Environmental Microbiology (2007) 9(10), 2486–2495 doi:10.1111/j.1462-2920.2007.01367.x

The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone disrupts quorum sensing-regulated gene expression in *Vibrio harveyi* by decreasing the DNA-binding activity of the transcriptional regulator protein luxR

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Summary

This study aimed at getting a deeper insight in the molecular mechanism by which the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone disrupts quorum sensing in *Vibrio harveyi*. Bioluminescence experiments with signal molecule receptor double mutants revealed that the furanone blocks all three channels of the *V. harveyi* quorum sensing system. In further experiments using mutants with mutations in the quorum sensing signal transduction pathway, the compound was found to block quorum sensing-regulated bioluminescence by interacting with a component located downstream of the Hfq protein. Furthermore, reverse transcriptase real-time polymerase chain reaction with specific primers showed that there was no effect of the furanone on *luxR*$_{Vh}$ mRNA levels in wild-type *V. harveyi* cells. In contrast, mobility shift assays showed that in the presence of the furanone, significantly lower levels of the LuxR$_{Vh}$ response regulator protein were able to bind to its target promoter sequences in wild-type *V. harveyi*. Finally, tests with purified LuxR$_{Vh}$ protein also showed less shifts with furanone-treated LuxR$_{Vh}$, whereas the LuxR$_{Vh}$ concentration was found not to be altered by the furanone (as determined by SDS-PAGE). Therefore, our data indicate that the furanone blocks quorum sensing in *V. harveyi* by rendering the quorum sensing master regulator protein LuxR$_{Vh}$ unable to bind to the promoter sequences of quorum sensing-regulated genes.

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Introduction

Vibrio harveyi is a Gram-negative, luminescent marine bacterium that can be found free-living in the water column as well as in association with marine animals (Thompson *et al.*, 2004). With the rapid development of the aquaculture industry, the species is becoming increasingly recognized as an important pathogen of marine vertebrates and invertebrates (Austin and Zhang, 2006). Because of the development and spread of antibiotic resistance in these bacteria, antibiotic treatments are becoming inefficient (Karunasagar *et al.*, 1994; Moriarty, 1998) and therefore, alternative control strategies are being developed. One of these strategies is the disruption of bacterial cell-to-cell communication, called quorum sensing (Defoirdt *et al.*, 2004).

Unlike many other Gram-negative bacteria, *V. harveyi* has been reported to use a multichannel quorum sensing system (Fig. 1). The first channel of this system is mediated by the Harveyi Autoinducer 1 (HAI-1), an acylated homoserine lactone (AHL) (Cao and Meighen, 1989). The second channel is mediated by the so-called Autoinducer 2 (AI-2), which is a furanosyl borate diester (Chen *et al.*, 2002). The chemical structure of the third autoinducer, called Cholerae Autoinducer 1 (CAI-1) is still unknown (Henke and Bassler, 2004a). All three autoinducers are detected at the cell surface and activate or inactivate target gene expression by a phosphorylation/dephosphorylation signal transduction cascade. Phenotypes that were found to be controlled by this quorum

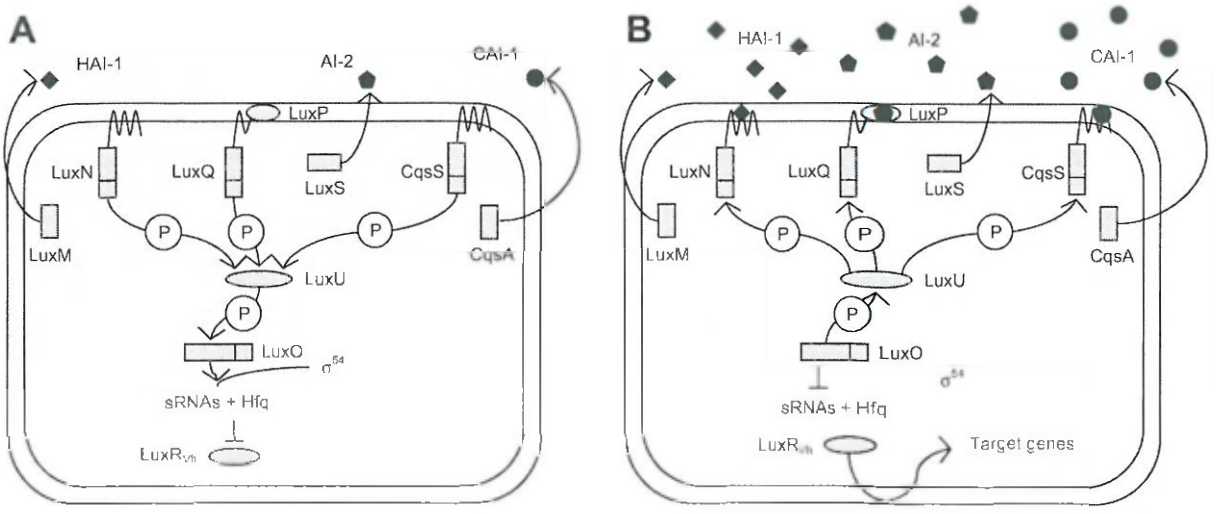

Fig. 1. Quorum sensing in *Vibrio harveyi*. The LuxM, LuxS and CqsA enzymes synthesize the autoinducers HAI-1, AI-2 and CAI-1 respectively. These autoinducers are detected at the cell surface by the LuxN, LuxP-LuxQ and CqsS receptor proteins respectively.
A. At low signal molecule concentration, the receptors autophosphorylate and transfer phosphate to LuxO via LuxU. Phosphorylation activates LuxO, which together with σ^{54} activates the production of small regulatory RNAs (sRNAs). These sRNAs, together with the chaperone Hfq, destabilize the mRNA encoding the response regulator $LuxR_{Vh}$. Therefore, in the absence of autoinducers, the $LuxR_{Vh}$ protein is not produced.
B. In the presence of high concentrations of the autoinducers, the receptor proteins switch from kinases to phosphatases, which results in dephosphorylation of LuxO. Dephosphorylated LuxO is inactive and therefore, the sRNAs are not formed and the response regulator $LuxR_{Vh}$ is produced (adapted from Henke and Bassler, 2004a).

