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ENVIRONMENTAL BIOTECHNOLOGY

Isolation of AHL-degrading bacteria from micro-algal cultures and their impact on algal growth and on virulence of *Vibrio campbellii* to prawn larvae

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Abstract Inactivation of quorum sensing (QS) signal molecules, such as acylhomoserine lactones (AHLs) of pathogenic bacteria, has been proposed as a novel method to combat bacterial diseases in aquaculture. Despite the importance of micro-algae for aquaculture, AHL degradation by bacteria associated with micro-algal cultures has thus far not been investigated. In this study, we isolated *Pseudomonas* sp. NFMI-T and *Bacillus* sp. NFMI-C from open cultures of the micro-algae *Tetraselmis suecica* and *Chaetoceros muelleri*, respectively. An AHL degradation assay showed that either monocultures or co-cultures of the isolates were able to degrade the AHL *N*-hexanoyl-L-homoserine lactone. In contrast, only *Bacillus* sp. NFMI-C was able to inactivate *N*-hydroxybutanoyl-L-homoserine lactone, the AHL produced by *Vibrio campbellii*. The isolated bacteria were able to persist for up to 3 weeks in conventionalized micro-algal cultures, indicating that they were able to establish and maintain themselves within open algal cultures. Using gnotobiotic algal cultures, we found that the isolates did not affect growth of the micro-algae from which they were isolated, whereas a mixture of both isolates increased the growth of *Tetraselmis* and decreased the growth of *Chaetoceros*. Finally, addition of *Bacillus* sp. NFMI-C to the rearing water of giant river prawn (*Macrobrachium rosenbergii*) larvae significantly improved survival of the larvae when challenged with pathogenic *V. campbellii*, whereas it had no effect on larval growth.

Keywords Quorum quenching · Acylase · Lactonase · Micro-algae · *Macrobrachium rosenbergii* · Vibriosis

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Introduction

Aquaculture, the farming of aquatic animals and plants (algae) in marine, brackish and freshwater environments, is the fastest-growing food-producing industry worldwide (Bostock et al. 2010). The giant river prawn, *Macrobrachium rosenbergii*, is an important crustacean species from an economic perspective, with an annual global production of more than 200 000 t (New et al. 2010; FAO 2013). Disease outbreaks are considered to be amongst the major obstacles to produce healthy and high quality seed for the further expansion of giant river prawn culture (Nhan et al. 2010). Previous studies have shown that *Vibrio* spp., including *Vibrio campbellii*, are a major cause of disease in the early life stages (larvae and postlarvae) of *M. rosenbergii* (Tonguthai 1997; Kennedy et al. 2006; New et al. 2010; FAO 2013).

Bacterial diseases in aquaculture have thus far mainly been tackled by using antibiotics, but unfortunately, the use of these compounds has not been very successful and has led to the development and spread of resistant pathogens (Defoirdt et al. 2011a). Therefore, there is a need for novel methods to control bacterial disease. As virulence gene expression in many bacterial pathogens is controlled by quorum sensing, interference

with this cell-to-cell communication mechanism has been proposed as a novel biocontrol strategy (Defoirdt et al. 2004). *V. campbellii* contains a three-channel quorum sensing system, with three different types of signal molecules, namely Harveyi autoinducer-1 (HAI-1), autoinducer-2 (AI-2) and Cholerae autoinducer-1 (CAI-1), which feed a shared signal transduction cascade (Ruwandeepika et al. 2012). Quorum sensing has been reported to control the expression of different virulence genes in *V. campbellii*, and we recently reported that the HAI-1 and the AI-2-mediated channels of the *V. campbellii* quorum sensing system are essential for full virulence to giant river prawn larvae (Pande et al. 2013).

The use of signal molecule-degrading bacteria is one of the most intensively studied strategies to interfere with quorum sensing (LaSarre and Federle 2013; Tang and Zhang 2014). The ability to inactivate acylhomoserine lactones (AHLs), one of the types of quorum sensing molecules, is widely distributed in the bacterial kingdom (Dong et al. 2007). Two major classes of AHL-inactivating enzymes have been described: lactonases, which are, e.g. produced by *Bacillus* spp., cleave the lactone ring of the signal molecules into acylated homoserine, whereas acylases, e.g. produced by *Pseudomonas* spp., cleave the AHL molecule into homoserine lactone and a fatty acid (Fast and Tipton 2012). As *V. campbellii* HAI-1 is an AHL and as this signal is essential for full virulence towards giant river prawn larvae (Pande et al., 2013), the use of AHL-degrading bacteria might be an effective strategy to protect the larvae from the pathogen. This kind of bacteria has been isolated from various environments, including the digestive tract of healthy shrimp (Tinh et al. 2007) and fish (Cam et al. 2009a).

