RESEARCH ARTICLE

N-acyl homoserine lactone-degrading microbial enrichment cultures isolated from *Penaeus vannamei* shrimp gut and their probiotic properties in *Brachionus plicatilis* cultures

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Abstract

Three bacterial enrichment cultures (ECs) were isolated from the digestive tract of Pacific white shrimp *Penaeus vannamei*, by growing the shrimp microbial communities in a mixture of *N*-acyl homoserine lactone (AHL) molecules. The ECs, characterized by denaturing gradient gel electrophoresis analysis and subsequent rRNA sequencing, degraded AHL molecules in the degradation assays. Apparently, the resting cells of the ECs also degraded one of the three types of quorum-sensing signal molecules produced by *Vibrio harveyi in vitro* [i.e. harveyi autoinducer 1 (HAI-1)]. The most efficient AHL-degrading ECs, EC5, was tested in *Brachionus* experiments. EC5 degraded the *V. harveyi* HAI-1 autoinducer *in vivo*, neutralizing the negative effect of *V. harveyi* autoinducer 2 (AI-2) mutant, in which only the HAI-1- and CAI-1-mediated components of the quorum-sensing system are functional on the growth of *Brachionus*. This suggests that EC5 interferes with HAI-1-regulated metabolism in *V. harveyi*. These AHL-degrading ECs need to be tested in other aquatic systems for their probiotic properties, preferably in combination with specific AI-2-degrading bacteria.

Introduction

Quorum sensing is a mechanism by which bacteria coordinate gene expression in a density-dependent manner. This process depends on the production, release and detection of chemical signal molecules called autoinducers (Miller & Bassler, 2001). Many bacterial processes are regulated by quorum sensing, including symbiosis, virulence, bioluminescence, antibiotic production and biofilm formation (Lazdunski *et al.*, 2004). Highly specific and universal quorum-sensing languages exist that allow intra- and interspecies communication (Schauder *et al.*, 2001). Gram-negative bacteria use *N*-acyl homoserine lactones (AHLs) as autoinducers, while Gram-positive bacteria use oligopeptides to communicate (Miller & Bassler, 2001; Whitehead *et al.*, 2001). The most extensively investigated intercellular signaling molecules are the AHLs.

AHLs are associated with the quorum-sensing processes in various pathogens (Bruhn *et al.*, 2005). Quorum sensing in *Vibrio 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 three autoinducers, namely harveyi autoinducer 1 (HAI-1), autoinducer 2 (AI-2) and CAI-1, which regulate the expression of bioluminescence (Bassler *et al.*, 1993, 1994, 1997; Chen *et al.*, 2002) and other virulence factors (Henke & Bassler, 2004). HAI-1 is an AHL and was identified as *N*-(β-hydroxybutanoyl) homoserine lactone (Cao & Meighen, 1989). AI-2 is a furanosyl borate diester (Chen *et al.*, 2002), a universal signal that is used by many 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, a *Vibrio cholerae*-like autoinducer CAI-1, was discovered in *V. harveyi* (Henke & Bassler, 2004). CAI-1 has not been structurally characterized, but the *V. cholerae* CAI-1-mediated quorum-sensing pathway functions analogously to that of *V. harveyi* (Miller *et al.*, 2002).

Disruption of quorum sensing was suggested as a new antiinfective strategy to control pathogenic bacteria without interfering with their growth (Finch *et al.*, 1998; Hentzer *et al.*, 2003), and it may be a particularly useful method in aquaculture (Defoirdt *et al.*, 2004). One of the approaches proposed for quorum-sensing disruption is the isolation of bacteria that degrade signal molecules involved in quorum sensing. A number of bacteria utilize AHL molecules as sole sources of carbon and nitrogen; thus, they can be used as potential quenchers of quorum-sensing-regulated functions in pathogenic bacteria. *Pseudomonas* strains PAI-A and PA01 degraded 3-oxododecanoyl homoserine lactone and other long-acyl groups, but not short-acyl, as an energy source (Huang *et al.*, 2003). Twenty-five isolates that degrade *N*-hexanoyl homoserine lactone were isolated from a tobacco rhizosphere (Uroz *et al.*, 2003), and one of these, *Rhodococcus erythropolis* strain W2, was used to quench quorum-sensing-regulated functions of other microorganisms. Introduction of the plasmid-borne *aiiA* gene encoding a lactonase enzyme confers the ability to degrade AHLs on the rhizosphere isolate *Pseudomonas fluorescens* P3 (Molina *et al.*, 2003). This transformed strain significantly reduced potato soft rot and tomato crown gall diseases that were caused by plant pathogenic bacteria. The evidence for AHL-degrading bacterial isolates in the aquatic environment is, however, scarce, in spite of the progress made in the study of terrestrial species.

