Inhibition of Luminescence and Virulence in the Black Tiger Prawn (*Penaeus monodon*) Pathogen *Vibrio harveyi* by Intercellular Signal Antagonists

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Expression of luminescence in the *Penaeus monodon* pathogen *Vibrio harveyi* is regulated by an intercellular quorum sensing mechanism involving the synthesis and detection of two signaling molecules, one of which is N-hydroxy butanoyl-L-homoserine lactone and the other of which is uncharacterized. Indirect evidence has suggested that virulence, associated with a toxic extracellular protein, an enteric organism in the enteric contents of marine animals (11, 22). Recognized as a primary pathogen of many commercially cultured invertebrate species, such as the black tiger prawn (*Penaeus monodon*), *V. harveyi* can cause up to 100% mortality of larvae in the hatchery stage of penaeid culture (12, 16).

Virulence in *V. harveyi* (strain 47666-1) has been attributed to the production of an extracellular protein referred to as toxin T1 with a molecular mass of approximately 100 kDa (10, 19). The extracellular protein is produced during the mid-exponential phase of growth and has sequence similarity to virulence-associated proteins in *Salmonella*, *Shigella*, and *Bacillus* species (10).

As suggested by the name of the disease phenomenon commonly referred to as luminous vibrosis, the expression of luminescence in *V. harveyi* has long been associated with virulence in pathogenic strains of this organism (14). Expression of the luminescence phenotype in *V. harveyi* is controlled at the transcriptional level by an atypical quorum sensing system (1). In the model quorum sensing system of *Vibrio fischeri*, a diffusible N-acylated-L-homoserine lactone (AHL) molecule is employed to link expression of the luminescence phenotype to bacterial population density. AHL, synthesized by the LuxI protein, accumulates throughout the population and at high concentrations interacts with a regulatory protein (LuxR), which then transcriptionally activates expression of the structural genes (*luxCDABEG*) encoding the luminescence phenotype (23). Other AHL quorum sensing systems, such as those regulating expression of virulence factors in *Pseudomonas aeruginosa* and *Erwinia carotovora*, also rely on LuxI and LuxR homologues for AHL production and detection, respectively (8).

In this study we investigated the ability of a halogenated furanone to inhibit the quorum sensing regulated expression of the *lux* structural genes (*luxCDABEG*) by stimulating phosphatase activity in a sensory protein (LuxN), which acts to dephosphorylate the transcriptional repressor LuxO (6) via a phosphorelay protein named LuxU (7). A second, uncharacterized quorum sensing signal, known as Al-2, stimulates phosphatase activity in a second sensory protein named LuxQ, the activity of which converges at LuxU with the HBHL-regulated phosphorelay channel (7). Following inactivation of the LuxO repressor protein, transcription of the *lux* structural genes is stimulated by a signal-independent transcriptional activator protein (24).

Recently it has been shown that halogenated furanones produced by the red, benthic, marine macroalga *Delisea pulchra* interfere with AHL-regulated gene expression. These algal metabolites are active against the luminescence phenotype of *V. fischeri* (9). Halogenated furanones are thought to act by displacing AHL from its receptor protein (LuxR or LuxR homologue), thus inhibiting transcriptional activation of genes encoding the quorum sensing phenotype (18).

In this study we investigated the ability of a halogenated furanone (Fig. 1B) to inhibit the quorum sensing regulated luminescence phenotype of the pathogenic *V. harveyi* strain.
Preparation of concentrated cell supernatant extracts and analysis of toxin T1 production. To determine the effect of the algal metabolites on toxin T1 production, 100-ml duplicate cultures of *V. harveyi* were grown to an OD$_{600}$ of 0.4 in the presence or absence of 100 μM halogenated furanone. Cells were pelleted, and supernatants were filtered through a 0.22-μm-pore-size filter. The resulting cell-free supernatants were then concentrated 100-fold with Ultrafuge 15 centrifugal filters, which retain proteins with masses above 10 kDa (Millipore). Native polyacrylamide gel electrophoresis (PAGE) with a 10% separating gel (15) was used to visualize the concentrated cell supernatant extracts from furanone-treated and untreated cultures. Relative concentrations of toxin T1 were quantified by standard densitometry using Multi-Analyst software (Bio-Rad). Toxin T1 activity, a 10-fold dilution of concentrated supernatant extract prepared from the filtered high-density supernatant, a 10-fold dilution of concentrated supernatant extract prepared from the filtered high-density supernatant, was then concentrated as described above.

