

ORIGINAL ARTICLE

Interference with the quorum sensing systems in a *Vibrio harveyi* strain alters the growth rate of gnotobiotically cultured rotifer *Brachionus plicatilis*

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Abstract

Aims: To evaluate the effect of *Vibrio harveyi* strains on the growth rate of the gnotobiotically cultured rotifer *Brachionus plicatilis*, and to establish whether quorum sensing is involved in the observed phenomena.

Methods and Results: Gnotobiotic *B. plicatilis* sensu strictu, obtained by hatching glutaraldehyde-treated amictic eggs, were used as test organisms. Challenge tests were performed with 11 *V. harveyi* strains and different quorum sensing mutants derived from the *V. harveyi* BB120 strain. Brominated furanone [(5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone] as a quorum sensing inhibitor was tested in *Brachionus* challenge tests. Some *V. harveyi* strains, such as strain BB120, had a significantly negative effect on the *Brachionus* growth rate. In the challenge test with MM77, an isogenic strain of BB120 in which the two autoinducers (HAI-1 and AI-2) are both inactivated, no negative effect was observed. The effect of single mutants was the same as that observed in the BB120 strain. This indicates that both systems are responsible for the growth-retarding (GR) effect of the BB120 strain towards *Brachionus*. Moreover, the addition of an exogenous source of HAI-1 or AI-2 could restore the GR effect in the HAI-1 and AI-2 nonproducing mutant MM77. The addition of brominated furanone at a concentration of 2.5 mg l⁻¹ could neutralize the GR effect of some strains such as BB120 and VH-014.

Conclusions: Two quorum sensing systems in *V. harveyi* strain BB120 (namely HAI-1 and AI-2-mediated) are necessary for its GR effect on *B. plicatilis*. With some other *V. harveyi* strains, however, growth inhibition towards *Brachionus* does not seem to be related to quorum sensing.

Significance and Impact of the Study: Interference with the quorum sensing system might help to counteract the GR effect of some *V. harveyi* strains on *Brachionus*. However, further studies are needed to demonstrate the positive effect of halogenated furanone in nongnotobiotic *Brachionus* cultures and eventually, in other segments of the aquaculture industry.

Introduction

The rotifer *Brachionus plicatilis* is used as valuable and indispensable live food organisms in the industrial larval culture of many marine fish and shrimp species (Dhert *et al.* 2001; Liao *et al.* 2001; Lubzens *et al.* 2001; Shields 2001; Marte 2003). One of the constraints in rotifer cul-

ture is the large diversity of microbiota associated with this filter-feeding organism (Skjeremo and Vadstein 1993; Verdonck *et al.* 1997; Savas *et al.* 2005), which increases the risk of contaminating the fish larvae with opportunistic pathogens such as *Vibrio* sp., *Aeromonas* sp. and *Pseudomonas* sp. (Gatesoupe 1990). As the gastrointestinal microbiota of the early life stages of fish larvae is influenced

by the microbiota associated with the live feed (Ringø and Birkbeck 1999), the opportunistic bacteria associated with the enrichment process may cause detrimental effects when rotifers are fed to the fish larvae (Skjermo and Vadstein 1993).

The microbiota associated with rotifer production systems were considered to play a major role in the instability and variability of rotifer cultures (Hirayama 1987; Skjermo and Vadstein 1993; Harzevilli *et al.* 1997). Dhert *et al.* (2001) suggested that the problem of unexplained crashes in batch cultures can be partly solved by bacterial management. In a recent study, Tinh *et al.* (2006) demonstrated that a standing microbial community of a crash culture is not necessarily responsible for a rotifer crash, and that food type strongly interferes with the behaviour of the microbial communities in rotifer cultures.

