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Quorum Sensing-Disrupting Brominated Furanones Protect the Gnotobiotic Brine Shrimp *Artemia franciscana* from Pathogenic *Vibrio harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* Isolates†

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Autoinducer 2 (AI-2) quorum sensing was shown before to regulate the virulence of *Vibrio harveyi* towards the brine shrimp *Artemia franciscana*. In this study, several different pathogenic *V. harveyi*, *Vibrio campbellii*, and *Vibrio parahaemolyticus* isolates were shown to produce AI-2. Furthermore, disruption of AI-2 quorum sensing by a natural and a synthetic brominated furanone protected gnotobiotic *Artemia* from the pathogenic isolates in in vivo challenge tests.

Bacteria belonging to the species *Vibrio harveyi* and the closely related *Vibrio campbellii* and *Vibrio parahaemolyticus* are important pathogens in the intensive rearing of mollusks, finfish, and shrimp (1, 7, 10, 11, 14, 19, 25). The traditional control of bacterial disease in aquaculture relies on the use of antibiotics (3, 21). However, the frequent use of these compounds, in many cases even when pathogens are not evident, has led to the development and spread of resistance (3, 10, 13, 23, 24). Therefore, there is an urgent need for alternative control techniques. Disruption of quorum sensing, bacterial cell-to-cell communication by means of small signal molecules, has been suggested as a new anti-infective strategy for aquaculture (4).

The quorum sensing system of *V. harveyi* has been shown to consist of three channels (8). Recently, we found that the autoinducer 2 (AI-2)-mediated channel of the system regulates the virulence of the bacterium towards the brine shrimp *Artemia franciscana* in vivo (5). Interestingly, halogenated furanones were found to interfere with AI-2 quorum sensing in *Escherichia coli* (16, 17) and were shown before to block quorum sensing-regulated extracellular toxin production in *V. harveyi*, resulting in a reduced toxicity of cell-free culture fluids to *Penaeus* shrimp (12). Therefore, in this study, we aimed at investigating whether these halogenated furanones could reduce the virulence of *V. harveyi* and closely related bacteria in our model system with gnotobiotic *Artemia franciscana*.

Detection of AI-2 production by the pathogenic isolates. In a first experiment, we aimed at detecting AI-2 production by the different pathogenic *V. harveyi*, *V. campbellii*, and *V. parahaemolyticus* isolates. The isolates tested are described in Table 1. Cell-free culture fluids of the isolates were prepared, and autoinducers were detected by a bioluminescent reporter assay as described before (22). The autoinducer receptor double mutant JMH597 (sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) (8) was used as a reporter strain. The culture fluids of all isolates significantly induced bioluminescence in the reporter strain (data not shown), indicating that they all produced AI-2. The detection of AI-2 in cell-free culture supernatants of *V. harveyi* and *V. parahaemolyticus* confirms the report of Bassler et al. (2), who used the HAI-1 receptor mutant BB170 as a reporter strain. To the best of our knowledge, this is the first report mentioning AI-2 production by *V. campbellii*.

Disruption of AI-2 quorum sensing in *V. harveyi* by the natural furanone. A second in vitro experiment aimed at determining whether the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone could disrupt AI-2 quorum sensing in *V. harveyi* (Fig. 1), using bioluminescence as a representative of other quorum sensing-regulated phenotypes. The furanone, synthesized as described previously (18), blocked bioluminescence of the *V. harveyi* receptor double mutant JMH597 (sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) in a concentration-dependent way (Fig. 2). Importantly, halogenated furanones were shown before not to block bioluminescence in a constitutive system (6), indicating that the biochemical function of the Lux proteins is not affected. Our data confirm the report by Ren et al. (17), who determined the impact of the furanone on HAI-1 and AI-2 quorum sensing by using single mutants with a mutation in the AI-2 or HAI-1 receptor protein, which thus were still responsive to both HAI-1 and CAI-1 and both AI-2 and CAI-1, respectively (the CAI-1-mediated channel was not yet discovered at that time).