Micro-algae are an important constituent of many aquaculture systems, especially the so-called greenwater systems, in which the animals are cultured in water containing 10^5 to 10^7 cells of micro-algae per ml (Coutteau and Sorgeloos 1992; Hargreaves 2006). Greenwater systems are used to culture various animals, including giant river prawn larvae (FAO 2013). Micro-algal cultures and greenwater used in aquaculture are not axenic and contain bacteria. However, the potential beneficial effects of bacteria associated with micro-algae remain largely unexplored (Natrah et al. 2014). In this study, we aimed at investigating (1) whether AHL-degrading bacteria can be isolated from micro-algal cultures, (2) whether they have any impact on algal growth, and (3) whether they can protect giant river prawn larvae against *V. campbellii*.

Materials and methods

Micro-algal strains and culture conditions

Axenic *Tetraselmis suecica* CCAP66/4 and *Chaetoceros muelleri* CCMP1316 were obtained from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, Scotland) and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, USA), respectively. The algae were grown in Guillard's F/2 medium (Sigma) (with silicate addition for C. *muelleri*) in sterile 250-ml Schott bottles provided with 0.22-μm filtered aeration. All parameters for algal culture were kept constant (pH 7, continuous light of 100 μmol photons $m^{-2}s^{-1}$, temperature of 24 °C and 30 g l^{-1} salinity). The density of axenic cultures was measured using a Bürker haemocytometer and a spectrophotometer (Thermo Spectronic) at $OD_{550\ nm}$. Axenity tests were done by plating the supernatant on marine agar and Luria-Bertani (LB) agar 30 g l^{-1} salinity (bacterial contamination test) and potato dextrose agar (fungal contamination test). Samples were also routinely checked microscopically at ×1000 magnification with oil immersion, immediately before harvesting.

Isolation of AHL-degrading bacteria from micro-algal cultures

Open cultures of *T. suecica* and *C. muelleri* were grown under similar conditions as described above for strains CCAP66/4 and CCMP1316. Fifty millilitres of the cultures was transferred to sterile Erlenmeyer flasks containing 5 ml of sterile NaCl solution (30 g l^{-1}) containing 50 mg l^{-1} *N*-hexanoyl-L-homoserine lactone (HHL). The Erlenmeyer flasks were covered with aluminium foil to prevent the growth of the micro-algae and were incubated on a shaker (120 rpm) at 24 °C. The isolation was performed in four consecutive cycles (72 h for the first cycle and 48 h for the second to fourth cycle). At the end of each cycle, 50 μl of the suspension was transferred to a new flask. At the end of the fourth cycle, the suspensions were spread-plated on Luria-Bertani agar containing 30 g l^{-1} NaCl (LB_{30}), and after 48 h incubation at 24 °C, colonies were picked, suspended in a 30 g l^{-1} NaCl solution and plated again. After three rounds of purification, isolates were grown in LB_{30} broth for 24 h at 24 °C and grown cultures were stored at −80 °C in 40 % glycerol. Two isolates, NFMI-T and NFMI-C isolated from *T. suecica* and *C. muelleri*, respectively, were used in further experiments. The isolates were submitted to the BCCM-LMG culture collection (http://bccm.belspo.be) under the numbers LMG 28858 (*Bacillus* sp. NFMI-C) and LMG 28859 (*Pseudomonas* sp. NFMI-T).

Bacterial strains and culture conditions

V. campbellii BB120 (ATCC BAA-1116) (Bassler et al. 1997), its mutant JAF548 (Freeman and Bassler 1999) and the AHL-degrading isolates were stored at −80 °C in 40 % glycerol. The stocks were streaked onto LB agar, and after 24 h of incubation at 28 °C, a single colony was picked and inoculated into 5 ml of fresh LB broth and incubated overnight at 28 °C under

constant agitation (100 min^{-1}). For the preparation of inocula for giant river prawn challenge tests, strains were grown in LB broth containing 12 g l^{-1} NaCl (LB_{12}).