Conventional methods for microbial control are based on the use of antibiotics or bacteriostatic compounds that kill or inhibit the growth of bacteria. However, this approach has led to the development of bacterial resistance to antibiotics. The interference with the quorum sensing systems, a means of bacterial communication, has been advocated as a novel strategy to control pathogenic bacteria without interfering with their growth (Hentzer *et al.* 2003). Disruption of quorum sensing was suggested as a new anti-infective strategy in general (Finch *et al.* 1998) and with particular potential for use in aquaculture (Defoirdt *et al.* 2004). Quorum sensing is known as a mechanism by which bacteria coordinate gene expression in a density-dependent manner. This process depends on the production, release and detection of signal molecules called autoinducers (Miller and Bassler 2001). By far, the most extensively investigated family of intercellular signalling molecules are the *N*-acyl homoserine lactones (AHL). These molecules are associated with the quorum sensing processes in various human and plant pathogens, such as *Pseudomonas aeruginosa* (Rumbaugh *et al.* 2000), *Erwinia carotovora*, *Agrobacterium tumefaciens* (Whitehead *et al.* 2001), as well as *Vibrio harveyi* (Manefield *et al.* 2000) and other fish pathogens (Bruhn *et al.* 2005). Quorum sensing in *V. harveyi*, a pathogen of many aquatic organisms (Gomez-Gil *et al.* 2004), is regulated via a multichannel phosphorylation/dephosphorylation cascade. This bacterium produces and responds to the three signal molecules, HAI-1 (Harveyi Autoinducer 1), AI-2 (Autoinducer 2) and CAI-1 (Cholerae Autoinducer 1), which regulate the expression of genes, among others, responsible for bioluminescence (Bassler *et al.* 1993, 1994, 1997). HAI-1 is an AHL and was identified as *N*-(β -hydroxybutyryl)homoserine lactone (Cao and Meighen 1989). AI-2 is a furanosyl bor-

ate diester (Chen *et al.* 2002), an universal signal that could be used by a variety of bacteria for communication among and between species (Cloak *et al.* 2002; Ohtani *et al.* 2002; Kim *et al.* 2003). Recently, a third quorum sensing component was discovered in *V. harveyi*, which involves a *Vibrio cholerae* autoinducer CAI-1 (Henke and Bassler 2004a).

Application of quorum sensing antagonists is one of the techniques that have been investigated for bacterial control in aquaculture. Both natural and synthetic halogenated furanone compounds, which are secondary metabolites in the marine red alga *Delisea pulchra* (Manefield *et al.* 1999), have been investigated as promising quorum sensing antagonists. The unicellular alga *Chlamydomonas reinhardtii* also secretes substances that mimic the activity of AHL signal molecules and thus interfere with quorum sensing effects in naturally encountered bacteria (Teplitski *et al.* 2004).

Rotifers, being an important element of the aquaculture food chain, can be used as an experimental *in vivo* system for studying the quorum sensing-mediated virulence in *V. harveyi*. This investigation is facilitated by the establishment of methods to obtain gnotobiotic rotifer cultures either from disinfected resting eggs (Douillet 1998; Rombaut *et al.* 1999) or from disinfected amictic eggs (Martinez-Diaz *et al.* 2003; Watanabe *et al.* 2005; Tinh *et al.* 2006), as they eliminate the effects of microbiota that are naturally present in the culture environment. In a first approach, we were interested in establishing whether *V. harveyi* strains display any negative effect towards *Brachionus*. Second, we wanted to find out whether quorum sensing disruption can interfere with *Vibrio-Brachionus* interactions.

Materials and methods

Rotifer culture

Brachionus plicatilis (clone 10) was obtained from CIAD (Centro de Investigación en Alimentación y Desarrollo, Mazatlan Unit for Aquaculture) in Mexico. This rotifer strain was identified as *B. plicatilis* *sensu strictu*, using the methodology described by Gomez *et al.* (2002). The rotifer stock was maintained at 25°C, a constant light intensity of 2000 lx, 25 g l⁻¹ seawater and fed with the microalga *Tetraselmis suecica*.

Preparation of food for rotifers

The wild-type strain of baker's yeast (*Saccharomyces cerevisiae*) (BY4741; genotype *Mat a*; *his 3ΔI*; *leu 2Δ0*; *met 15Δ0*; *ura 3Δ0*) was used as food for the rotifers in the experiments. It was obtained from EUROSCARF (Insti-

tute of Microbiology, University of Frankfurt) in Germany. Axenic yeast culture was grown in sterile Erlenmeyer flask on a shaker at 150 rev min⁻¹ and 30°C. The culture medium used was yeast extract peptone dextrose (YEPD), which contains yeast extract (Sigma, Belgium, 1% w/v), peptone (MP Biomedicals, France, 1% w/v) and D-glucose (Sigma, 2% w/v). This medium was prepared in 25 g l⁻¹ 0.22-µm filtered and autoclaved seawater (FASW).

Yeast culture in exponential growth phase was harvested by centrifugation (1600 g for 5 min). Cell pellets were resuspended twice in sterile falcon tubes (technical rubber product; TRP®, γ-irradiated) with 10 ml of 25 g l⁻¹ FASW. Cell density was determined under a microscope, using a Bürker haemocytometer.

Axenic yeast was fed to the rotifers at a level of 2.4 × 10⁵ cells per rotifer. Feeding was carried out twice (at the start of the experiment and 24 h after the challenge) under a laminar flow hood, using MultiGuard™ Barrier pipette tips (Sorenson BioScience, West Salt Lake City, UT, USA).