sensing system include bioluminescence (Bassler *et al.*, 1993) and the production of several virulence factors such as a type III secretion system (Henke and Bassler, 2004b), extracellular toxin (Manefield *et al.*, 2000) and a siderophore (Lilley and Bassler, 2000). Recently, we found that virulence of the bacterium towards the brine shrimp *Artemia franciscana* is also regulated by its quorum sensing system (Defoirdt *et al.*, 2005).

One of the mechanisms to disrupt bacterial quorum sensing is the use of halogenated furanones, such as the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone. These furanones (natural occurring compounds as well as synthetic derivatives) were found to disrupt the expression of different AHL-regulated phenotypes in several Gram-negative species, without affecting their growth (Hentzer and Givskov, 2003; Rasmussen and Givskov, 2006). Because of the structural similarity between AHL molecules and the furanones, it was originally hypothesized that these compounds disrupt AHL-mediated quorum sensing in the LuxI/$LuxR_{VI}$-type of quorum sensing system by competitively binding to the AHL receptor site of the *Vibrio fischeri* $LuxR_{VI}$ protein (Givskov *et al.*, 1996). Later on, it was shown that the halogenated furanones promote rapid turnover of the $LuxR_{VI}$-type AHL receptor protein, reducing the amount of $LuxR_{VI}$ available to interact with AHL and to act as transcriptional regulator (Manefield *et al.*, 2002). More recently, Koch and colleagues (2005) used site-directed mutagenesis to study interactions between $LuxR_{VI}$ and halogenated furanones. The authors could not conclude that the furanones bind to the AHL-binding cavity and suggested that furanones do not compete in a classic way with the signal molecules.

Meanwhile, several research groups provided evidence that halogenated furanones also disrupt quorum sensing-regulated gene expression in *V. harveyi* (Manefield *et al.*, 2000; Ren *et al.*, 2001; McDougald *et al.*, 2003; Defoirdt *et al.*, 2006). However, because the quorum sensing system of *V. harveyi* is quite different from the LuxI/$LuxR_{VI}$-type of quorum sensing system and does not contain a $LuxR_{VI}$ homologue (Milton, 2006), the molecular mechanism of quorum sensing disruption in this species must be different from that in the LuxI/$LuxR_{VI}$-type of system. Consequently, in this study, we aimed at defining the molecular mechanism by which the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (Fig. 2) disrupts quorum sensing-regulated gene expression in this different type of quorum sensing system.

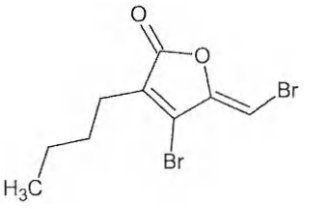

Fig. 2. Structure of the natural furanone used in this study.

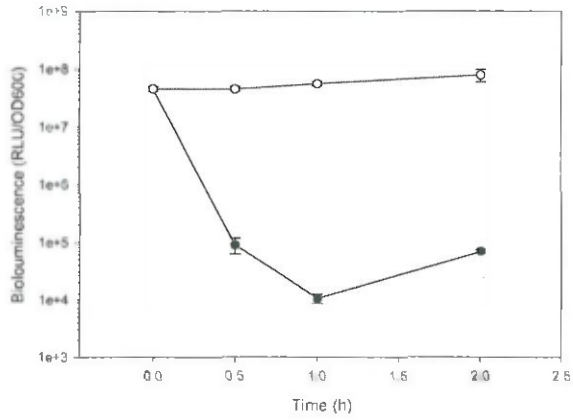

Fig. 3. Bioluminescence per unit cell density in wild-type *Vibrio harveyi* BB120 as a function of time, without (open symbols) and with (filled symbols) the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (50 mg l^{-1}). The error bars represent the standard deviation of three replicates. Note that RLU is the relative unit of luminescence reported by the Lumac Biocounter M2500 luminometer.

Results

Impact of the furanone on quorum sensing-regulated bioluminescence of wild-type V. harveyi

Bioluminescence is one of the phenotypes that is regulated by the *V. harveyi* quorum sensing system and therefore, in a first experiment, the impact of the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone on the bioluminescence of wild-type *V. harveyi* was determined. Strain BB120 was grown in LB_{20} medium to high cell density in order to activate quorum sensing-regulated bioluminescence, after which the furanone was added to the medium at 50 mg l^{-1}. The compound blocked bioluminescence of BB120, with over 3 log units difference between the furanone-treated and the untreated cultures already 0.5 h after the addition of the furanone (Fig. 3). Consistent with this, luciferase activity in protein lysates of furanone-treated BB120 cells was found to have decreased with approximately 1 log unit 0.5 h after the addition of 50 mg l^{-1} furanone (Fig. 4). The bacterial alkaline phosphatase activities of the protein lysates were also measured in order to verify that the furanone had no effect on phenotypes that are not regulated by the quorum sensing system. As expected, there was no significant difference in bacterial alkaline phosphatase activities between furanone-treated and untreated cells (Fig. 4).