In vivo protection of *Artemia* from the pathogenic isolates by the natural furanone. We previously showed that the virulence of *V. harveyi* BB120 towards *Artemia* is regulated by the AI-2-mediated channel of its quorum sensing system (5), which we showed here to be blocked by the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone. Conse-

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TABLE 1. Bacterial strains used in this study[a]

Strain	Relevant features and/or synonyms	Reference(s) or source
V. campbellii		
LMG21361	= CAIM415 = Z1; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	7, 20
LMG21362	= CAIM333 = M1; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	7, 20
LMG21363	= CAIM372 = PN9801; isolated from the lymphoid organ of diseased shrimp (*Penaeus* spp.) juveniles, Philippines	7, 20
LMG22888	= CAIM416 = Z2; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	7, 20
LMG22889	= CAIM417 = Z3; isolated from seawater from shrimp (*Litopenaeus* spp.) broodstock tank, Mexico	7, 20
LMG22890	= CAIM395 = STD3-131; isolated from diseased shrimp (*Litopenaeus* spp.) postlarvae, Ecuador	7, 20
LMG22895	= CAIM223; isolated from the hepatopancreas of diseased shrimp (*Litopenaeus* spp.), Mexico	Bruno Gomez-Gil
V. harveyi		
LMG22891	= CAIM88; isolated from the hemolymph of shrimp (*Litopenaeus* spp.), Mexico	Bruno Gomez-Gil
LMG22893	= CAIM148; isolated from the hemolymph of diseased shrimp (*Penaeus* spp.), Mexico	Bruno Gomez-Gil
LMG22894	= CAIM151; isolated from the hemolymph of diseased shrimp (*Penaeus* spp.), Mexico	Bruno Gomez-Gil
BB120		2
V. parahaemolyticus		
CAIM170	Isolated from the hemolymph of diseased shrimp (*Penaeus* spp.), Mexico	Bruno Gomez-Gil

[a] LMG, Laboratory of Microbiology Collection (Ghent University, Ghent, Belgium); CAIM, Collection of Aquacultural Important Micro-organisms (CIAD/Mazatlán Unit for Aquaculture, Mazatlán, Mexico).

quently, we investigated whether the natural furanone could protect gnotobiotic *Artemia* from pathogenic *V. campbellii*, *V. harveyi*, and *V. parahaemolyticus* in in vivo challenge tests. Challenge tests were performed as described in the work of Defoirdt et al. (5). In a first test, we aimed at determining the best dosage of the natural furanone, and therefore, we investigated the impact of the furanone added in different concentrations (5, 10, 20, and 50 mg/liter) on the virulence of the opportunistic strain *V. harveyi* BB120 (causing mortality only in nauplii cultured under suboptimal conditions [5]) and the virulent strain *V. campbellii* LMG21363. The survival of *Artemia* was proportional to the concentration of furanone for the lower concentrations (5 to 20 mg/liter), whereas high mortality was noted after treatment with 50 mg/liter of furanone (Fig. 3). For both pathogens, the furanone significantly enhanced survival of the nauplii when added at 20 mg/liter ($P < 0.05$). The protection was complete for *V. harveyi* BB120 (no significant difference in survival from unchallenged nauplii), whereas significant mortality still occurred in furanone-treated nauplii challenged with the more virulent *V. campbellii* LMG21363 ($P < 0.01$). In further challenges, we investigated whether the natural furanone, at 20 mg/liter, could offer some protection against the different pathogenic isolates. The compound significantly reduced mortality in *Artemia* for all isolates except for LMG22889, where differences were not significant (Table 2). The protection was complete for all *V. harveyi* strains and

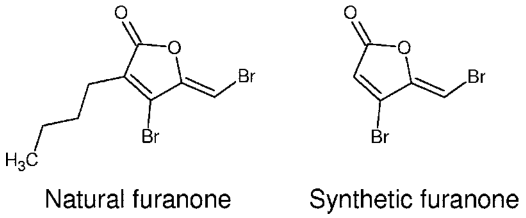

FIG. 1. Structures of the natural and synthetic furanones used in this study.