To determine the effect of HBHL and AI-2 on toxin T1 production, 100-ml duplicate cultures of *V. harveyi* were grown to an OD$_{600}$ of 0.1 in the presence and absence of 200 nM HBHL or a 10% filtered high-density (OD$_{600}$ = 0.4) *V. harveyi* culture supernatant. Toxin T1 preparations were made from these cultures and analyzed as described above. To ensure that the presence of toxin T1 in low-density cultures was not left over from addition of the 10% high-density supernatant, a 10-fold dilution of concentrated supernatant extract prepared from the filtered high-density (OD$_{600}$ = 0.4) *V. harveyi* culture employed was included in the PAGE analysis.

**Results**

Inhibition of luminescence in *V. harveyi* by a halogenated furanone. Given the ability of halogenated furanones to interfere with AHL-mediated gene expression in other organisms such as *V. fischeri* and *Serratia liquefaciens* (9, 18), it was of interest to test the effect of the algal metabolites on the genetically distinct signal-mediated gene expression of *V. harveyi*. Cultures of *V. harveyi* were grown from low to high cell density in the presence and absence of 50, 100, or 200 μM halogenated furanone (Fig. 1B). In this strain, the characteristic autoinduced increase in the expression of the luminescence phenotype is associated with an OD$_{600}$ of 0.2 to 0.4. Figure 2A shows the concentration-dependent inhibition of the luminescence phenotype, most apparent after 4 h of incubation at an OD$_{600}$ of approximately 0.3, by the halogenated furanone. At the concentrations tested, no alteration in the growth of the bacterium was observed as measured by optical density (Fig. 2B). This suggested that the AHL-dependent luminescence phenotype of *V. harveyi*, as with *V. fischeri*, is susceptible to regulation by halogenated furanones.

**Inhibition of toxin T1 production.** Virulence in *V. harveyi* has been attributed to a partially characterized 100-kDa protein referred to as toxin T1 (10). PAGE analysis of concentrated cell supernatant extracts has shown that the toxin ap-
pears in culture supernatants at an optical density between 0.2 and 0.4, corresponding with the density at which luminescence is expressed. The putative link between expression of luminescence and virulence prompted us to test the effect of a halogenated furanone on the production of toxin T1 in *V. harveyi*. Cultures were grown in the presence and absence of 100 μM halogenated furanone to an optical density of 0.4. Concentrated cell supernatant extracts of the untreated and furanone-treated cultures contained 3.9 mg and 3.8 mg of total protein/ml, respectively. These concentrates were analyzed by PAGE to assess the resultant effect of the furanone on toxin production (Fig. 3A). Densitometric analysis revealed a 50% reduction in the presence of toxin T1 in the supernatants of cultures treated with the halogenated furanone (Fig. 3B). The simultaneous down-regulation of luminescence and toxin T1 production adds further indirect evidence to the putative coregulation of luminescence and virulence in this pathogenic *V. harveyi* strain.

In vivo toxicity of cell supernatant extracts is reduced when the extracts are prepared from halogenated furanone-treated

**V. harveyi cultures.** Harris and Owens (10) have previously found that cell supernatant extracts from *V. harveyi*, administered by intraperitoneal injection, are toxic to CBA mice, with an LD₅₀ of 3.9 μg of total protein per g of body weight. Similarly, such extracts, administered by intramuscular injection, are toxic to *P. monodon*, with an LD₅₀ of 3.5 μg per g of prawn. To determine whether in vivo toxicity of *V. harveyi* cell supernatant extracts to mice and prawns is reduced by furanones, extracts were prepared from *V. harveyi* cultures incubated in the presence and absence of 100 μM halogenated furanone.