In the present work, three different microbial enrichment cultures (ECs), namely EC3, EC4 and EC5, isolated from the shrimp gut, were tested for AHL-degrading properties *in vitro*. Additionally, the ability to attenuate the quorum-sensing-dependent negative effect of *V. harveyi* on gnotobiotic rotifers *Brachionus plicatilis* was examined.

Materials and methods

Preparation of microbial communities

Microbial communities (MCs) were collected from the digestive tract of healthy Pacific white shrimp juveniles *Penaeus vannamei*, maintained in culture on formulated feeds at Ghent University, Belgium. The digestive tract was removed from the shrimp body after dissection and was homogenized by means of a stomacher blender (Seward, UK). After homogenizing, the suspensions were centrifuged at 1600 *g* for 5 min, and then the supernatant was preserved at −80 °C in 20% glycerol.

These MCs were used as seed material for isolation of AHL-degrading strains. Two hundred microliters of the MC suspension was inoculated into 20 mL of a minimal culture medium, which contained 9 g L^{-1} NaCl and 5 mg L^{-1} of a mixture of AHL molecules (in equal weight for all compounds), which are commercially available (Fluka, Germany) (Table 1). The cultures were placed on a shaker (120 r.p.m.) at 28 °C.

The isolation was performed in six consecutive cycles; each cycle lasted 48 h. At the end of each cycle, 200 μL of each sample was transferred to a new flask containing 20 mL of fresh medium. The cell densities at the start and at the end of each cycle were determined, by measurement of $OD_{550\,nm}$ wavelength and by plating the samples on Marine Agar (Difco, Detroit).

Three ECs, originating from three different shrimp individuals, were obtained at the end of the sixth cycle. The ECs were maintained at −80 °C in 20% glycerol for further characterization.

Bacterial strains

Chromobacterium violaceum strain CV026, a mini-Tn5 mutant derived from the *C. violaceum* strain ATCC31532 (McClean *et al.*, 1997), was used as an AHL reporter. This strain cannot produce AHL, but can detect and respond to a range of AHL molecules (with an acyl side chain of four to eight carbons) by inducing the synthesis of the purple pigment violacein. Strain P3/pME6863, a transformant of a soil bacterium *P. fluorescens*, was used as a positive control in the degradation assays. Plasmid pME6863 carries the *aiiA* gene from a soil bacterium *Bacillus* sp. A24 that encodes a lactonase enzyme (Molina *et al.*, 2003).

Vibrio harveyi strain BB120 and its mutants were obtained from the Department of Molecular Biology, Princeton University, New Jersey, USA (Table 2).

Table 1. AHL molecules used in the study

AHL molecule	Abbreviation
N-butyryl-DL-homoserine lactone	C_4-AHL
N-butyryl-DL-homocysteine thiolactone	C_4-AHT
N-hexanoyl-DL-homoserine lactone	C_6-AHL
N-heptanoyl-DL-homoserine lactone	C_7-AHL
N-octanoyl-DL-homoserine lactone	C_8-AHL

Table 2. *Vibrio harveyi* strains used in the study

Strain	Characteristic	Reference
BB120	Wild-type strain	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)
JMH606	Dysfunctional AI-2 and CAI-1 synthase	Henke & Bassler (2004)
JMH612	Dysfunctional AI-2 and CAI-1 receptor	Henke & Bassler (2004)

Culture media

Marine Broth (Difco, Detroit) was used as a universal medium to grow bacterial ECs and *V. harveyi* strains. P3/pME6863 strain was grown in Luria–Bertani (LB) medium, containing tryptone (BD, France, 1% w/v) and yeast extract (Sigma, Germany, 0.5% w/v) and NaCl (0.4% w/v). CV026 strain was grown in LB medium supplemented with 20 mg L^{-1} of kanamycin, in order to maintain the plasmid carrying the gene responsible for violacein production.