Selection of natural rifampicin-resistant mutants of the isolates

Rifampicin-resistant mutants of the isolates were selected as described by Pande et al. (2013). Briefly, 100 μl of densely grown cultures (OD_{600} of 1) was inoculated into 5 ml of fresh LB_{12} broth containing 50 mg l^{-1} rifampicin (Sigma) and incubated for 5 days at 24 °C under constant agitation (100 min^{-1}). The grown cultures were inoculated into fresh LB broth with 50 mg l^{-1} rifampicin. The grown cultures were stored at −80 °C in 40 % glycerol until use.

Quantification of *N*-hexanoyl-L-homoserine lactone

A stock solution of HHL was prepared by dissolving HHL (Fluka) in 200 μl of ethanol (95 %) and then further diluted to a final concentration of 2500 mg l^{-1} by adding sterile distilled water. A plate diffusion method was used for quantitative detection of HHL using *Chromobacterium violaceum* CV026 as a reporter (Defoirdt et al. 2011b). Briefly, CV026 was grown to an optical density of around 2 at 550 nm in buffered (2 g/l MOPS) LB medium (pH 6.5) containing 20 mg l^{-1} kanamycin and spread over buffered (pH 6.5) LB plates. Subsequently, 10 μl of sample solution was applied to the centre of the plates and the plates were incubated at 28 °C for 48 h. After the incubation, the zone of purple-pigmented CV026 was measured and the concentration of HHL in the sample was calculated based on a standard curve.

AHL degradation assay

AHL degradation by the isolates was studied as reported previously (Defoirdt et al. 2011b). Briefly, the isolates (either single isolates or a 1:1 mixture) were inoculated at 10^8 CFU ml^{-1} in buffered LB_{30} medium (pH 6.5) supplemented with 10 mg l^{-1} HHL. At regular time intervals, 1-ml samples from each culture was taken and filtered over a 0.2-μm filter. The HHL concentration in the cell-free supernatants was determined as described above using *C. violaceum* CV026. *Pseudomonas* sp. P3/pME6000 and *Pseudomonas* sp. P3/pME6863 (= pME6000 + the *Bacillus* AHL lactonase gene *aiiA*; Molina et al. 2003), grown under the same conditions, were used as negative and positive control, respectively.

Identification of the isolates by 16S rRNA gene sequencing

PCR targeting a 1500-bp fragment of the 16S rRNA gene of the isolates was performed according to Boon et al. (2002) using the primer pair GM3f and GM4r (Biolegio, Nijmegen, The Netherlands). PCR was performed with a GeneAmp PCR system 2700 thermal cycler (PE Applied Biosystems, Nieuwerkerken a/d Ijssel, The Netherlands) using the program: 95 °C for 5 min, 32 cycles of 94 °C for 1 min, 42 °C for 1 min, 72 °C for 3 min and finally an extension period of 72 °C for 10 min. DNA sequencing of the obtained PCR products (467 and 462 bp for NFMI-C and NFMI-T, respectively) was carried out at IIT Biotech (Bielefeld, Germany). The nucleotide sequences of the isolates were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/Genbank) under the accession numbers KM525666 and KM525667 for NFMI-C and NFMI-T, respectively. Homology searches were completed with the BLAST server of the National Centre for Biotechnology Information for the comparison of the nucleotide query sequence against a nucleotide sequence database (blastn).

Impact of the isolates on the growth of gnotobiotic micro-algae

Axenic *T. suecica* CCAP66/4 and *C. muelleri* CCMP1316 were inoculated in 250-ml Erlenmeyer flasks containing 50 ml F/2 medium (with silica for *C. muelleri*) at 10^4 cells ml^{-1}, with and without the isolates (either single isolates or a 1:1 mixture) at 10^2 CFU ml^{-1}. As controls, the isolates were inoculated in flasks without algae. The flasks were incubated on a shaker (120 rpm) with constant illumination of 100 μmol photons $m^{-2}s^{-1}$ at 24 °C for 15 days. All treatments were performed in triplicate. Growth of the micro-algae was monitored by measuring in vivo chlorophyll a fluorescence (exCitation 430 nm, emission 670 nm) using a Tecan Infinite 200 microplate reader (Tecan, Mechelen, Belgium).