CBA mice were challenged in triplicate with 100 μl of 1-in-4-dilution, 1-in-2-dilution, or undiluted extracts from untreated or furanone-treated cultures of *V. harveyi*. The resultant survival and symptoms of treated mice were recorded every 12 h for 96 h after injection. All mice treated with undiluted extracts (13.7 μg/g) or 1-in-2 dilutions of extracts (~7.5 μg/g) from untreated or furanone-treated cultures died within 96 h of injection (Table 1). It was noted that mice injected with a 1-in-2 dilution of extract from the halogenated furanone-treated culture had delayed mortality (1 mouse deceased after 96 h, 2 survivors recovered after 96 h) compared with mice

FIG. 2. Effect of the halogenated furanone at 0 μM (diamonds), 50 μM (circles), 100 μM (triangles), and 200 μM (squares) on specific luminescence response reported in relative light units (RLU) (A) and growth as determined by OD₆₀₀ of *V. harveyi* (B).

FIG. 3. Effect of 100 μM halogenated furanone on the presence of toxin T1 in *V. harveyi* supernatants as determined by native PAGE and densitometry. (A) The gel shows purified toxin T1 (lane 1), a twofold-dilution series of extracts prepared from control cultures (lanes 2, 4, and 6), and a twofold-dilution series of extracts prepared from halogenated furanone-treated cultures (lanes 3, 5, and 7). (B) Bar graph representing the band intensity of toxin T1 in each lane as a percentage of the untreated, undiluted toxin T1 band (lane 2).
injected with equivalent dilutions of extract from the untreated culture (3 mice deceased after 48, 48, and 60 h). Mice injected with a 1-in-4 dilution of an extract (4.5 µg/g) from the halogenated furanone-treated culture displayed a higher survival rate (100%) than mice treated with the same dilution of extract (3.6 µg/g) from an untreated V. harveyi culture (33%). Based on these data, groups of 10 mice were injected with a 1-in-4 dilution (~4.5 µg/g) of freshly prepared extracts from untreated or furanone-treated cultures and were observed as described above. Table 1 reveals a significant reduction in mortality rates for CBA mice treated with a 1-in-4 dilution of extracts prepared from furanone-treated (90% survival) versus untreated (20% survival) V. harveyi cultures ($G = 9.404; df = 1; P = 0.0022$).

As with the CBA mice, P. monodon specimens were challenged with extracts from furanone-treated and untreated V. harveyi cultures. Significant differences in survival were not observed between prawns injected with pure extracts (~15 µg/g) or 1-in-2 dilutions of extracts (~7 µg/g) from untreated or furanone-treated cultures (Table 1). A significant difference was observed, however, when prawns were injected with a 1-in-4 dilution (~3.5 µg/g) of the extracts ($G = 4.487, df = 1, P = 0.1698$). Eighty-two percent of prawns challenged with a 1-in-4 dilution of extract from the furanone-treated culture survived the treatment, compared with a 36% survival rate for prawns treated with the equivalent dilution of extract prepared from untreated V. harveyi cultures. No significant differences were observed when 1-in-8 dilutions of the two extracts were used.

The LD$_{50}$ of the dilution series of extracts from furanone-treated cultures for P. monodon was calculated to be 7.11 µg of protein per g of prawn, which was significantly higher than that with the extract prepared from untreated cultures, calculated to be 3.45 µg/g. These data suggest that the production, stability, or activity of the extracellular virulence factor produced by V. harveyi is reduced by the presence of 100 µM halogenated furanone.

To ensure that this reduction in in vivo toxicity was not the result of the halogenated furanone interfering directly with the stability or activity of the extracellular virulence factor, samples of cell supernatant from an untreated V. harveyi culture were treated with 100 µM halogenated furanone, and others were left untreated. These samples were then concentrated and injected into CBA mice and P. monodon specimens. At all dilutions tested, no significant differences in toxicity for mice or prawns were observed between furanone-treated and untreated cell supernatants (Table 2). This suggests that the halogenated furanone acts on the production of the virulence factor.

**Stimulation of toxin T1 production with the supernatant of a high-density culture.** Based on indirect evidence suggesting that toxin T1 production is regulated by the quorum sensing mechanism of V. harveyi, the effects of HBHL and spent-culture supernatants on toxin T1 production were tested. HBHL at 200 nM or 10% conditioned media were added to V. harveyi cultures, which were analyzed by PAGE for the presence of toxin T1 at a low density to check for an early induction. As shown in Fig. 4, toxin T1 was barely detectable in extracts from untreated cultures at low densities (OD$_{600}$ = 0.1). Culturing in the presence of HBHL did not increase toxin T1 production at this density. Addition of 10% high-density culture supernatant, however, caused production of toxin T1 to be induced even at this low cell density (Fig. 4). This strongly suggests that toxin T1 production is regulated by intercellular signals.