Impact of the furanone on bioluminescence of V. harveyi *autoinducer receptor double mutants*

The *V. harveyi* quorum sensing system consists of three channels, with each channel being activated by a different type of signal molecule (Fig. 1). In order to determine the impact of the furanone on the different channels, the signal molecule receptor double mutants JAF375 (sensor $HAI\text{-}1^-$, sensor $AI\text{-}2^-$, sensor $CAI\text{-}1^+$), JMH597 (sensor $HAI\text{-}1^+$, sensor $AI\text{-}2^+$, sensor $CAI\text{-}1^-$) and JMH612 (sensor $HAI\text{-}1^+$, sensor $AI\text{-}2^-$, sensor $CAI\text{-}1^-$) were used. Because of the mutated receptors, bioluminescence in these mutants is only responsive to one of the three signal molecules (Henke and Bassler, 2004a). The mutants were grown to high cell densities, and after furanone addition, bioluminescence was found to be blocked in all three double mutants in a concentration-dependent way similar to the one obtained for the wild type (Fig. 5). This indicates that all three channels of the quorum sensing system were blocked.

Impact of the furanone on bioluminescence of constitutively luminescent V. harveyi *mutants with mutations in the quorum sensing signal transduction cascade*

Because the three signal molecules have quite different chemical structures (Henke and Bassler, 2004a), we suspected that the furanone did not block quorum sensing-regulated bioluminescence by competing with the autoinducers for receptor sites but rather by interfering with the quorum sensing signal transduction. In order to test this hypothesis, the effects of the furanone on bioluminescence of mutants that were fixed in the high cell-density configuration at different stages in the quorum sensing signal transduction cascade were investigated. The mutants JAF553 and JAF483 contain a point mutation in the *luxU* and *luxO* genes, respectively, that render the LuxU and LuxO proteins incapable of phosphorelay (Freeman and

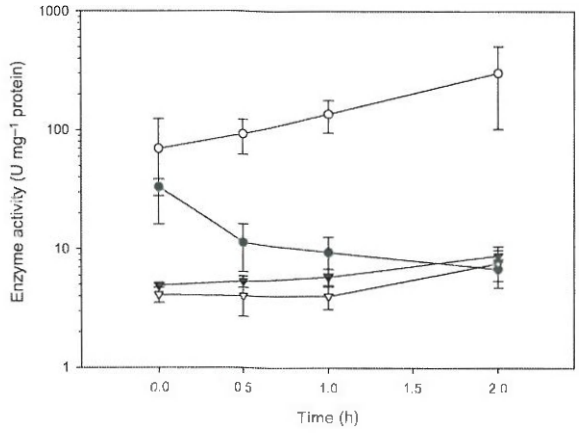

Fig. 4. Lucilerase (circles) and bacterial alkaline phosphatase (triangles) activities in protein lysates of wild-type *Vibrio harveyi* BB120 as a function of time, without (open symbols) and with (filled symbols) the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (50 mg l^{-1}). The error bars represent the standard deviation of four independent experiments.

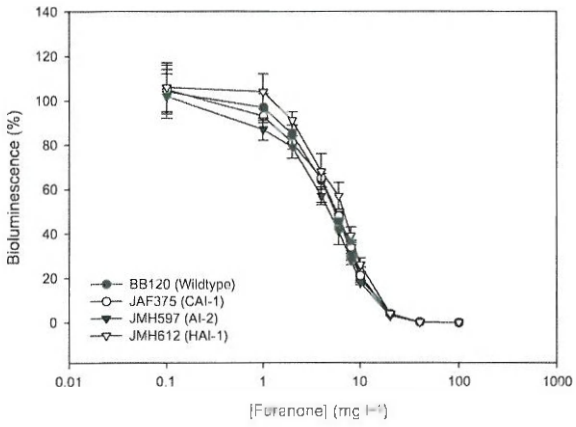

Fig. 5. Bioluminescence of the *Vibrio harveyi* wild type (BB120) and the double mutants JAF375 (sensor HAI-1$^+$, sensor AI-2$^-$, sensor CAI-1$^+$), JMH597 (sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) and JMH612 (sensor HAI-1$^+$, sensor AI-2$^-$, sensor CAI-1$^-$) as a function of the concentration of the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone. Luminescence measurements were performed 0.5 h after the addition of the furanone. For each strain, the bioluminescence without the addition of furanone was set at 100% and the other samples were normalized accordingly. The error bars represent the standard deviation of three replicates.

Bassler, 1999a,b). Strain BNL258 has a Tn*5* insertion in the *hfq* gene, resulting in a non-functional Hfq protein (Lenz *et al.*, 2004). Hence, because of the nature of these mutations, the three mutants are constitutively luminescent and therefore, blocking luminescence in one of them would indicate that the furanone acts downstream of the mutated component. The furanone, at 50 mg l^{-1}, blocked luminescence in all three mutants (Fig. 6). In wild-type *V. harveyi*, the Hfq protein acts together with small regulatory RNAs to destabilize the mRNA of the master regulator $LuxR_{Vh}$ (see Fig. 1). In the mutant BNL258, a transposon insertion has rendered the Hfq protein non-functional and therefore, in this mutant, the $luxR_{Vh}$ mRNA cannot be destabilized by the quorum sensing signal transduction system, resulting in a constitutively expressed bioluminescence (Lenz *et al.*, 2004). The fact that the furanone blocked bioluminescence in this mutant indicates that it acts downstream of Hfq, i.e. at the level of the $luxR_{Vh}$ mRNA and/or the $LuxR_{Vh}$ protein.