Persistence of the isolates in conventionalized micro-algal cultures

Conventionalized cultures of *T. suecica* CCAP66/4 and *C. muelleri* CCMP1316 were obtained by inoculating axenic cultures with 10^5 CFU ml^{-1} of microbiota taken from the open cultures of the respective micro-algae (obtained by taking the supernatant of cultures centrifuged at 300×*g* for 5 min). The micro-algae were inoculated in 250-ml Erlenmeyer flasks containing 50 ml F/2 medium (with silica for *Chaetocerosmuelleri*) at 10^4 cells ml^{-1}. A 1:1 mixture of the rifampicin-resistant mutants of NFMI-C and NFMI-T was inoculated at the start of the experiment at a total density of 10^5 CFU ml^{-1}. The flasks were incubated on a shaker (120 rpm) with constant illumination (4000 lx) at 24 °C. All treatments were performed in triplicates. The density of the AHL degraders was determined after 2 and 3 weeks of incubation by plating on LB_{30} agar containing 100 mg l^{-1}

rifampicin. Conventionalized algal cultures without the addition of the isolates were used as controls.

Giant river prawn challenge test

Giant river prawn challenge tests were performed as described by Pande et al. (2013). Briefly, larvae were obtained from a single oviparous female breeder. A matured female which had just completed its pre-mating molt was mated with a hard-shelled male. The female with fertilized eggs was then maintained for 20 to 25 days to undergo embryonic development. When fully ripe (indicated by dark grey colour of the eggs), the female was transferred to a hatching tank (30 l) containing slightly brackish water (containing 6 g l^{-1} Instant Ocean synthetic sea salt, Aquarium System Inc., Sarrebourg, France). The water temperature was maintained at 28 °C by a thermostat heater. After hatching, the newly hatched larvae with yolk were left for 24 h in the hatching tank. The next day, prawn larvae with absorbed yolk were distributed in groups of 25 larvae in 200-ml glass cones containing 100 ml fresh autoclaved brackish water (12 g l^{-1} synthetic sea salts). The glass cones were placed in a rectangular tank containing water maintained at 28 °C and was provided with aeration. The larvae were fed daily with 5 *Artemia* nauplii/larva and acclimatized to the experimental conditions for 24 h.

During the experiments, water quality parameters were kept at minimum 5 mg l^{-1} dissolved oxygen, maximum 0.5 mg l^{-1} ammonium-N and maximum 0.05 mg l^{-1} nitrite-N. Larvae were challenged by adding 10^6 CFU ml^{-1} of *V. campbellii* BB120 to the rearing wateron the day after first feeding, and *Bacillus* sp. NFMI-C was added at 10^5 CFU ml^{-1}. Survival was counted daily in the treatment receiving *V. campbellii* BB120 only, and the challenge test was stopped when more than 50 % mortality was achieved. At this time point, larval survival was determined in all treatments by considering that only those larvae presenting movement of appendages were alive.

The larval stage index (LSI) was determined according to Maddox and Manzi (1976) by randomly sampling five larvae from each treatment and calculated as:

$LSI = \Sigma\, Si/N$

Si : stage of the larva (*i* = 1 to 12)

N : the number of larvae examined

Statistical data analysis

Statistical analyses were performed using the SPSS software, version 20. Giant river prawn survival data were arcsin transformed in order to satisfy normal distribution and homoscedasticity requirements. Data were analysed by one-way ANOVA, followed by Tukey multiple range tests with a significance level set at 0.05.

Results

Isolation of AHL-degrading bacteria from micro-algal cultures

AHL-degrading strains were isolated from open cultures of *T. suecica* and *C. muelleri* by sequentially culturing the mixed microbial community in a medium containing *N*-hexanoyl-L-homoserine lactone (HHL) as the sole carbon and nitrogen source, followed by selecting individual colonies on LB agar plates. Two isolates, NFMI-T and NFMI-C (isolated from *T. suecica* and *C. muelleri*, respectively), were used in further experiments. The isolates were inoculated at 10^8 CFU ml^{-1} in buffered LB medium supplemented with 10 mg l^{-1} HHL in order to determine whether they were able to degrade AHLs in a nutrient-rich background, simulating the presence of high levels of other nutrients as is the case in a gastrointestinal environment. Both isolates were able to degrade HHL to below detection limit within 12 h when grown in monoculture (Fig. 1). However, degradation proceeded faster when inoculated in a 1:1 mixture (total density 10^8 CFU ml^{-1}), with the HHL level being decreased to below detection limit after 6 h (Fig. 1). The HHL degradation rates of NFMI-C monoculture, NFMI-T monoculture and an NFMI-C NFMI-T co-culture were 0.75, 0.77 and 1.67 mg l^{-1} h^{-1}, respectively.

Identification of the isolates

The isolates were identified by sequencing of the 16S rDNA. An NCBI BLAST search revealed that isolate NFMI-C was most closely related to *Bacillus* spp. and that isolate NFMI-T was most closely related to *Pseudomonas* spp. (Table 1).

