. The limited resolution of the assay failed to reproducibly distinguish between the activity of these three compounds. Finally, compound 30 (inhibition index of 0.16) had the greatest impact on $LuxR$ turnover compared to the other treatments. These results supported a correlation between furanone activity, $LuxR$ concentration and P_{luxI} - gfp (ASV) expression.

Finally, to confirm the relationship between the furanone-induced decreases in fluorescence expression from the $LuxR$ -controlled P_{luxI} - gfp (ASV) fusion and the furanone-accelerated turnover of the $LuxR$ protein, we assayed samples of *E. coli* MT102(pJBA89) cultures treated with 100 nM 3-oxo-C6-HSL and 0, 0.5, 1, 5 or 10 μ M compound 30 for fluorescence every 10 min. When distinct differences between the fluorescence

Fig. 4. Western blots comparing the rate of decay of the LuxR protein in XL-1 Blue(pHK724)(pGroESL) cultures treated with different halogenated furanones (50 μ M). Treatments are: A, an untreated negative control; B, 50 μ M compound 2; C, 50 μ M compound 4; D, 50 μ M compound 8; E, 50 μ M compound 30; and F, 50 μ M compound 56. The relative effect of each compound on the half-life of the LuxR protein corresponds with the activity of each in the LuxR-based GFP assays. These blots are representative of two separate experiments.

signal from the various treatments were apparent (90 min after furanone addition) samples were assayed for LuxR levels by means of Western blotting. Fig. 5 illustrates the correlation between decreases in LuxR levels and decreases in fluorescence. It was apparent that incremental decreases in the fluorescence levels associated with increasing compound 30 concentrations are concurrent with the loss of the LuxR protein.

Fig. 5. Correlation between changes in GFP expression and LuxR concentration. MT102(pJBA89) cultures treated with 100 nM 3-oxo-C6-HSL and various concentrations of compound 30 were sampled in triplicate for GFP activity and the quantity of LuxR. The specific GFP activity (RFU/OD₆₀₀) (black bars) and the relative LuxR quantity (white bars) of the negative control culture (treated with 3-oxo-C6-HSL only) were arbitrarily set to 1. This graph is representative of two separate experiments. Error bars represent standard deviation.

Furanone-promoted degradation of LuxR is protease independent

Comparable results were obtained when the above experiments were conducted in a *clpP* strain and in a *lon* strain. In both cases activity was tested [LuxR controlled P_{luxI} -gfp(ASV)] and protein half-life was investigated (data not shown). This indicated that neither the *clpP* protease nor the *lon* protease affected stability of the LuxR protein. We therefore tested expression of Gfp from pJBA89 in a *clp lon* background as well as in the isogenic *clp⁺ lon⁺* parent. Strains KY2347 and MG1655 grew more slowly than our preferred MT102 strain. In MG1655 the IIX₄₀ value for compound 30 was found to be 0.23 (compared to 0.16 in MT102). In the KY2347 (*clp lon*) the IIX₄₀ value for compound 30 was found to be 0.34.

Fig. 6. Western blot following the decay of the LuxR protein over time (5–30 min) in XL-1 Blue(pHK724)(pGroESL) cultures in the presence and absence of 3-oxo-C6-HSL and compound 30. Treatments are: A, an untreated negative control; B, 2 μ M 3-oxo-C6-HSL; C, 10 μ M compound 30 + 2 μ M 3-oxo-C6-HSL (in that order); and D, 2 μ M 3-oxo-C6-HSL + 10 μ M compound 30 (in that order). Addition of 3-oxo-C6-HSL prior to treatment with the halogenated furanone protects the protein from degradation. This blot is representative of three separate experiments.

The presence of 3-oxo-C6-HSL protects the LuxR protein against furanone promoted degradation

Whilst analysing the expression of the LuxR controlled P_{luxI} -*gfp*(ASV) fusion we realized that increasing 3-oxo-C6-HSL concentrations reduced the inhibitory activity of the furanones on the fluorescence phenotype (data not shown). To assess whether this was a manifestation of the protection of LuxR by 3-oxo-C6-HSL we tested the effect of the AHL on compound 30 induced LuxR decay. Fig. 6 (c) illustrates that the addition of 2 μ M 3-oxo-C6-HSL approximately 60 s after the addition of 10 μ M compound 30 did not afford LuxR any protection from furanone-induced degradation. Fig. 6 (d) however, illustrates that if 2 μ M 3-oxo-C6-HSL was added 60 s prior to the addition of 10 μ M compound 30, the furanone-induced degradation was retarded by at least 10 min. Note that in the fluorescence assay employed to calculate the inhibition index for each compound, halogenated furanones and 3-oxo-C6-HSL were added simultaneously.

These data indicate that once compound 30 induced degradation of the LuxR protein is initiated, presumably through a direct interaction between the compound and the protein, it is not reversible by addition of 3-oxo-C6-HSL. However, prior addition of 3-oxo-C6-HSL offers some protection to LuxR from compound 30.

DISCUSSION

From the few biochemical studies that have been performed on the function of LuxR-type regulatory proteins, a model of how AHLs cause transcription of specific genes is beginning to emerge (Zhu & Winans, 2001; Welch *et al.*, 2000; Qin *et al.*, 2000; Zhu & Winans, 1999). *In vitro* investigations with the CarR protein of *E. carotovora* and the TraR protein of *A. tumefaciens*, including DNA bandshift, fluorescence quenching and tryptic digestion experiments, have revealed that cognate AHLs directly interact with and induce conformational changes in these regulatory proteins (Zhu & Winans, 2001; Welch *et al.*, 2000). What remains uncertain is the effect these conformational changes have on the behaviour of the protein.