Method to obtain axenic rotifers

Amictic rotifer eggs were disinfected in 100 ppm of glutaraldehyde for 2 h at 28°C (Tinh *et al.* 2006). After disinfection, the dead rotifers and the amictic eggs were transferred to new sterile falcon tubes containing fresh 25 g l⁻¹ FASW. The falcon tubes were placed on a rotor for incubation for 3 h to allow the amictic eggs to hatch. The culture was allowed to stand for 5 min to let most of the dead adult rotifers settle out. The sterile newly hatched neonates were collected from the water column and distributed to sterile falcon tubes containing 20 ml of 25 g l⁻¹ FASW, in order to obtain a density of eight rotifers per millilitre at the start of the experiment. All the manipulations were performed under a laminar flow hood in order to maintain axenicity of the rotifer cultures.

Axenicity test

Axenicity of the control treatment (where no bacteria were added) was tested on the starting day (day 1) and on the last day (day 4) of the experiment. Fifty microlitre of rotifer culture from each replicate was spread on a marine agar (Difco, Detroit, MI, USA) plate, and bacterial growth was checked after incubation of the plate at 28°C for 48 h. Axenicity of the rotifer culture, as well as of the food, was also checked by bacterial staining. Each sample was treated with MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma, 0.5% w/v) in a sterile microcentrifuge tube (one part of MTT to nine parts of sample) and incubated at 30°C for 30 min.

Under a light microscope (1000× magnification), the sample was checked for the presence of blue-stained viable cells (Sladowski *et al.* 1993). All results were discarded if contamination was found in any replicate of the control treatment.

Vibrio harveyi strains

Eleven *V. harveyi* strains and five mutants derived from BB120 strain were used in the challenge tests (Table 1). Ten *V. harveyi* strains (except BB120) had been isolated from shrimp hatcheries with disease outbreaks or from seawater at different locations, and were provided by the Department of Fishery Microbiology, University of Agricultural Sciences, Mangalore, India. They were identified as *V. harveyi* based on a series of biochemical tests, as described by Karunasagar *et al.* (1994). The BB120 strain and its mutants were obtained from the Department of Molecular Biology, Princeton University, New Jersey, USA. All the strains were preserved in 20% glycerol at -80°C. Before starting an experiment, the bacterial cultures were inoculated into fresh marine broth (Difco) and incubated at 28°C for 24 h on a shaker (120 min⁻¹). After incubation, the bacterial suspensions were centrifuged at 4500 g for 10 min; subsequently, the cells were resuspended with 25 g l⁻¹ FASW and stored at 4°C before use (for maximum 3 h).

Preparation of cell-free washwater of MM30 and BB152 strains

The MM30 and BB152 strains were grown in marine broth as described earlier (the optical density reached approximately 1 at 600 nm). After incubation, the cultures were centrifuged at 4500 g for 10 min and the pellets were resuspended in 25 g l⁻¹ FASW. The suspensions were centrifuged a second time after incubation for 15 min on a shaker (120 min⁻¹). The supernatants were subsequently filter-sterilized over 0.22-µm Millipore filters (Bedford, MA, USA) and stored at -30°C until use (for maximum 1 month). Two millilitre of each washwater was added to 18 ml of rotifer culture water.

Challenge tests

The experiments took place in sterile 50-ml falcon tubes containing 20 ml of 25 g l⁻¹ FASW. The falcon tubes were put on a rotor which was placed inside a temperature-controlled room (28°C, 2000 lx). Each treatment was performed in four replicates and each experiment was repeated twice. *Vibrio harveyi* strains were added to the culture water after the first feeding, at 5 × 10⁶ CFU ml⁻¹. No bacteria were added in the control treatment. Rotifers