Impact of the furanone on $luxR_{Vh}$ *mRNA levels*

In order to verify whether the furanone indeed affects the quorum sensing master regulator, an experiment was set up in which the impact of the addition of the compound on $luxR_{Vh}$ mRNA was studied. Wild-type *V. harveyi* BB120 was grown to high cell density, after which the natural furanone was added at 50 mg l^{-1}. The addition of the furanone resulted in a rapid decrease in luminescence (Fig. 3). $luxR_{Vh}$ mRNA levels were quantified relatively by reverse transcriptase real-time polymerase chain reaction (PCR) with specific primers and the RNA polymerase A subunit (*rpoA*) mRNA was analysed as a control of a non-quorum sensing-regulated gene. For both genes, the mRNA levels followed a similar trend, with no significant difference between furanone-treated and untreated cells (Fig. 7). From these results, we conclude that the furanone has no effect on $luxR_{Vh}$ mRNA levels.

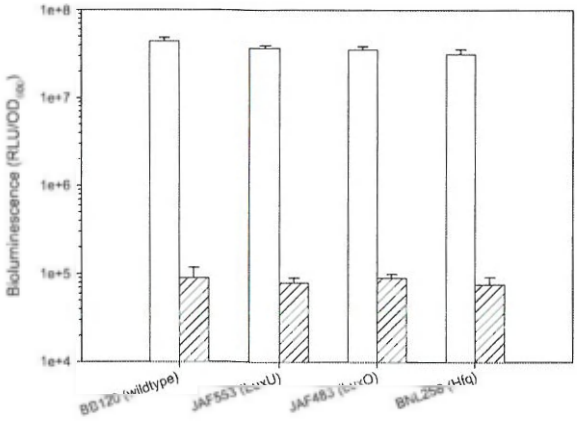

Fig. 6. Bioluminescence of wild-type *Vibrio harveyi* BB120 and the mutants JAF553 (*luxU* H58A), JAF483 (*luxO* D47A) and BNL258 (*hfq*::Tn*5lacZ*) without (white bars) and with (striped bars) the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (50 mg l^{-1}). Measurements were performed 0.5 h after the addition of the furanone. The error bars represent the standard deviation of three replicates. Note that RLU is the relative unit of luminescence reported by the Lumac Biocounter M2500 luminometer.

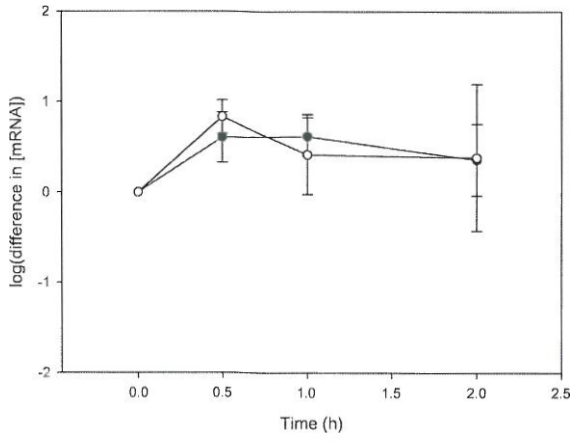

Fig. 7. Difference in $luxR_{Vh}$ (filled symbols) and *rpoA* (open symbols) mRNA levels between wild-type *Vibrio harveyi* BB120 cells treated with the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone (50 mg l^{-1}) and untreated cells, as determined by reverse transcriptase real-time PCR with specific primers. The error bars represent the standard deviation of two independent experiments.