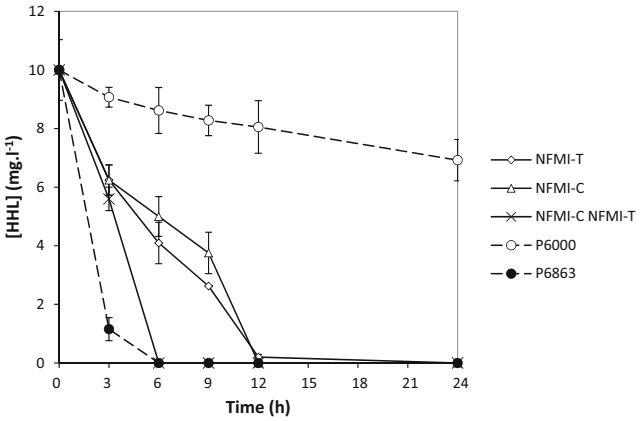

Fig 1 *N*-hexanoyl-L-homoserine lactone (HHL) degradation by the isolates NFMI-C and NFMI-T, either alone or as a 1:1 mixture, in Luria-Bertani broth containing 10 mg l^{-1}HHL. *Pseudomonas* sp. P3/pME6000 was used as a negative control; *Pseudomonas* sp. P3/pME6863 (containing the *Bacillus* sp. AHL lactonase gene *aiiA*) was used as a positive control. All strains were inoculated at a total density of 10^8 CFU ml^{-1}

Table 1 Identification of the isolates. Closest relatives based on NCBI BLAST performed using partial 16S rDNA sequences

Isolate	Source	Accession no.[a]	Closest relative (accession no.)	Similarity (%)
Bacillus sp. NFMI-C	*Chaetoceros muelleri*	KM525666	*Bacillus cereus* LNE7 (AM397642.1)	100
Pseudomonas sp. NFMI-T	*Tetraselmis suecica*	KM525667	*Pseudomonas putida BACD4* (KF413409.1)	99

[a] GenBank accession numbers

Impact of the isolates on growth of axenic micro-algae

Axenic *T. suecica* CCAP66/4 and *C. muelleri* CCMP1316 were inoculated in fresh algal growth medium, with and without the isolates (either single isolates or a 1:1 mixture) and growth of the micro-algae was monitored by measuring in vivo chlorophyll a fluorescence. *Pseudomonas* sp. NFMI-T alone had no effect on the growth of *T. suecica* CCAP66/4, whereas the addition of a mixture of both isolates resulted in higher chlorophyll fluorescence after 12 and 15 days of culture (Fig. 2a). In case of *C. muelleri* CCMP1316, growth was not affected by *Bacillus* sp. NFMI-C alone, whereas the mixture of both isolates resulted in a decreased chlorophyll fluorescence throughout the experiment (Fig. 2b). Because differences could only be observed when using both isolates and because the mixture showed the best HHL degrading capacity, a 1:1 mixture of the isolates was used in further co-culture experiments with micro-algae.