Table 1 *Vibrio harveyi* strains used in this study

| Strain | Characteristic | Luminescence on marine agar | Reference |
|--------------------|--|-----------------------------|-----------------------------|
| VH-011 | Isolated in shrimp hatcheries in Ecuador | – | Karunasagar* |
| VH-012 | Isolated in shrimp hatcheries in Ecuador | – | Karunasagar* |
| VH-013 | Isolated in shrimp hatcheries in Ecuador | – | Karunasagar* |
| VH-014 | Isolated in shrimp hatcheries in Ecuador | – | Karunasagar* |
| VH-017 | Isolated in shrimp hatcheries in Ecuador | – | Karunasagar* |
| VH-021 | Isolated from diseased shrimp in China | – | Karunasagar* |
| VH-023 (LMG 04044) | Isolated from dead amphipod in USA | – | Karunasagar* |
| VH-039 (LMG 11659) | Isolated from seawater in USA | – | Karunasagar* |
| VH-040 (LMG 11660) | Isolated from seawater in USA | + | Karunasagar* |
| VH-042 (LMG 13949) | Isolated in shrimp hatcheries in Thailand | + | Karunasagar* |
| BB120 | Wild-type strain from which MM30, BB152, BB886, BB170 and MM77 are derived | + | Bassler et al. (1997) |
| MM30 | Dysfunctional AI-2 synthase | + | Surette et al. (1999) |
| BB152 | Dysfunctional HAI-1 synthase | + | Bassler et al. (1994) |
| BB886 | Dysfunctional AI-2 receptor | + | Bassler et al. (1997) |
| BB170 | Dysfunctional HAI-1 receptor | + | Bassler et al. (1993, 1997) |
| MM77 | Dysfunctional HAI-1 and AI-2 synthase | + | Mok et al. (2003) |

*These strains were provided by Dr. Indrani Karunasagar, Department of Fishery Microbiology, University of Agricultural Sciences, Mangalore, India.

Abbreviations: HAI-1, Harveyi Autoinducer 1; AI-2, Autoinducer 2.

were fed twice (see previous para), immediately after distribution of the newly hatched rotifers (day 1) and 24 h after challenging with *V. harveyi* (day 2). Rotifer density was monitored daily until day 4 (72 h after challenge with *V. harveyi*).

Furanone preparation

(5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone was synthesized as described by Ren and Wood (2004). The furanone was dissolved in absolute ethanol and stored at -30°C until use. It was added to the culture water following the addition of *V. harveyi* strains. Ethanol was added in the control treatment, corresponding to the amount added in the treatment with highest furanone concentration.

Data collection and analysis

Two subsamples of 500 μl were withdrawn daily from each replicate to estimate the rotifer density. The population growth rate (μ) was calculated as follows:

$$\mu = (\ln N_t - \ln N_0)/t$$

where N_0 is the initial rotifer density (rotifers per millilitre), N_t is the rotifer density on day t of the culture and t is the duration in days.

Parametric assumptions were evaluated using Shapiro–Wilk's test for normality and Levene's test for homogeneity of variances. For each experiment, the growth rates

on day 4 were compared between treatments using one-way ANOVA, followed by a Tukey test. All the tests were performed using the SPSS program version 11.5.

Results

Challenge tests with different *Vibrio harveyi* strains

Different *V. harveyi* strains were used in our study to verify their effect on the growth performance of *B. plicatilis*. Eleven strains were tested in the experiments (Tables 2–4), and their interference with *Brachionus* growth was different. Only three strains (VH-014, VH-012, BB120) consistently caused a significant reduction in the growth

Table 2 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with *Vibrio harveyi* strains

| Treatment | Experiment 1 | Experiment 2 |
|-----------|------------------------------|-------------------------------|
| Control | 0.40 \pm 0.08 ^b | 0.30 \pm 0.03 ^b |
| VH-014 | 0.19 \pm 0.06 ^a | 0.08 \pm 0.05 ^a |
| VH-017 | 0.44 \pm 0.05 ^b | 0.32 \pm 0.08 ^b |
| VH-021 | 0.46 \pm 0.03 ^b | 0.24 \pm 0.10 ^{ab} |
| VH-023 | 0.49 \pm 0.07 ^b | 0.35 \pm 0.01 ^b |
| VH-039 | 0.41 \pm 0.11 ^b | 0.37 \pm 0.06 ^b |
| VH-040 | 0.46 \pm 0.03 ^b | 0.27 \pm 0.03 ^b |
| VH-042 | 0.43 \pm 0.04 ^b | 0.31 \pm 0.05 ^b |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). All the strains were added at 5×10^6 CFU ml^{-1} . Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

Table 3 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with *Vibrio harveyi* strains

| Treatment | Experiment 1 | Experiment 2 |
|-----------|-------------------------------|-------------------------------|
| Control | 0.28 \pm 0.02 ^b | 0.31 \pm 0.05 ^b |
| VH-011 | 0.12 \pm 0.06 ^{ab} | 0.28 \pm 0.08 ^b |
| VH-012 | 0.07 \pm 0.05 ^a | 0.07 \pm 0.01 ^a |
| VH-013 | 0.16 \pm 0.09 ^{ab} | 0.20 \pm 0.07 ^{ab} |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). All the strains were added at 5×10^6 CFU ml⁻¹. Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