Preparation of cell-free washwater of *V. harveyi* strains

Vibrio harveyi strains were grown in Marine Broth until the OD reached *c.* 1 at 600 nm. The culture was centrifuged at 4500 ***g*** for 10 min and the pellet was resuspended in 0.22 μm filtered and autoclaved 20 g L^{-1} NaCl solution (pH = 7.0). The suspension was centrifuged a second time after incubation at 28 °C for 30 min on a shaker (120 r.p.m.). The supernatant was subsequently filter sterilized over a 0.22 μm Millipore filter (Bedford, MA) and stored at −30 °C until use (for maximum 1 month).

Denaturing gradient gel electrophoresis (DGGE) and DNA sequencing

Total DNA of the ECs was extracted using standard methods (Boon *et al.*, 2000). 16S rRNA gene fragments were amplified with the primers PRBA338fGC and P518r (Muyzer *et al.*, 1993) and analyzed by DGGE with a denaturing gradient ranging from 45% to 60% (Boon *et al.*, 2002).

16S rRNA gene fragments were cut out of the DGGE gel with a clean scalpel and added in 50 μL of PCR water. After 12 h of incubation at 4 °C, 1 μL of the PCR water was reamplified with the primer set P338F and P518r. Five microliter of the PCR product was loaded on a DGGE gel. DNA sequencing of the ca. 180-bp fragments was carried out by ITT Biotech-Bioservice (Bielefeld, Germany). Analysis of DNA sequences and homology searches were completed with standard DNA sequencing programs and the BLAST server of the National Center for Biotechnology Information (NCBI) using the BLAST algorithm (Altschul *et al.*, 1997) and the Ribosomal Database project (Cole *et al.*, 2005). The sequence data of the ECs have been submitted to the GenBank databases under accession numbers EF177457–EF177459 and EF635911.

AHL degradation assays

Microtiter assay

The wells of a 96-well microplate were filled with two layers. The bottom layer consisted of 100 μL of semisolid LB agar (1% agar, pH = 6.5) supplemented with 1 mg L^{-1} of *N*-hexanoyl-DL-homoserine lactone (HHL). Fifty microliters of suspension of an EC containing 3×10^6 CFU mL^{-1} was added to the well before the agar solidified. The top layer contained 50 μL of CV026 strain (10^6 CFU mL^{-1}) in LB medium, which was added to each well after 24 h of incubation. Sixteen replicates were performed for each EC. The negative control wells and positive control wells contained no bacteria or a suspension of P3/pME6863 strain in the bottom layer, respectively. The microplate was incubated at 28 °C for 24 h after CV026 was added. The degradation of HHL molecule by the ECs was assessed by observation of the nonappearance of a purple color in the wells.

Correlation between the HHL concentration and the diameter of violacein-induced halo

Before starting the degradation kinetics assay, a standard curve correlating the diameter of the purple-pigmented halo produced by the CV026 strain with the HHL concentration was established. An overnight-grown culture of CV026 was diluted in fresh LB medium to obtain an OD of *c.* 0.1. Fifty microliters of this suspension was spread on an LB agar plate. Ten microliters of an HHL solution was subsequently applied to the center of the plate. Five concentrations were tested: 10; 5; 2.5; 1; and 0.5 mg L^{-1}. Each concentration was made in triplicate. The diameters of the purple-pigmented halos produced by the CV026 strain were measured after incubation of the plates at 28 °C for 24 h.