**TABLE 1.** Survival rates of CBA mice and juvenile P. monodon specimens challenged with concentrated cell supernatant extracts prepared from cultures of V. harveyi 47666-1 grown in the presence and absence of 100 µM halogenated furanone.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Supernatant extracts from:</th>
<th>Untreated culture$^a$</th>
<th>Furanone-treated culture$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. surviving/ dose (%)</td>
<td>No. surviving/ dose (%)</td>
<td></td>
</tr>
<tr>
<td>CBA mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/3 (0)</td>
<td>13.7</td>
<td>0/3 (0)</td>
<td>13.7</td>
</tr>
<tr>
<td>0/3 (0)</td>
<td>7.5</td>
<td>0/3 (0)</td>
<td>7.7</td>
</tr>
<tr>
<td>1/3 (33)</td>
<td>3.6</td>
<td>3/3 (100)</td>
<td>4.5</td>
</tr>
<tr>
<td>2/10 (20)</td>
<td>4.4</td>
<td>$9/10$ (90)</td>
<td>4.5</td>
</tr>
<tr>
<td>P. monodon specimens</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/7 (0)</td>
<td>16.5</td>
<td>0/7 (0)</td>
<td>14.3</td>
</tr>
<tr>
<td>1/7 (14)</td>
<td>7.4</td>
<td>4/7 (57)</td>
<td>7.1</td>
</tr>
<tr>
<td>4/11 (36)</td>
<td>3.5</td>
<td>9/11 (82)</td>
<td>3.6</td>
</tr>
<tr>
<td>6/8 (75)</td>
<td>1.5</td>
<td>7/7 (100)</td>
<td>1.9</td>
</tr>
</tbody>
</table>

$^a$ Total protein concentration = 3.8 µg/ml.

$^b$ Total protein concentration = 3.9 µg/ml.

**TABLE 2.** Survival rates of CBA mice and juvenile P. monodon specimens challenged with concentrated cell supernatant extracts prepared from cultures of V. harveyi 47666-1 and treated postextraction with 100 µM halogenated furanone.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Untreated extract$^a$</th>
<th>Furanone-treated extract$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. surviving/ dose (%)</td>
<td>No. surviving/ dose (%)</td>
</tr>
<tr>
<td>CBA mice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0/3 (0)</td>
<td>10.6</td>
<td>0/3 (0)</td>
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<tr>
<td>1/3 (33)</td>
<td>6.1</td>
<td>0/3 (0)</td>
</tr>
<tr>
<td>1/3 (33)</td>
<td>4.2</td>
<td>1/3 (33)</td>
</tr>
<tr>
<td>0/3 (0)</td>
<td>2.9</td>
<td>1/3 (33)</td>
</tr>
<tr>
<td>P. monodon specimens</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/9 (22)</td>
<td>13.9</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>3/9 (33)</td>
<td>6.1</td>
<td>2/8 (25)</td>
</tr>
<tr>
<td>5/9 (56)</td>
<td>5.6</td>
<td>5/8 (63)</td>
</tr>
<tr>
<td>4/7 (57)</td>
<td>4.1</td>
<td>6/7 (86)</td>
</tr>
<tr>
<td>7/7 (100)</td>
<td>3.5</td>
<td>6/7 (86)</td>
</tr>
</tbody>
</table>

$^a$ Total protein concentration = 4.0 µg/ml.

$^b$ Total protein concentration = 3.8 µg/ml.
DISCUSSION

The quorum sensing mechanism regulating luminescence in *V. harveyi* differs from that of *V. fischeri*, as initially was indicated by the inability of culture supernatants from either species to stimulate luminescence in the other (5). It was found that intercellular signals (HBHL and AI-2) of *V. harveyi* do not interact with a transcriptional activator directly but rather stimulate a phosphorelay, which culminates in the deactivation of a transcriptional repressor protein (2, 6).