Table 4 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with *Vibrio harveyi* mutants

| Treatment | Mutation in | Experiment 1 | Experiment 2 |
|-----------|-------------------------|-------------------------------|-------------------------------|
| Control | – | 0.28 \pm 0.04 ^c | 0.40 \pm 0.09 ^c |
| BB120 | – | 0.18 \pm 0.03 ^b | 0.22 \pm 0.06 ^{ab} |
| MM30 | AI-2 synthase | 0.13 \pm 0.02 ^{ab} | 0.15 \pm 0.03 ^a |
| BB152 | HAI-1 synthase | 0.15 \pm 0.05 ^{ab} | 0.30 \pm 0.05 ^b |
| BB886 | AI-2 receptor | 0.11 \pm 0.05 ^{ab} | 0.16 \pm 0.05 ^a |
| BB170 | HAI-1 receptor | 0.10 \pm 0.04 ^a | 0.16 \pm 0.03 ^a |
| MM77 | HAI-1 and AI-2 synthase | 0.25 \pm 0.08 ^c | 0.39 \pm 0.04 ^c |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). All the strains were added at 5×10^6 CFU ml⁻¹. Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

rate ($P < 0.05$) of *Brachionus* during 72 h of challenge, while the other strains did not provoke any negative effect, although some of them were isolated from diseased shrimp.

Challenge tests with *Vibrio harveyi* mutants

The challenge tests were set up to determine whether the growth-retarding (GR) effect of *V. harveyi* towards *Brachionus* has any relation with the bacterial quorum sensing system. *Vibrio harveyi* strain BB120 and its isogenic mutants, which are deficient in one of the two quorum sensing components (HAI-1 or AI-2-mediated), were added to *Brachionus* cultures. A mutation in either the HAI-1-mediated components (BB152 and BB170 strains) or the AI-2-mediated components (MM30 and BB886 strains) did not change the effect of *V. harveyi* (Table 4). In contrast, the deletion of both components (CAI-1-mediated component is still present) neutralized the GR effect of *V. harveyi*, as the *Brachionus* growth rate in the presence of the MM77 mutant was similar to that in the control treatment ($P > 0.05$).

In a further series of experiments, the cell-free wash-water of MM30 strain (AI-2-negative mutant) or BB152 strain (HAI-1-negative mutant) as an exogenous source

Table 5 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with the MM77 strain (HAI-1 and AI-2 synthase mutant), with and without the addition of the washwater of the MM30 (AI-2 synthase mutant) or the BB152 (HAI-1 synthase mutant) strain

| Treatment | Experiment 1 | Experiment 2 |
|------------------------|------------------------------|------------------------------|
| Control | 0.31 \pm 0.04 ^b | 0.54 \pm 0.04 ^b |
| MM77 | 0.27 \pm 0.10 ^b | 0.51 \pm 0.05 ^b |
| MM77 + MM30 washwater | 0.15 \pm 0.02 ^a | 0.30 \pm 0.06 ^a |
| MM77 + BB152 washwater | 0.12 \pm 0.03 ^a | 0.28 \pm 0.08 ^a |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). The MM77 strain was added at 5×10^6 CFU ml⁻¹. Washwater was added at 10% of the culture volume. Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

Abbreviations: HAI-1, *Harveyi* Autoinducer 1; AI-2, Autoinducer 2.

of HAI-1 or AI-2 molecules, respectively, was added to the culture water concomitantly with the challenge with the MM77 strain (double mutant in HAI-1 and AI-2 synthase). We found that the addition of the washwater of the MM30 or BB152 strains could restore the GR effect of MM77 strain (Table 5).

Effect of brominated furanone on the GR effect of *Vibrio harveyi* strain BB120 towards gnotobiotic *Brachionus*

In this series of experiments, we investigated whether brominated furanone can be used as a quorum sensing disrupting compound in *Brachionus* cultures. Furanone was added to the culture water at five different concentrations, with and without challenge with the wild-type *V. harveyi* strain BB120. Furanone added at 7.5 mg l⁻¹ and 10 mg l⁻¹ was shown to be toxic to *Brachionus*, as the growth rate was significantly reduced ($P < 0.05$) compared with that in the treatment without furanone (Table 6). In the presence of BB120 strain, we found that the growth rate of *Brachionus* was highest in the treat-