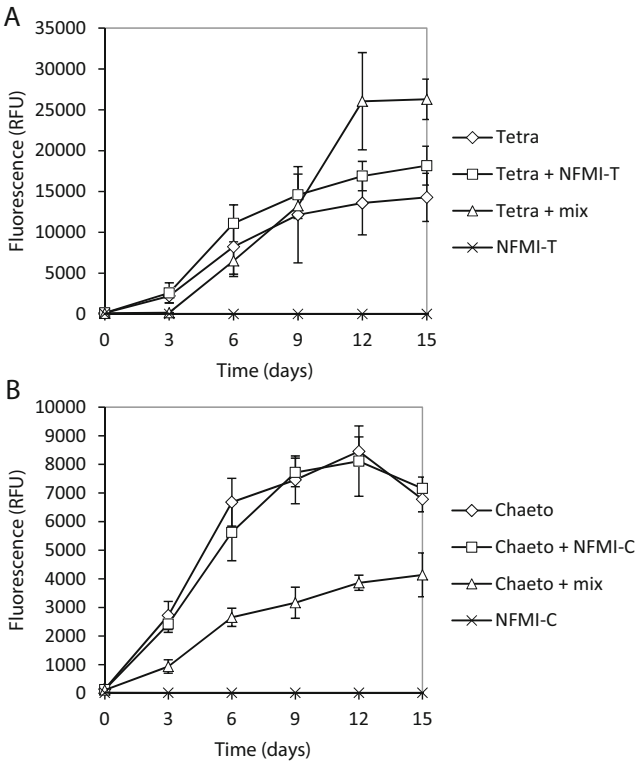

Fig. 2 Impact of the isolates on growth of micro-algae in Guillard's F/2 medium. **a** Chlorophyll fluorescence of *Tetraselmis suecica* CCAP66/4 (Tetra), with or without *Pseudomonas* sp. NFMI-T or a 1:1 mixture of both isolates. **b** Chlorophyll fluorescence of *Chaetoceros muelleri* CCMP1316 (Chaeto), with or without *Bacillus* sp. NFMI-C or a 1:1 mixture of both isolates. Cultures of the isolates without micro-algae were used as controls

Persistence of the isolate mixture in conventionalized micro-algal cultures

Conventionalized cultures of *T. suecica* CCAP66/4 and *C. muelleri* CCMP1316 were obtained by inoculating axenic cultures with 10^5 CFU ml^{-1} of microbiota taken from the open cultures of the respective micro-algae. A 1:1 mixture of natural rifampicin-resistant mutants of *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T was inoculated at the start of the experiment at a total density of 10^5 CFU ml^{-1}, and the density of the AHL degraders was determined after 2 and 3 weeks of incubation by plating on LB_{30} agar containing 100 mg l^{-1} rifampicin. The isolates were detected after 2 and 3 weeks of culture in both conventionalized *T. suecica* CCAP66/4 and *C. muelleri* CCMP1316 cultures (Table 2), where they had increased to approximately 10^6 CFU ml^{-1}.

Impact of the isolates on AHL quorum sensing in *V. campbellii*

Because bioluminescence is one of the phenotypes that are regulated by quorum sensing in *V. campbellii*, we used bioluminescence as a read-out of quorum sensing activity and determined the impact of the isolates on bioluminescence of wild-type *V. campbellii* BB120. In order to compensate for the competition for nutrients, we mixed BB120 with its mutant JAF548 as a control. JAF548 has a completely inactive quorum sensing system (and therefore is not luminescent; Freeman and Bassler 1999). The results revealed that *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, decreased quorum sensing-regulated luminescence of *V. campbellii* in co-culture (Fig. 3). However, there were no such effects when *V. campbellii* was grown in the presence of cell-free supernatants of the isolate (data not shown). Importantly, neither of the isolates affected the growth of *V. campbellii* in co-culture (Table 3) and the cell-free supernatants of the isolates also did not affect the growth of the pathogen (data not shown).

Table 2 Persistence of the 1:1 mixture of the AHL-degrading isolates NFMI-C and NFMI-T in conventionalized micro-algal cultures after 2 and 3 weeks of culture

Treatment	Plate counts ($\times 10^6$ CFU ml^{-1})	
	Week 2	Week 3
Conventionalized *Tetraselmis suecica* CCAP66/4 + isolates	1.1 ± 0.2	1.2 ± 0.9
Conventionalized *Tetraselmis suecica* CCAP66/4	ND	ND
Conventionalized *Chaetoceros muelleri* CCMP1316 + isolates	1.7 ± 0.2	1.6 ± 0.1
Conventionalized *Chaetoceros muelleri* CCMP1316	ND[1]	ND[1]

Plate counts on LB agar containing 100 mg l^{-1} rifampicin. The conventionalized micro-algal cultures were obtained by inoculating axenic cultures with 10^5 CFU ml^{-1} of microbiota taken from open cultures of the respective micro-algae. A 1:1 mixture of rifampicin-resistant isolates was inoculated at the start of the experiment at a total density of 10^5 CFU ml^{-1}

ND not detected

Impact of *Bacillus* sp. NFMI-C on the survival and growth of giant river prawn larvae challenged with *V. campbellii*

Our previous research showed that AHL quorum sensing is essential for full virulence of *V. campbellii* towards giant river prawn larvae (Pande et al. 2013). Hence, since *Bacillus* sp. NFMI-C was able to interfere with AHL quorum sensing in *V. campbellii*, we went further to investigate whether this isolate was able to protect giant river prawn larvae from the pathogen. Addition of *Bacillus* sp. NFMI-C to the rearing water indeed resulted in a significantly improved survival of challenged prawn larvae when compared to untreated challenged larvae (Table 4). Consistent with our previous work, we found no differences in growth (as assessed by determining the larval stage index) between the different treatments.