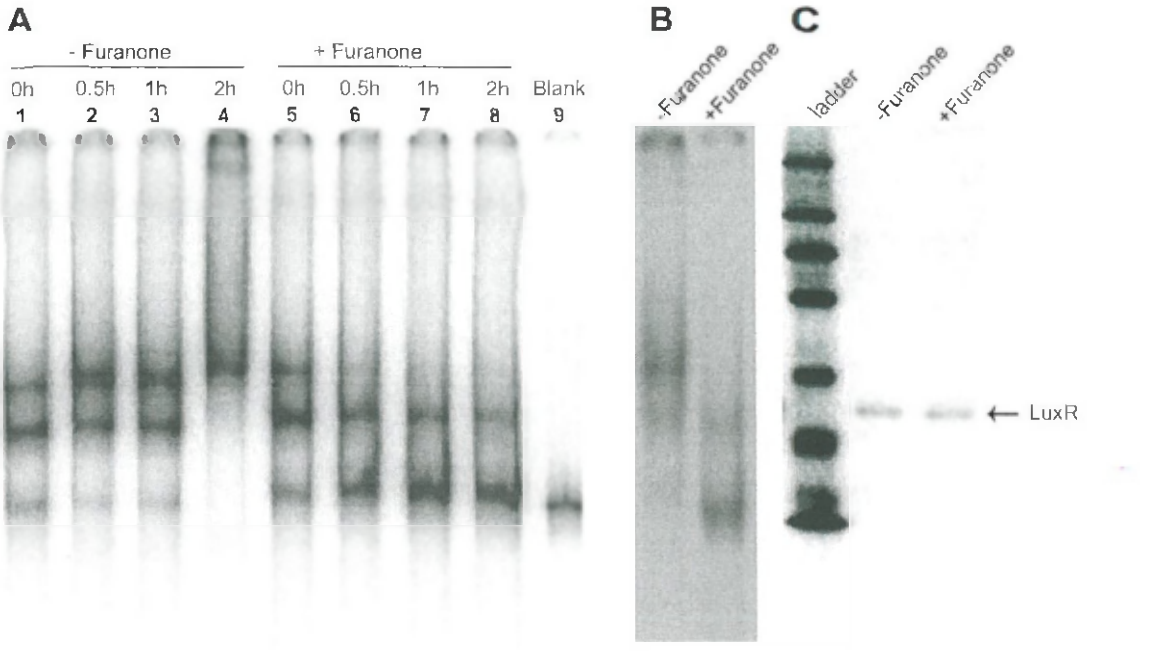

Fig. 8. $LuxR_{Vh}$ DNA binding as determined by mobility shifts.
A. Autoradiograph after 5% polyacrylamide gel electrophoresis of 5′ ^{32}P-labelled $luxR_{Vh}$ promoter DNA containing the $LuxR_{Vh}$ binding sites, mixed with 0.5 μg of protein lysates from wild-type *Vibrio harveyi* BB120: lanes 1–4, addition of lysates taken at different time points from an untreated culture; lanes 5–8, addition of lysates taken at different time points from a culture treated with 50 mg l^{-1} of the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone; last lane, blank (addition of lysate from the *luxR*-negative strain MM1130).
B. Autoradiograph after 5% polyacrylamide gel electrophoresis of 5′ ^{32}P-labelled $luxR_{Vh}$ promoter DNA containing the $LuxR_{Vh}$ binding sites, mixed with purified $LuxR_{Vh}$, which was incubated *in vitro* for 1 h with or without the furanone (50 mg l^{-1}).
C. SDS-PAGE of the same samples as in panel B.

Impact of the furanone on $LuxR_{Vh}$ protein levels and $LuxR_{Vh}$ binding to its target promoter sequences

Mobility shifts using radiolabelled $luxR_{Vh}$ promoter DNA containing the $LuxR_{Vh}$ binding sites showed less shifts with cell lysates from furanone-treated cells when compared with untreated cells (Fig. 8A), indicating that there were significantly lower levels of the protein able to bind the promoter DNA. In order to quantify the difference in bound $LuxR_{Vh}$ levels between furanone-treated and untreated cells, a titration for mobility shifts of $luxR_{Vh}$ promoter DNA using the 2-h samples was performed. This titration revealed that 0.025 μg lysate of untreated cells gave the same shift as 0.5 μg lysate of furanone-treated cells (data not shown), which indicates that there was a 20-fold difference in $LuxR_{Vh}$ levels bound to the promoter DNA. The *luxCDABEGH* promoter DNA was also used to check for $LuxR_{Vh}$ shifts, with similar findings (Fig. 9). These observations could be explained by the furanone either decreasing translation or increasing turnover of $LuxR_{Vh}$, or altering the protein in such a way that it is rendered unable to bind to its target promoter. Some additional tests were performed to clear this up. First, 50 mg l^{-1} chloramphenicol was added to *V. harveyi* BB120 cultures in order to stop translation of $LuxR_{Vh}$. In these cultures, there was no effect on $LuxR_{Vh}$ mobility shifts in the absence of furanone throughout the 1 h of incubation, whereas in the presence of furanone, again less shifts occurred (data not shown). This suggests that $LuxR_{Vh}$ has quite a slow turnover and that the furanone acts on pre-existing $LuxR_{Vh}$. Finally, the furanone was added to purified $LuxR_{Vh}$, and after subsequent incubation of the mixture, again significantly lower levels of $LuxR_{Vh}$ were found to bind to the promoter DNA when compared with untreated $LuxR_{Vh}$ (Fig. 8B). Interestingly, SDS-PAGE showed that the concentration of $LuxR_{Vh}$ in the mixtures was not affected by the furanone (Fig. 8C). Together, these data lead to the conclusion that the furanone compound renders $LuxR_{Vh}$ unable to bind to its target promoter sequences, without degrading the protein.

Discussion

Disease caused by antibiotic resistant luminescent vibrios is a serious problem in the aquaculture industry (Austin and Zhang, 2006). Recent investigations have pointed out

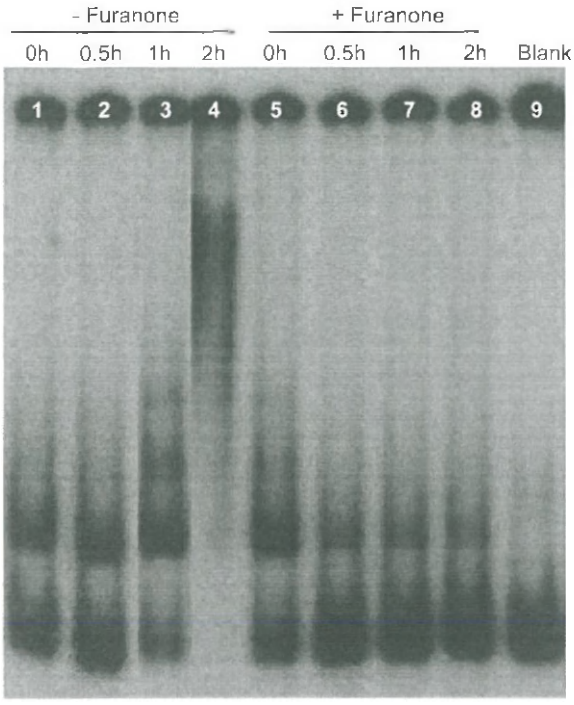

Fig. 9. $LuxR_{Vh}$ DNA binding as determined by mobility shifts. Autoradiograph after 5% polyacrylamide gel electrophoresis of 5′ ^{32}P-labelled *luxCDABEGH* promoter DNA containing the $LuxR_{Vh}$ binding sites, mixed with 0.5 μg of protein lysates from wild-type *Vibrio harveyi* BB120: lanes 1–4, addition of lysates taken at different time points from an untreated culture; lanes 5–8, addition of lysates taken at different time points from a culture treated with 50 mg l^{-1} of the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone; last lane, blank (addition of lysate from the *luxR*-negative strain MR1130).