Table 6 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of the addition of (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone

| Treatment | Experiment 1 | Experiment 2 |
|---------------------------------|-------------------------------|-------------------------------|
| Control | 0.56 \pm 0.12 ^b | 0.38 \pm 0.10 ^b |
| 1 mg l ⁻¹ furanone | 0.38 \pm 0.09 ^b | 0.35 \pm 0.07 ^b |
| 2.5 mg l ⁻¹ furanone | 0.36 \pm 0.10 ^{ab} | 0.27 \pm 0.05 ^{ab} |
| 5 mg l ⁻¹ furanone | 0.34 \pm 0.07 ^{ab} | 0.25 \pm 0.09 ^{ab} |
| 7.5 mg l ⁻¹ furanone | 0.14 \pm 0.08 ^a | 0.15 \pm 0.05 ^a |
| 10 mg l ⁻¹ furanone | 0.14 \pm 0.09 ^a | 0.10 \pm 0.05 ^a |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

Table 7 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with the BB120 (wild-type) strain followed by the addition of (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone

| Treatment | Experiment 1 | Experiment 2 |
|---|-------------------------------|-------------------------------|
| Control | 0.51 \pm 0.05 ^d | 0.30 \pm 0.05 ^c |
| BB120 | 0.39 \pm 0.07 ^{bc} | 0.19 \pm 0.03 ^b |
| BB120 + 1 mg l ⁻¹ furanone | 0.48 \pm 0.04 ^{cd} | 0.25 \pm 0.02 ^{bc} |
| BB120 + 2.5 mg l ⁻¹ furanone | 0.54 \pm 0.09 ^d | 0.32 \pm 0.03 ^c |
| BB120 + 5 mg l ⁻¹ furanone | 0.34 \pm 0.10 ^{ab} | 0.20 \pm 0.03 ^b |
| BB120 + 7.5 mg l ⁻¹ furanone | 0.26 \pm 0.06 ^a | 0.05 \pm 0.01 ^a |
| BB120 + 10 mg l ⁻¹ furanone | 0 | 0 |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). The BB120 strain was added at 5×10^6 CFU ml⁻¹. Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

ment with 2.5 mg l⁻¹ of furanone and was comparable with that in the control treatment ($P > 0.05$) (Table 7). A furanone concentration of 10 mg l⁻¹ in the presence of BB120 strain resulted in total mortality of *Brachionus* within 24 h after the treatment.

In separate experiments, we tested the effect of furanone, added at 2.5 mg l⁻¹, on the growth rate of *Brachionus*, which were challenged with the *V. harveyi* strains BB120, VH-012 and VH-014. These strains inhibited the growth of *Brachionus* in previous experiments. The alteration of GR effect was strain-dependent (Table 8). Furanone could nullify the negative effect of the BB120 and VH-014 strains ($P < 0.05$). On the other hand, this compound did not show any effect towards the VH-012 strain ($P > 0.05$).

Table 8 Growth rate of *Brachionus plicatilis* (mean \pm SD) over 72 h: effect of challenge with *Vibrio harveyi* strains followed by the addition of 2.5 mg l⁻¹ of (5Z)-4-bromo-5-(bromomethylene)-3-butyl-2(5H)-furanone

| Treatment | Experiment 1 | Experiment 2 | Experiment 3 |
|--|------------------------------|-------------------------------|-------------------------------|
| Control | 0.36 \pm 0.05 ^b | 0.47 \pm 0.09 ^c | 0.48 \pm 0.09 ^{bc} |
| BB120 | 0.15 \pm 0.03 ^a | 0.22 \pm 0.03 ^{ab} | 0.18 \pm 0.02 ^a |
| BB120 + 2.5 mg l ⁻¹ furanone | 0.34 \pm 0.06 ^b | 0.43 \pm 0.07 ^c | 0.41 \pm 0.06 ^b |
| VH-012 | 0.11 \pm 0.07 ^a | 0.18 \pm 0.08 ^{ab} | 0.18 \pm 0.05 ^a |
| VH-012 + 2.5 mg l ⁻¹ furanone | 0.15 \pm 0.10 ^a | 0.21 \pm 0.08 ^{ab} | 0.23 \pm 0.10 ^a |
| VH-014 | 0.11 \pm 0.07 ^a | 0.12 \pm 0.02 ^a | 0.24 \pm 0.05 ^a |
| VH-014 + 2.5 mg l ⁻¹ furanone | 0.30 \pm 0.01 ^b | 0.40 \pm 0.01 ^b | 0.52 \pm 0.01 ^c |

Treatments with different superscripts in each experiment are significantly different from each other (Tukey test, $P < 0.05$). *Vibrio harveyi* strains were added at 5×10^6 CFU ml⁻¹. Rotifers were fed with axenic yeast twice, at the start of experiment and after 24 h.