Because HBHL is unable to stimulate transcriptional activation in the LuxR protein of *V. fischeri* and because the intercellular signal receptors of *V. harveyi* are not LuxR homologues, it was of interest to see if halogenated furanones, known to displace N-3-oxohexanoyl-l-homoserine lactone from the LuxR protein of *V. fischeri*, could interfere with quorum sensing in *V. harveyi*. This was tested in *V. harveyi* strain 47666-1, a known pathogen of the commercially farmed prawn species *P. monodon*.

Structural similarities between HBHL and halogenated furanones suggest that the two molecules could interact with receptor proteins in similar fashions. The halogenated furanone tested has a molecular weight of 309.97, whereas the molecular weight of HBHL is 187.18. Both molecules have acyl chains of four carbons extending from five-membered lactone rings. The differences between the structures are that the halogen metabolite possesses a furan ring rather than a homoserine lactone ring and that the two possess different side groups in different positions. HBHL has a ketone and a hydroxyl group extending from carbons 1 and 3 of the acyl chain, respectively. The halogenated furanone has a bromine atom and a brominated exocyclic double bond extending from atoms 4 and 5 of the ring, respectively. Such differences do not render the furanones incapable of antagonizing AHL activity but do reduce their affinity for AHL receptor proteins relative to the native signals (18).

This study reveals that in the presence of the halogenated furanone, the luminescence phenotype of *V. harveyi* was reduced with no impact on the growth of the organism. This result, in conjunction with similar results in *V. fischeri* and other bacterial species, may indicate that halogenated furanones possess broader antagonist activity than AHLs. The present study also demonstrates that the production of an extracellular toxin, which appears in the supernatant of *V. harveyi* cultures concurrently with expression of the luminescence phenotype, is inhibited by the halogenated furanone. Cell density-dependent expression of virulence factors is not uncommon (18). In support of the putative regulatory link between expression of luminescence and virulence in this organism, we found that the addition of high-density culture supernatant of *V. harveyi* to low-density cultures causes an early induction of toxin T1 synthesis. HBHL did not have this effect.

If indeed the toxin T1 structural gene, like the lux cassette, is under transcriptional repression by the LuxO protein, then toxin T1 production is dependent on switching the kinase activity of the signal receptors, LuxN and LuxQ, to phosphatase activity (6). Based on the inhibition of toxin T1 production by the halogenated furanone, we initially expected addition of HBHL to affect this switch, causing an early induction of toxin T1 synthesis at low cell density. Closer examination of the *V. harveyi* quorum sensing mechanism, however, suggested that this would not be the case.

Freeman and Bassler (6) have demonstrated that a luxN mutant has the wild-type capacity to phosphorylate LuxU, producing a wild-type luminescence response, and that a luxQ mutant, in contrast, has much-reduced kinase activity. This suggests that at low cell densities, repression of the lux cassette and any other LuxO-repressed genes is largely mediated by the kinase activity of phosphorylated LuxQ and that derepression requires AI-2. This potentially explains why, at low cell density, toxin T1 production is not responsive to HBHL alone but requires the addition of both signals, in the form of a high-density culture supernatant. Reduced toxin T1 production, via interference with HBHL binding by the halogenated furanone at a higher cell density, is then as expected. Alternatively, the halogenated furanone may exert its effect on luminescence and toxin T1 synthesis through interference with AI-2 activity. This interpretation, however, remains highly speculative, given that the structure of AI-2 is unresolved.

In vivo assays demonstrated that the toxicity of *V. harveyi* supernatant extracts is reduced when cultures contain the halogenated furanone. This reduction in toxicity was found not to be the result of a direct furanone interaction with the toxin leading to inactivation or degradation; it is most probable that it is due to inhibition of transcription of the toxin T1 structural gene. These data suggest that there exists potential for the use of furanones to limit *V. harveyi* infections in *P. monodon*. Delivery and efficacy of the algal metabolites at the site of *V. harveyi* infection in *P. monodon* are currently under investigation.

The results of this study have shown that a halogenated furanone from the alga *D. pulchra* inhibits the concurrent expression of luminescence and toxin production in the prawn pathogen *V. harveyi*. Additionally, toxin production was found to be induced at low density by a high-density culture supernatant. Besides the potential biotechnological application, these findings suggest that virulence in *V. harveyi*, like luminescence, is at least partially regulated by the quorum sensing mechanism.

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