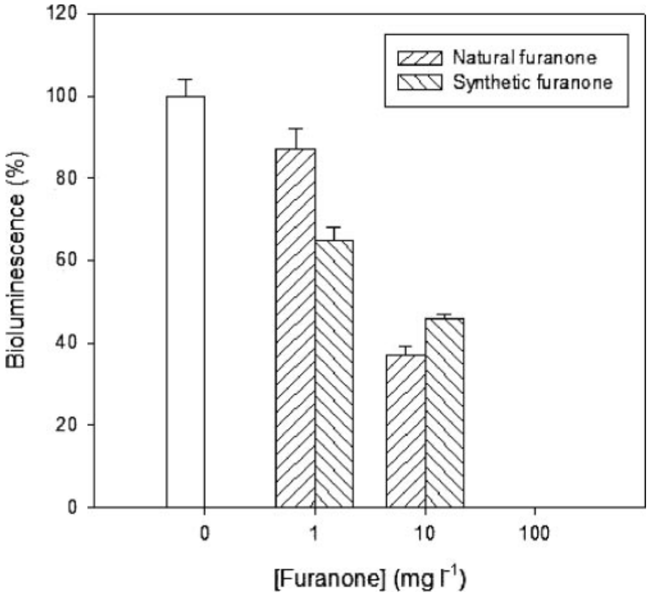

FIG. 2. Bioluminescence of the *V. harveyi* reporter strain JMH597 (sensor HAI-1$^-$, sensor AI-2$^+$, sensor CAI-1$^-$) as a function of the concentration of the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone and the synthetic furanone (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone. The luminescence without the addition of furanone was set at 100% (white bar), and the other samples were normalized accordingly. The error bars represent the standard deviations of three replicates.

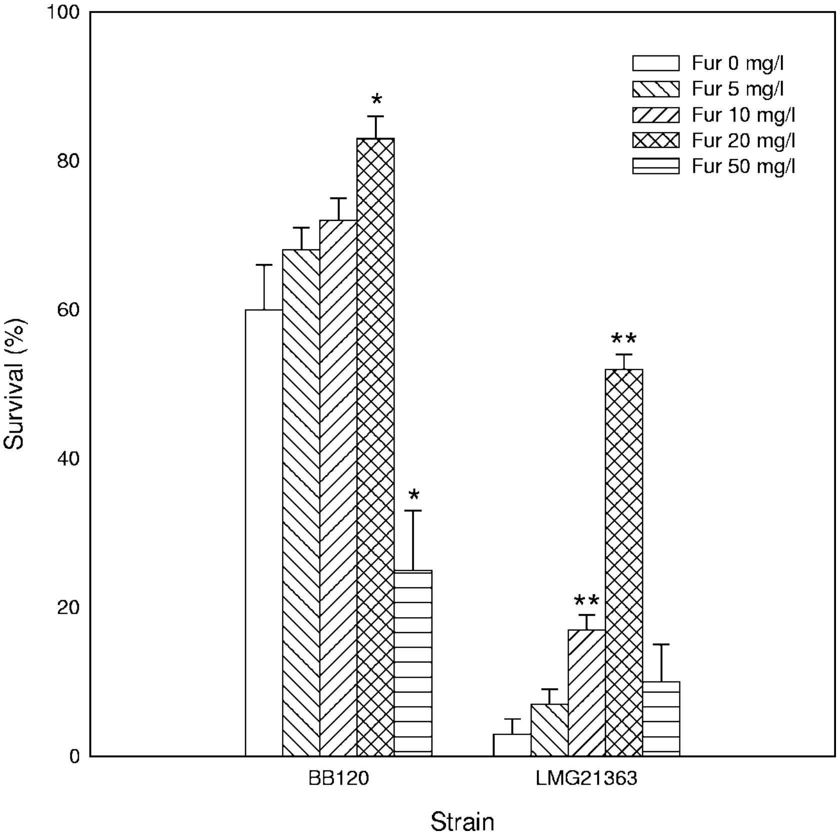

FIG. 3. Percent survival of *Artemia* after 48 h of challenge with *V. harveyi* BB120 and *V. campbellii* LMG21363, with and without the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (Fur). The error bars represent the standard errors of three replicates. The survival of unchallenged nauplii was 83% ± 3%. *, significantly different from the treatment with the same pathogen and without furanone ($P < 0.05$); **, significantly different from the treatment with the same pathogen and without furanone ($P < 0.01$).