Discussion

The ability to interfere with bacterial quorum sensing by degrading AHL molecules is widely distributed in the bacterial kingdom (LaSarre and Federle 2013), and because of their potential as novel disease control agents, AHL-degrading bacteria have been isolated from various aquatic environments, including the water column, sediment, seaweed and the intestinal tract of healthy aquatic organisms (Tang and Zhang 2014). However, despite the importance of micro-algae for, e.g. aquaculture (Natrah et al. 2014), AHL degradation by bacteria associated with micro-algal cultures has thus far not been investigated. In this study, we report the isolation of two AHL-degrading strains, *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T, from open cultures of *C. muelleri* and *T. suecica*, respectively. Both strains showed HHL degradation rates that were similar to those of *Bacillus* sp. strains isolated from the intestinal tract of shrimp and fish (0.7–0.9 mg l^{-1} h^{-1}) (Defoirdt et al. 2011b). However, a co-culture of both strains degraded HHL approximately twice as fast. HHL was used as test compound because it is relevant to aquaculture. Indeed, it is produced by pathogenic bacteria such as *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Edwardsiella tarda* and *Vibrio salmonicida* (Swift et al. 1997; Morohoshi et al. 2004; Bruhn et al. 2005). Furthermore, *Bacillus* sp. NFMI-C was also found to interfere with quorum sensing in *V. campbellii* (most probably by degradation of the AHL *N*-3-hydroxybutanoyl-L-homoserine lactone) in co-culture.

Several *Bacillus* species have been reported to produce AHL lactonases, which inactivate AHLs by hydrolysing the lactone ring. Lactonases are intracellular enzymes capable of inactivating a wide range of AHLs, varying in acyl chain length and substitution (Dong et al. 2007). *Pseudomonas* species, in contrast, have been reported to produce AHL acylases, which cleave AHLs by amino hydrolysis. Unlike lactonases, acylases exhibit substrate

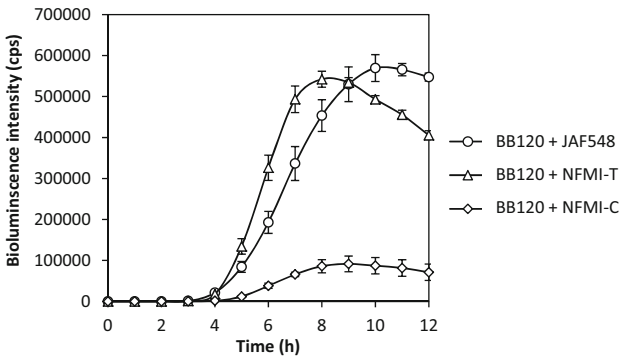

Fig. 3 Quorum sensing-regulated bioluminescence of *V. campbellii* BB120 in co-culture with the AHL-degrading isolates NFMI-C or NFMI-T. A co-culture with the dark mutant of BB120, JAF548, served as control. *Error bars* represent the standard deviation of six replicates.

Table 3 Impact of the isolates on cell density of *V. campbellii* BB120 after 12 h of co-culture as determined by plate counting of luminescent cells

Treatment	Cell density ($\times 10^8$ CFU ml^{-1})
V. campbellii BB120 + *V. campbellii* JAF548	4.7 ± 0.5
V. campbellii BB120 + *Bacillus* sp. NFMI-C	4.6 ± 0.2
V. campbellii BB120 + *Pseudomonas* sp. NFMI-T	4.5 ± 0.2

Table 4 Survival and growth (as expressed by the larval stage index—LSI) of giant river prawn larvae after 6 days of challenge with *V. campbellii* BB120 (average ± standard deviation of five prawn cultures)

Treatment	Survival (%)[a]	LSI[a]
Control	83 ± 7c	4.4 ± 0.5A
V. campbellii BB120	42 ± 8a	4.4 ± 0.5A
V. campbellii BB120 + *Bacillus* sp. NFMI-C	67 ± 5b	4.6 ± 0.5A

"Control" refers to unchallenged larvae that were otherwise treated in the same way as the other larvae

[a] Values in the same column with different letters are significantly different ($P < 0.05$)

specificity depending on the acyl side chain length and the substitution at the β-position of the acyl chain (Tang and Zhang 2014). This might explain why *Pseudomonas* sp. NFMI-T was not able to interfere with quorum sensing in *V. campbellii* although it was able to degrade HHL.