that disruption of the quorum sensing system of these bacteria by using halogenated furanones could be a promising alternative biocontrol strategy (Manefield *et al.*, 2000; Defoirdt *et al.*, 2006). In view of the potential practical applications of this type of compounds, it is of significant interest to define the mode of action of the furanones in these bacteria. Hence, in this study, we aimed at elucidating the molecular mechanism of quorum sensing disruption by the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone in *V. harveyi*.

In a first series of experiments, the impact of the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone on quorum sensing-regulated bioluminescence of *V. harveyi* wild type and quorum sensing mutants was determined. Importantly, halogenated furanones were shown before not to block bioluminescence when expressed from an external promoter (Givskov *et al.*, 1996), indicating that the biochemical function of the Lux proteins is not affected. Consistent with the work of Manefield and colleagues (2000), the compound was found to block bioluminescence in wild-type *V. harveyi* BB120 in a concentration-dependent way. Moreover, luciferase activity was significantly decreased in protein lysates of furanone-treated BB120 cells. In order to determine the effect of the furanone on the three different channels of the *V. harveyi* quorum sensing system, its impact on bioluminescence of the signal molecule receptor double mutants JAF375, JMH597 and JMH612 (Henke and Bassler, 2004a) was studied. The compound was shown to block bioluminescence in all three double mutants in a pattern similar to the one obtained for the wild type. Because of the mutated receptors, bioluminescence in these mutants is only responsive to one of the three signal molecules, which implies that all three channels of the quorum sensing system were blocked. These data confirm the reports by Ren and colleagues (2001) and McDougald and colleagues (2003), who determined the impact of the furanone on the AI-2- and/or HAI-1-mediated channels of the system by using the AI-2 and/or HAI-1 receptor mutants BB886 and BB170 (which thus were still responsive to both HAI-1 and CAI-1 and AI-2 and CAI-1 respectively). Moreover, our results indicate that the furanone also blocks the CAI-1-mediated channel of the *V. harveyi* quorum sensing system.

Previously, the hypothesis that prevailed in literature was that the furanones disrupt quorum sensing in *V. harveyi* by displacing the signal molecules from their receptors (Manefield *et al.*, 2000; Ren *et al.*, 2001). However, because the three signal molecules have quite different chemical structures (although the exact structure of CAI-1 is still unknown; Henke and Bassler, 2004a), we suspected that the furanone did not block quorum sensing-regulated bioluminescence by competing with the autoinducers for receptor sites but rather by interfering with the quorum sensing signal transduction. In order to test this hypothesis, the effects of the furanone on bioluminescence of mutants that were fixed in the high cell-density configuration at different stages in the quorum sensing signal transduction cascade were investigated (i.e. LuxU, LuxO and Hfq). We found that the furanone blocked bioluminescence in the *hfq* mutant BNL258. Hfq is a chaperone protein that acts together with small regulatory RNAs to destabilize the mRNA of the master regulator $LuxR_{Vh}$. The Hfq protein is non-functional in strain BNL258, resulting in constitutively expressed bioluminescence (Lenz *et al.*, 2004). The fact that the furanone blocked bioluminescence in this mutant indicates that it acts downstream of Hfq, i.e. at the level of the $luxR_{Vh}$ mRNA and/or the $LuxR_{Vh}$ protein and not by displacing the signal molecules from their receptors.

The quorum sensing master regulator protein $LuxR_{Vh}$ has been shown before to be a transcriptional activator that is required for expression of the *lux* operon (Swartzman *et al.*, 1992; Swartzman and Meighen, 1993).

Consequently, in a final series of experiments, the effect of the furanone on this response regulator protein was investigated. Reverse transcriptase real-time PCR with primers specific for *V. harveyi luxR*$_{Vh}$ revealed that the furanone has no effect on the concentration of the *luxR*$_{Vh}$ mRNA. In contrast, mobility shift assays showed that the concentration of the LuxR$_{Vh}$ response regulator protein able to bind to its target promoter sequences significantly decreased after furanone addition, both in intact cells and in purified LuxR$_{Vh}$ extracts. Interestingly, the concentration of the LuxR$_{Vh}$ protein was shown not to be affected by the furanone (as determined by SDS-PAGE analysis). LuxR$_{Vh}$ is a member of the TetR family of transcriptional regulators containing a helix–turn–helix DNA binding domain (Ramos *et al.*, 2005). Members of the TetR family that have been studied in depth, have been shown to bind DNA as dimers, but this has not yet been proven for LuxR$_{Vh}$. Because halogenated furanones are known to be very reactive compounds (Hentzer and Givskov, 2003), we hypothesize that the furanone reacts with the LuxR$_{Vh}$ protein, thereby altering it in such a way that it cannot bind the DNA anymore, either by changing the structure of the DNA binding domain or the regions involved in dimer formation (that is, if LuxR$_{Vh}$ also needs to dimerize in order to bind DNA). However, the elucidation of the exact biochemical reaction mechanism between halogenated furanones and LuxR$_{Vh}$ (with the aid of mass spectrometry) will be part of future work at our laboratories.