TABLE 2. Percent survival of *Artemia* (means ± standard errors of three replicates) after 48 h of challenge with different *V. campbellii*, *V. harveyi*, and *V. parahaemolyticus* isolates, with and without the natural furanone (5*Z*)-4-bromo-5-(bromomethylene)-3-butyl-2(5*H*)-furanone (20 mg/liter)

Treatment	Expt 1		Expt 2	
	− Furanone	+ Furanone[d]	− Furanone	+ Furanone[d]
V. campbellii[a]				
LMG21361	43 ± 4	62 ± 4*	43 ± 3	80 ± 3**
LMG21362	32 ± 4	75 ± 3**	38 ± 3	72 ± 4**
LMG21363	18 ± 4	58 ± 4**	12 ± 4	55 ± 5**
LMG22888	48 ± 4	63 ± 2*	48 ± 6	77 ± 6*
LMG22889	50 ± 3	63 ± 4	48 ± 4	62 ± 6
LMG22890	38 ± 3	67 ± 4**	45 ± 6	70 ± 5*
LMG22895	52 ± 6	73 ± 4*	50 ± 5	83 ± 2**
V. harveyi[b]				
BB120	52 ± 4	87 ± 3**	60 ± 3	78 ± 2**
LMG22891	62 ± 4	83 ± 3*	53 ± 6	73 ± 2*
LMG22893	55 ± 3	78 ± 2**	57 ± 3	75 ± 3*
LMG22894	48 ± 4	75 ± 5*	43 ± 4	72 ± 6*
V. parahaemolyticus[c]				
CAIM170	55 ± 6	82 ± 4*	57 ± 2	78 ± 4*

[a] Survival of unchallenged nauplii was 85% ± 3% in the first experiment and 75% ± 3% in the second experiment.

[b] Survival of unchallenged nauplii was 77% ± 2% in the first experiment and 83% ± 3% in the second experiment.

[c] Survival of unchallenged nauplii was 85% ± 5% in the first experiment and 83% ± 6% in the second experiment.

[d] *, significantly different from treatment with the same pathogen and without furanone ($P < 0.05$); **, significantly different from treatment with the same pathogen and without furanone ($P < 0.01$).