In addition to the AHL degradation capacity of the isolates, we also investigated their impact on micro-algal growth. *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T had no effect on growth of *C. muelleri* and *T. suecica*, respectively, when added alone. However, a mixture of both strains increased the growth of *T. suecica*, whereas it decreased the growth of *C. muelleri*. Interactions between bacteria and micro-algae are fairly complex and not yet completely understood. The exact mechanisms by which bacteria stimulate micro-algae are largely unknown, although a few compounds responsible for such effects (including vitamins and hormones) have been identified (Natrah et al. 2014). The negative impact of the NFMI-C NFMI-T mixture on the growth of *C. muelleri* might be due to competition for nutrients. Indeed, bacteria have been reported to decrease the growth of another diatom, *Cylindrotheca fusiformis*, at low phosphate concentrations (suggesting that the bacteria scavenge phosphate better than the micro-algae) (Amin et al. 2012). Another possibility is that *Pseudomonas* sp. NFMI-T produces a compound that inhibits the growth of *Chaetoceros*, without affecting the growth of *Tetraselmis* (from which it was isolated). Indeed, some bacteria (including *Pseudomonas aeruginosa*) are able to produce algicidal compounds (Natrah et al. 2014). This algicidal activity can be caused either by the release of algicidal compounds in the environment or by lysis of the micro-algal cells following attachment (Mayali and Azam 2004).

Our in vivo challenge test revealed that *Bacillus* sp. NFMI-C significantly increased the survival of giant river prawn larvae challenged with *V. campbellii*, whereas the isolate had no effect on larval growth. This is consistent with our previous work showing that AHL quorum sensing is essential for full virulence of *V. campbellii* towards giant river prawn larvae (Pande et al. 2013) and previous reports documenting that AHL degraders are able to improve the survival of prawn larvae (Cam et al. 2009b) and turbot larvae (*Scophthalmus maximus* L.) (Tinh et al. 2008) in the presence of exogenous AHL (which caused mortality in both species; probably by triggering pathogenicity mechanisms in pathogenic bacteria that were naturally present in the cultures). We found that isolate NFMI-C was most closely related to *Bacillus* spp. Strains belonging to *Bacillus* species such as *B. subtilis*, *B. cereus*, *B. coagulans*, *B. clausii*, *B. megaterium* and *B. licheniformis* are the most frequently used probiotics in aquaculture (Wang et al. 2008). Hence, the use of *Bacillus* sp. strains able to degrade AHL molecules might be an interesting new type of probiotics for aquaculture with a defined mode of action (which does not necessarily mean that AHL degradation is the sole beneficial effect). Along this line, AHL-degrading *Bacillus* sp. have been shown to inhibit the protease production, haemolytic activity and biofilm formation of *A. hydrophila* strain YJ-1 and to significantly improve the survival of zebrafish (*Danio rerio*) challenged with this pathogen (Chu et al. 2014). AHL-degrading enzymes could be administered to the aquaculture system in several ways, and this will depend on the nature of the specific system. Indeed, AHL-degrading bacteria capable of establishing themselves in algal cultures (as described in this study) might be the agents of choice in greenwater systems, whereas in other systems, it might be more efficient to use purified (preferentially heat stable) AHL-degrading enzymes that are administered via the feed (as e.g. described in Chen et al., 2010; Cao et al., 2012).

In conclusion, in this study, we isolated two AHL-degrading strains, *Bacillus* sp. NFMI-C and *Pseudomonas* sp. NFMI-T, from cultures of the micro-algae *C. muelleri* and *T. suecica*, respectively. Both strains were able to quickly degrade AHLs in a nutrient-rich background. The isolates were able to maintain themselves in conventionalized algal cultures, and their impact on algal growth was dependent on the tested micro-algal species. *Bacillus* sp. NFMI-C, but not *Pseudomonas* sp. NFMI-T, was able to decrease quorum sensing-regulated luminescence of *V. campbellii* and improved the survival of giant river prawn larvae when challenged with *V. campbellii*. These AHL-degrading isolates might have potential as novel biocontrol strains for use in aquaculture. Further research will reveal the efficacy of the isolates in different aquaculture host-pathogen systems. Indeed, although *Pseudomonas* sp. NFMI-T was not able to interfere with quorum sensing in *V. campbellii*, it might be well capable of protecting aquatic hosts from bacteria that use another type of AHL molecule such as *Aeromonas* spp. and *Edwardsiella* spp., which use *N*-hexanoyl-L-homoserine lactone (HHL). Furthermore, a combination of both isolates might even be more effective since a co-

culture showed a higher HHL degradation rate than the single cultures of the isolates.

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Conflict of interest The authors declare that they have no competing interests.

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