The fact that the furanone affects the master regulator rather than selectively blocking one of the channels of the *V. harveyi* quorum sensing system is quite important with respect to possible practical applications because there seems to be a difference in the relative importance of the three channels for a successful infection of different hosts. Indeed, disrupting only the AI-2-mediated channel of the *V. harveyi* quorum sensing system has been shown to significantly increase survival of the brine shrimp *A. franciscana*, whereas the HAI-1-mediated channel had no effect on infection of the shrimp (Defoirdt *et al.*, 2005). In contrast, both the HAI-1- and AI-2-mediated channel needed to be disrupted in order to decrease mortality of gnotobiotic rotifers (*Brachionus plicatilis*) caused by *V. harveyi* (Tinh *et al.*, 2007). Because the furanone blocks all three channels of the system at once by acting at the end of the quorum sensing signal transduction cascade, it will not be necessary to develop different furanone compounds to protect different hosts. Consistent with this, the natural furanone was shown to protect both brine shrimp and rotifers from luminescent vibrios (Defoirdt *et al.*, 2006; Tinh *et al.*, 2007). In addition to this, several human pathogens, including *Vibrio cholerae, Vibrio parahaemolyticus* and *Vibrio vulnificus*, have been found to contain a LuxR$_{Vh}$ homologue (Jobling and Holmes, 1997; McCarter, 1998; McDougald *et al.*, 2000). Moreover, the LuxR$_{Vh}$ homologues have been shown before to regulate virulence factor production in these species as well (Henke and Bassler, 2004b; Milton, 2006) and consequently, the furanones might be useful to control infections caused by these human pathogens.

In addition to affecting both *V. fischeri* LuxR$_{Vf}$ (Manefield *et al.*, 2002) and *V. harveyi* LuxR$_{Vh}$ (this study), covalent binding of this natural furanone to the *Escherichia coli* LuxS protein has been reported (Ren *et al.*, 2004). Hence, it can be hypothesized that these furanones react with certain reactive groups which are present in different regulatory proteins. It therefore seems that the macro-alga *Delisea pulchra* has evolved a sophisticated quorum sensing disruption-based defence system, producing compounds that affect different types of signal regulators in a non-growth inhibitory way. In view of the broad range of bacteria that can potentially colonize marine organisms, the production of broad spectrum quorum sensing-disrupting compounds by the alga probably confers a strong evolutionary advantage.

In conclusion, the results presented in this article show that the natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone disrupts quorum sensing-regulated gene expression in *V. harveyi* by decreasing the DNA-binding activity of the quorum sensing master regulator protein LuxR$_{Vh}$. These results are of significant practical interest because they suggest that the furanone could be used as a broad spectrum biocontrol agent to protect a variety of hosts from different pathogenic vibrios in which virulence factor production is regulated by a LuxR$_{Vh}$ homologue.

Experimental procedures

Furanone preparation

The natural furanone (*5Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(*5H*)-furanone was synthesized as described previously (Ren *et al.*, 2001). The furanone was dissolved and diluted in pure ethanol and stored at –20°C.

Bacterial strains and growth conditions

The strains that were used in this study are shown in Table 1. All strains were grown in Luria–Bertani medium containing 20 g l^{-1} NaCl (LB$_{20}$) at 28°C under constant agitation. Spectrophotometry at OD$_{600}$ was used to measure growth. Luminescence was measured with a Lumac Biocounter M2500 luminometer (Lumac b.v., Landgraaf, the Netherlands).

For all experiments, *V. harveyi* strains were grown to an OD$_{600}$ of approximately 0.75, after which the furanone was added to the cultures in the appropriate concentration and the cultures were further incubated at 28°C with shaking. Unless otherwise indicated, samples were taken in triplicate 0.5 h after furanone addition.

Table 1. Strains used in this study.

Strain	Phenotype	Reference
Vibrio harveyi BB120	Wild type from which strains BNL258, JAF375, JAF483, JAF553, JMH597 and JMH612 are derived	Bassler *et al.* (1997)
Vibrio harveyi BNL258	*hfq*::Tn*5lacZ*	Lenz *et al.* (2004)
Vibrio harveyi JAF375	*luxN*::Cm^R *luxQ*::Kan^R	Freeman and Bassler (1999a)
Vibrio harveyi JAF483	*luxO* D47A linked to Kan^R	Freeman and Bassler (1999a)
Vibrio harveyi JAF553	*luxU* H58A linked to Kan^R	Freeman and Bassler (1999b)
Vibrio harveyi JMH597	*luxN*::Tn5 *cqsS*::Cm^R	Henke and Bassler (2004a)
Vibrio harveyi JMH612	*luxPQ*::Tn5 *cqsS*::Cm^R	Henke and Bassler (2004a)
Vibrio harveyi MR1130	Isogenic *LuxR* mutant	Martin *et al.* (1989)

Protein assays

Wild-type *V. harveyi* BB120 was used in the luciferase and bacterial alkaline phosphatase assays. Immediately before and 0.5, 1 and 2 h after the addition of the furanone, 2 units [$OD_{600} \times$ vol (ml)] were pelleted, sonicated in 0.5 ml of 0.25 M Tris, pH 8 and the cellular debris removed (as described by Miyamoto *et al.*, 1990). A modified Lowry assay was used to determine the protein content (Markwell *et al.*, 1981). *In vitro* luciferase and bacterial alkaline phosphatase activities were determined as described by Gunsalus-Miguel and colleagues (1972) and Garen and Levinthal (1960).