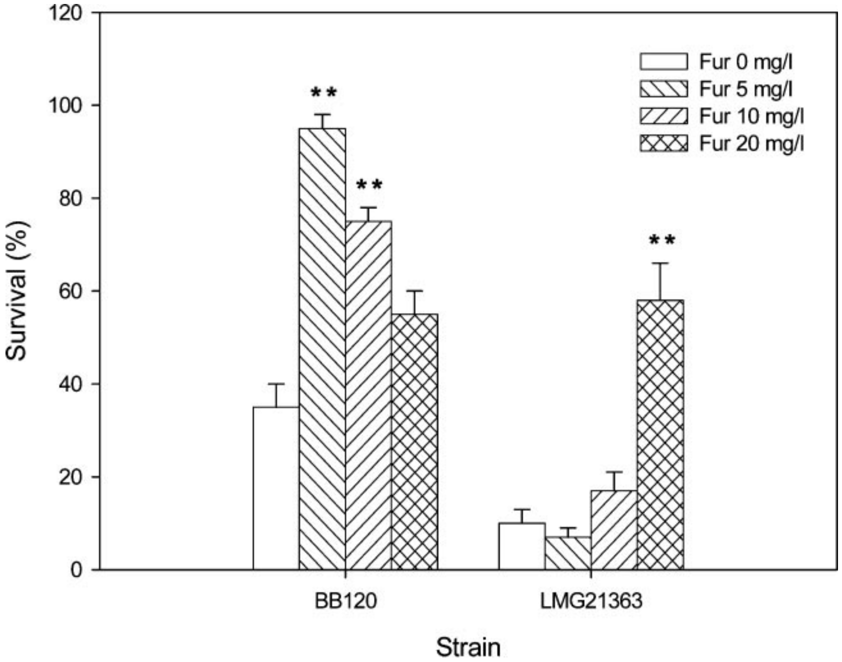

FIG. 4. Percent survival of *Artemia* after 48 h of challenge with *V. harveyi* BB120 and *V. campbellii* LMG21363, with and without the synthetic furanone (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (Fur). The error bars represent the standard errors of three replicates. The survival of unchallenged nauplii was 85% ± 3%. **, significantly different from the treatment with the same pathogen and without furanone ($P < 0.01$).

the *V. parahaemolyticus* strain, whereas for *V. campbellii* LMG21361, LMG22888, and LMG22889 there was still significant mortality in furanone-treated *Artemia* ($P < 0.05$) in the first experiment and for strain LMG21363 there was still significant mortality in furanone-treated *Artemia* in both experiments ($P < 0.05$).

Effect of the natural furanone on growth of the vibrios in vitro and in vivo. There was no effect of the natural furanone on growth of *V. harveyi* BB120 and *V. campbellii* LMG21363 in liquid growth medium as well as in the *Artemia* challenge tubes (data not shown), indicating that the protection offered by the natural furanone was not due to growth inhibition of the pathogens. We used plate counts of the *Artemia* culture water as an indicator of growth of the pathogens in vivo since it was not feasible to determine the bacterial concentration in/on infected *Artemia*. Our data are in accordance with those reported by Manefield et al. (12), who showed that there was no effect on the growth of *V. harveyi* strain 47666-1 for concentrations up to 200 μM (≈62 mg/liter) of the same furanone compound.

In vitro and in vivo disruption of AI-2 quorum sensing by the synthetic furanone. We also tested the quorum sensing-disrupting potential of the synthetic furanone (5*Z*)-4-bromo-5-(bromomethylene)-2(5*H*)-furanone (Fig. 1), which as far as we know is the compound with the highest quorum sensing-disrupting activity reported to date. The furanone, synthesized as described before (18), did not have a higher activity towards the *V. harveyi* quorum sensing system in vitro since it blocked bioluminescence at similar concentrations as did the natural furanone (Fig. 2). Finally, the impact of the synthetic furanone, added in different concentrations (5, 10, and 20 mg/liter), on the virulence of *V. harveyi* BB120 and *V. campbellii* LMG21363 towards gnotobiotic *Artemia* was investigated. The compound completely protected the *Artemia* nauplii from *V. harveyi* BB120 at 5 mg/liter, whereas 20 mg/liter was needed to obtain protection from the virulent *V. campbellii* strain (Fig. 4). As was the case for the natural furanone, the protection against the virulent strain was not complete since significant mortality still occurred in furanone-treated nauplii ($P < 0.01$). For *V. harveyi* BB120, the survival with 5 mg/liter was the highest. The survival decreased proportionally to the furanone concentration for higher concentrations, indicating that the compound is slightly more active and more toxic than the natural furanone. The concentration needed to disrupt the *V. harveyi* quorum sensing system is comparable to that in the report by Hentzer et al. (9), who mentioned a partial or complete suppression of the production of virulence factors in *Pseudomonas aeruginosa* in the presence of 2.5 mg/liter of the same synthetic furanone as used in this study. Rasch et al. (15), in contrast, found that the compound protected rainbow trout (*Oncorhynchus mykiss*) from *Vibrio anguillarum* at much lower concentrations (≈2.5 μg/liter). However, these authors mentioned that the effect that they observed might have been due to interaction of the furanone with the fish host rather than the *V. anguillarum* quorum sensing system. The fact that the furanones protected the *Artemia* nauplii from the pathogenic isolates in our in vivo tests at concentrations similar to those needed to block quorum sensing-regulated bioluminescence in *V. harveyi* in vitro indicates that the effect that we observed was probably due to quorum sensing disruption rather than interaction with the shrimp, although we cannot exclude the latter possibility.

Detailed experimental procedures can be found in the supplemental material.

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