Qin *et al.* (2000) have proposed a model in which the TraR protein is monomeric and membrane associated in the absence of 3-oxo-C8-HSL but dimeric, cytoplasmic and capable of transcriptional activation in its presence. Zhu & Winans (2001) have recently presented another model in which the TraR protein is monomeric and vulnerable to proteolysis in the absence of 3-oxo-C6-HSL but dimeric, resistant to proteolysis and capable of transcriptional activation in its presence. These models both draw attention to the relevance of the cytoplasmic concentration of TraR in the activation of target promoters.

In the present communication we have sought to further define the molecular mechanism by which halogenated furanones inhibit the AHL-mediated transcriptional activation of target genes. We have been unable to detect the formation of a stable complex between a tritiated

halogenated furanone and the LuxR protein overproduced in *E. coli*. We have however demonstrated that the cytoplasmic concentration of the LuxR protein is decreased in the presence of halogenated furanones. In the light of both these results and the demonstration that 3-oxo-C8-HSL protects the TraR protein in *E. coli* from proteolytic digestion (Zhu & Winans, 2001) it is suggested that halogenated furanones interact with the LuxR protein but that this interaction causes conformational changes that enlist the furanone–LuxR complex into rapid proteolytic degradation. This model is consistent both with the observed effects of furanones on the formation of the AHL–LuxR complex (Manefield *et al.*, 1999) and with the inability to detect a long-lived furanone–LuxR complex.

Is the loss of LuxR the result of proteolytic degradation? The effect of furanones on the LuxR concentration was comparable in a wild-type *E. coli* strain and a *clpP* and a *lon* *E. coli* strain. Zhu & Winans (2001) found that a substantial change in the rate of TraR degradation in *E. coli* required the simultaneous crippling of both the *clp* and *lon* proteases. We found no significant change in the inhibition index for compound 30 in the different strains. This indicated that not even the Clp and Lon proteases in concert could severely affect the LuxR stability. This however, does not rule out the possibility that other proteases could be involved in the proteolytic turnover of LuxR. The possibility that the LuxR protein becomes compartmentalized in a manner analogous to the proposal of Qin *et al.* (2000) in the presence of the furanones is unlikely given that whole cells (i.e. including membranes) were used in the Western procedure employed here.

We did not reproducibly detect an increase in LuxR stability in the presence of 3-oxo-C6-HSL as was demonstrated by Zhu & Winans (2001) for the TraR protein in the presence of 3-oxo-C8-HSL. In our experiments however 3-oxo-C6-HSL was always added after the cessation of stimulation of the *luxR* promoter (i.e. after removal of IPTG). Zhu & Winans (2001) found that the AHL-induced protection of TraR was dependent on the presence of 3-oxo-C8-HSL during synthesis of the protein and that addition of 3-oxo-C8-HSL to *E. coli* cells already harbouring the TraR monomer did not afford the protein any protection against proteolysis. Our observations with LuxR are therefore not inconsistent with those of Zhu & Winans (2001).

The ability of 3-oxo-C6-HSL to protect the LuxR protein from furanone-induced degradation was dependent on the addition of the AHL before the furanone. This result suggests that, unlike 3-oxo-C8-HSL and TraR, 3-oxo-C6-HSL binds mature LuxR and that in this state the halogenated furanones are less able to compete for the AHL binding site. It is possible that 3-oxo-C6-HSL is binding and protecting low levels of freshly translated LuxR from persistent *luxR* mRNA transcript. However, the addition of chloramphenicol to block translation after cessation of transcription did not

affect LuxR levels, indicating that residual translation was not occurring. Either way it is clear that 3-oxo-C6-HSL can protect the LuxR protein from furanone-induced degradation when present before the furanone, but is unable to rescue the protein if the furanone is present first and has already initiated degradation.

The superior activity of compounds lacking a carbon chain extending from the furan ring structure in the control of both P_{luxI} -*gfp*(ASV) expression and LuxR concentration was somewhat unexpected because the homoserine lactone ring without the acyl chain has been shown not to interact with the LuxR homologue CarR (Welch *et al.*, 2000). The significance of this will remain unresolved until more structural information regarding LuxR homologues and their AHL binding sites is available.

AHLs are required for the expression of Gram-negative bacterial phenotypes involved in many cases in an interaction with a higher organism. Amongst some of the best studied examples, including elastase production in *P. aeruginosa*, pectate lyase production in *E. carotovora* and conjugation of the plant-tumour-inducing Ti plasmid of *A. tumefaciens*, are behaviours with central roles in the success of bacterial infections of medical, agricultural and therefore economic significance. This investigation has demonstrated that halogenated furanones produced naturally by the marine alga *D. pulchra* can modulate the cellular concentration of the LuxR protein responsible for the reception of, and response to, AHLs. While this study is directed at the effects of furanones on the LuxR protein of *V. fischeri*, the findings highlight the potential for the use of halogenated furanones in the control of unwanted bacterial activity. Additionally these results lend support to a model of AHL function in which the metabolite regulates the steady state concentration of LuxR homologues by shielding the regulator from proteolytic degradation.

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