Primer design

Specific primers for the amplification of $luxR_{Vh}$ and RNA polymerase A subunit (*rpoA*) mRNA were designed using the Primer Express 2.0 software (Applied Biosystems, Foster City, USA). The primers were designed based on the consensus of the sequences that have been deposited before in GenBank. The combinations of the two primer sequences were blasted against GenBank. The primer sequences are shown in Table 2.

RNA extraction and reverse transcriptase real-time PCR

The effect of the furanone on $luxR_{Vh}$ mRNA concentrations was studied in wild-type *V. harveyi* BB120. Immediately before and 0.5, 1 and 2 h after furanone addition, luminescence and cell density (OD_{600}) of the cultures were measured and 0.5 ml of samples for RNA extraction were taken, which were immediately frozen in cold ethanol (–80°C). RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA extracts were treated with RNAse-free DNAse I (Fermentas, St. Leon-Rot, Germany), after which the RNA quantity was checked spectrophotometrically. RNA concentrations obtained were all around 300 ng μl^{-1}. RNA quality was confirmed by electrophoresis. cDNA was obtained by reverse transcription using the Qiagen One Step RT-PCR kit (Qiagen), according to the manufacturer's instructions.

Table 2. Primers used in this study.

Target	Primer	Sequence
rpoA	$rpoA_{forward}$	5′-CGTAGCTGAAGGCAAAGATGA-3′
	$rpoA_{reverse}$	5′-AAGCTGGAACATAACCACGA-3′
$luxR_{Vh}$	$LuxR_{forward}$	5′-TCAATTGCAAAGAGACCTCG-3′
	$LuxR_{reverse}$	5′-AGCAAACACTTCAAGAGCGA-3′

Real-time PCR was performed with the specific $luxR_{Vh}$ and *rpoA* primers. Amplicon lengths are 84 bp and 197 bp for $luxR_{Vh}$ and *rpoA* respectively. Amplification was performed in 25 µl reaction mixtures using the SYBR Green PCR Master Mix kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, the Netherlands), according to the manufacturer's instructions, in optical 96-well reaction plates with optical caps (Applied Biosystems). The thermal profile was as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 54°C for 1 min, and 60°C for 1 min. Amplicon dissociation curves were determined by constant fluorescent measurement during a final heating step at 60°C to 95°C at 0.1°C s^{-1} ramping speed. The melting temperatures for the amplicons were 77°C and 82°C for $luxR_{Vh}$ and *rpoA* respectively. The template DNA in the reaction mixtures was amplified and monitored with an ABI Prism SDS 7000 instrument (Applied Biosystems). The difference in mRNA levels between furanone-treated and untreated extracts was calculated as follows:

$$\log(\text{difference in concentration}) = \Delta C_t / \text{regression coefficient}$$

with ΔC_t the difference in C_t value between furanone-treated and untreated extracts. The regression coefficients between log(concentration) and C_t value were determined by analysing a 10-fold dilution series of a *V. harveyi* BB120 DNA extract and were –3.380 ($R^2 = 0.998$) and –3.597 ($R^2 = 0.993$) for $luxR_{Vh}$ and *rpoA* respectively.

Mobility shift assays

The effect of the furanone on $LuxR_{Vh}$ protein concentrations was studied in wild-type *V. harveyi* BB120. Immediately before and 0.5, 1 and 2 h after the addition of the furanone, 2 units [$OD_{600} \times$ vol (ml)] were pelleted, sonicated in 0.5 ml of 0.25 M Tris, pH 8 and the cellular debris removed (as described by Miyamoto *et al.*, 1990). In the experiment that aimed at testing whether the furanone affected translation or acted on pre-existing translates, cultures were treated with 50 mg l^{-1} chloramphenicol Sigma-Aldrich (Bornem, Belgium) before furanone addition. Mobility shift assays were conducted as described previously (Swartzman and Meighen, 1993) using radiolabelled $luxR_{Vh}$ promoter DNA (Chatterjee *et al.*, 1996) or *luxCDABEGH* promoter DNA (Miyamoto *et al.*, 1996) containing the $LuxR_{Vh}$ binding sites. The amount of retarded DNA was quantified as described in Miyamoto and colleagues (1996) using a Fuji bioimage.

Experiments with purified LuxR$_{Vh}$

LuxR$_{Vh}$ purification was carried out as described previously (Swartzman and Meighen, 1993). The purified LuxR$_{Vh}$ was stored in 50 mM $NaPO_4$, 300 mM NaCl, pH 8.0. The protein concentration (as determined by Bio-Rad protein assays) was 0.2 mg ml^{-1}. To glass tubes containing 40 µl of the protein preparation, 0.2 µl of absolute ethanol or 0.2 µl of furanone (10 mg ml^{-1} in ethanol) were directly added. The tubes were incubated in a 37°C water bath for 1 h and then stored at 4°C. For mobility shift analyses, the samples were diluted 10-fold in storage buffer and 1 µl (0.02 µg) was assayed. For SDS-PAGE, 1 µg of protein was applied and 10% SDS-PAGE was performed according to Maniatis and colleagues (1982).

Acknowledgements

We thank Bonnie Bassler for generously providing us with the *V. harveyi* mutants. This work was supported by the 'Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen' (IWT Grant no. 33205), the 'Fonds voor Wetenschappelijk Onderzoek' (project no. G0230.02N), the National Institutes of Health (ER003872-01A1) and the Canadian Institutes of Health Research (MT7672).

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