Discussion

In this study, gnotobiotically grown rotifers *B. plicatilis* were used as test organisms. In this way, the naturally occurring microbial communities could not interfere with the quorum sensing system of the tested *V. harveyi* strains. In a preliminary study, which was aimed at standardizing the conditions for a challenge test in *Brachionus*, we found that a *V. harveyi* (BB120 strain) density as high as 5×10^6 CFU ml⁻¹ is sufficient to cause an observable effect in the *Brachionus* culture. This density is much higher compared with that reported by Defoirdt *et al.* (2005) for *Artemia nauplii* (10^4 CFU ml⁻¹). We also found a suitable feeding regime in which *Brachionus* are fed only twice (at the start of the experiment and 24 h after the challenge) instead of being fed daily, as the GR effect was attenuated in the latter case (data not shown). Similarly, Defoirdt *et al.* (2005) tested two different feeding regimes (feeding once or twice) in *Artemia franciscana* nauplii, which were challenged with *V. harveyi* BB120. The authors found that the virulence of the BB120 strain was significantly reduced if *Artemia* was fed twice. These findings indicate that a good feeding regime may compensate for the negative effect caused by a bacterium on the test organism, which is consistent with the notion that *V. harveyi* is an opportunistic pathogen.

The *V. harveyi* strains used in the study were isolated from different sources (seawater, diseased shrimp, wild crustacean) at different geographical locations (Table 1). Four strains are bioluminescent on marine agar. However, there is no evidence of the presence of quorum-sensing signal molecules in these strains (except BB120 strain). Although most of the strains were isolated from shrimp tanks with observed symptoms of disease, only three strains were shown to inhibit *Brachionus* growth (Tables 2–4). This suggests that the GR effect of *V. harveyi* on *Brachionus* growth is strain-dependent. *Vibrio harveyi* was found to be an ubiquitous species in the aquacultural environments (Thompson *et al.* 2001). Different strains may produce different virulence factors, such as exotoxins (Manefield *et al.* 2000), siderophores (Lilley and Bassler 2000), type III protein secretion (Henke and Bassler 2004b), or metalloproteases (Mok *et al.* 2003), when invading the host. *Vibrio* sp. present in the rotifer culture tanks may be harmful to the rotifers but could also serve as food and contribute to their growth. Yu *et al.* (1990) reported that a *Vibrio alginolyticus* strain can cause the collapse of rotifer cultures. Balompapueng *et al.* (1997) found that *Flavobacterium* sp., *Aeromonas* sp. and *Vibrio* sp. isolated from the unstable collapsing rotifer cultures showed pathogenicity for the rotifer populations. On the other hand, in a study on the bioencapsulation of different bacteria belonging to Vibrionaceae in the gnoto-

biotic rotifer *B. plicatilis*, Martinez-Diaz *et al.* (2003) found that none of the tested bacteria negatively affected rotifer growth, rather they were used as food by the rotifers. However, it should be noticed that the food type used in that study (microalgae) is different from that used in our study (yeast). In a recent study, Tinh *et al.* (2006) stated that food type interferes with the performance of a microbial community, which is present in or added into a rotifer culture.

In the other series of experiments, we tested the effect of mutants which are derived from strain BB120. The mutant strains are defective in either the HAI-1-mediated or the AI-2-mediated quorum sensing system, but not in the CAI-1-mediated component. All the mutants were able to reduce *Brachionus* growth rate significantly over 72 h, except the double-mutant MM77 (Table 4). Moreover, the addition of MM30 or BB152 washwater (as an exogenous source of HAI-1 or AI-2 autoinducers, respectively) restored the GR effect of the MM77 strain (Table 5). These data strongly suggest that the action of either the HAI-1-mediated or the AI-2-mediated channel of the quorum sensing system in the *V. harveyi* strain BB120 is sufficient to alter the growth rate of *Brachionus*. On the other hand, the CAI-1-mediated component alone is not responsible for the GR effect in this strain. Although there is evidence that all three quorum sensing channels are responsible for bioluminescence induction in *V. harveyi* (Henke and Bassler 2004a), its GR effect towards *Brachionus* seems to be dependent on only two components of the quorum sensing system.

The results found in *Brachionus* are different from those found by Defoirdt *et al.* (2005). These authors performed challenge tests in *A. franciscana* nauplii using the BB120 strain and the same mutants that were used in our study. They found that only the AI-2-mediated component, and not the HAI-1-mediated component, controls the virulence of BB120 strain towards *Artemia*. Possibly, pathogenic *Vibrio* spp. behave physiologically different in different host organisms, adapting in this way to the different environments and/or the immune system of the hosts. There are many studies concerning the importance of AHL and AI-2 autoinducers on the virulence of pathogenic bacteria. AHL signals are involved in the regulation of virulence factors in some human and plant-pathogenic bacteria. They were found to be produced by many strains of fish-pathogenic bacteria (Bruhn *et al.* 2005). AI-2 is found in many genera and was also reported to control virulence factor production in many species (Federle and Bassler 2003). AI-2 signal was detected in *V. cholerae* (Miller *et al.* 2002), *Vibrio vulnificus* (Kim *et al.* 2003) and *Escherichia coli* (Anand and Griffiths 2003). AI-2 regulates starvation adaptation and stress resistance in *V. vulnificus* and *Vibrio angustum* (McDougald *et al.*

2003) and toxin production in *Clostridium perfringens* (Ohtani *et al.* 2002).

Finally, we investigated the effect of a brominated furanone as means of quorum sensing disruption. Halogenated furanones interfere with AHL-regulated biofilm formation and virulence in the human pathogen *Pseudomonas aeruginosa* (Hentzer *et al.* 2002, 2003), as well as with bioluminescence in *Vibrio fischeri* (Givskov *et al.* 1996). Ren *et al.* (2001, 2004) provided evidence that brominated furanone inhibited biofilm formation and swarming of *E. coli* by interfering with AI-2 signaling. In a recent study, Defoirdt *et al.* (2006) found that brominated furanone blocked all three channels of the *V. harveyi* quorum sensing system. In our study, the growth rate of *Brachionus* over 72 h after challenge with the BB120 strain was proportional to the furanone concentration and reached a maximum in the presence of 2.5 mg l⁻¹ of furanone (Table 6), indicating that this concentration of furanone is appropriate to completely block all quorum sensing pathways in the BB120 strain, and thus attenuate the GR effect of this strain towards *Brachionus*. On the other hand, furanone added at a concentration of 7.5 mg l⁻¹ and higher appeared to be toxic for *Brachionus*. *Vibrio harveyi* densities were determined at the start and at the end of experiments by plating the water samples on TCBS agar (thiosulfate citrate bile salt sucrose agar; Biokar Diagnostics, France). No reduction in *Vibrio* growth was noticed (data not shown), indicating that furanone, at the tested concentrations, did not interfere with the growth of *V. harveyi*. The same furanone compound was tested in gnotobiotic *Artemia* nauplii by Defoirdt *et al.* (2006). *Artemia* nauplii were only protected at a much higher furanone concentration (20 mg l⁻¹), and the lowest concentration of furanone found to be toxic to *Artemia* (50 mg l⁻¹) was higher than for *Brachionus*, indicating that *Brachionus* are more sensitive to furanone.

The effect of brominated furanone as a quorum sensing inhibitor was also tested for the *V. harveyi* strains which reduced the growth rate in *Brachionus*. Interestingly, furanone added at 2.5 mg l⁻¹ could neutralize the negative effect of the VH-014 strain, but not the effect of the VH-012 strain. These observations suggest that the GR effect in the VH-014 strain towards *Brachionus* might be regulated by a *V. harveyi*-type quorum sensing system, as in case of the BB120 strain. The mechanism of alteration of *Brachionus* growth by the VH-012 strain remains to be elucidated.

Halogenated furanones utilized at high concentrations can indeed have a toxic effect as was documented in mammalian cells. 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, a by-product of drinking water disinfection, has been found to be mutagenic in bacteria and to be a potent carcinogen in rats (Vaittinen *et al.* 1995; Komulainen *et al.* 1997; Komulainen 2004). This com-

pound and its analogue, mucochloric acid, are toxic to hepatocytes and gill epithelial cells in rainbow trout and to aquatic invertebrate *Daphnia magna* (Isomaa *et al.* 1995).

In conclusion, our results indicate that both quorum sensing systems in some *V. harveyi* strains are responsible for their GR effects towards *B. plicatilis* sensu strictu, a live food organism for many aquaculture species. Therefore, disruption of both components may help to alleviate these effects of *V. harveyi* in *Brachionus* cultures. On the other hand, the importance of quorum sensing in nongnotobiotic systems, and hence the beneficial effect of quorum sensing inhibitors in open *Brachionus* cultures, remain to be established. Finally, as *Vibrio* growth is not inhibited by halogenated furanone, the *Vibrio* number associated with *Brachionus* cultures is likely to remain unchanged, which might cause problems to their predators (such as fish larvae), unless quorum sensing inhibitors can also be utilized successfully at that stage of the aquaculture food chain.

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