Assay to determine the degradation kinetics

This assay was performed in 50 mL erlenmeyer's flasks containing 10 mL of LB medium supplemented with 5 mg L^{-1} of HHL. The ECs were inoculated into this medium at 10^6 CFU mL^{-1}. Each treatment was performed in triplicate. P3/pME6863 strain was inoculated into the positive control flasks. No bacteria were added in the negative control treatment. The flasks were placed on a shaker (120 r.p.m.).

Degradation of HHL was assessed at 12, 24, 36 and 48 h. At each sampling time, 1 mL of culture from each flask was 0.22 μm filtered. Subsequently, 10 μL of the filtrate was dropped in the center of an LB plate, on which 50 μL of a CV026 culture (at an OD of *c.* 0.1) had been spread plated. The plate was placed in an incubator at 28 °C. The diameter of the purple-pigmented halo produced by the CV026 strain was measured after a 24-h incubation. The residual concentration of HHL in the culture filtrate was determined based on the standard curve.

Degradation of *V. harveyi* HAI-1 autoinducer by the ECs

The cell-free washwater of the BB120 (wild-type) and JMH606 (dysfunctional AI-2 and CAI-1 synthase) strains

was prepared as described above. The resting cells of the ECs were inoculated into these washwaters at a density of 10^6 CFU mL^{-1}. Three replicates were performed for each treatment. The cultures were incubated at 28 °C with shaking (120 r.p.m.). After 24 and 48 h of incubation, the cultures were centrifuged at 4500 *g* for 10 min and subsequently 0.22 μm filter sterilized to remove the EC cells.

The levels of *V. harveyi* HAI-1 autoinducer remaining in the culture supernatants at each sampling time were quantified based on the ability to induce bioluminescence in the *V. harveyi* double-mutant JMH612, which was used as a reporter strain for HAI-1 (Table 2). The reporter strain was grown in Marine Broth at 28 °C with shaking (120 r.p.m.) to an $OD_{600\,nm}$ of *c.* 1 and was diluted 1/5000 in fresh medium. Fifty microliters of the diluted reporter culture was mixed with 50 μL of the culture supernatants in 3 mL test tubes. The washwaters incubated without ECs were used as positive controls. The test tubes were incubated at 28 °C for 4 h. The luminescence intensity of the cultures was measured in relative light unit (RLU), by means of a Biocounter M2500 luminometer (Lumac, The Netherlands).

Gnotobiotic rotifers as test organisms

Brachionus plicatilis (clone 10) was obtained from CIAD (Centro de Investigación en Alimentación y Desarrollo, Mazatlan Unit for Aquaculture) in Mexico and was confirmed to belong to the species *B. plicatilis* sensu stricto (Papakostas *et al.*, 2006). Amictic rotifer eggs were disinfected with 100 p.p.m. of glutaraldehyde for 2 h at 28 °C. The procedure for obtaining axenic rotifers is described in Tinh *et al.* (2006).

Challenge tests to evaluate the effect of EC5

The challenge tests took place in sterile 50 mL falcon tubes containing 20 mL of 25 g L^{-1} filtered and autoclaved seawater (FASW). The falcon tubes were placed on a rotor (4 r.p.m.) that was placed inside a temperature-controlled room (28 °C, 2000 Lx). Each treatment was performed in four replicates, and each experiment was repeated twice. An enrichment culture (EC5) was added to the culture water after the first feeding, at 5×10^6 CFU mL^{-1}. *Vibrio harveyi* strain BB120 and its mutants (MM30, BB152, BB886, BB170 and MM77) were added 3 h later, at the same density. No bacteria were added in the control treatment. Rotifers were fed twice with an axenic baker's yeast strain (*Saccharomyces cerevisiae*), at the start of each experiment (day 1) and 24 h after challenging with *V. harveyi* (day 2). The rotifer density was monitored daily until day 4 (72 h after the challenge with *V. harveyi*).

Two subsamples of 500 μL of rotifer culture were withdrawn daily from each replicate to estimate the rotifer density. Population growth rate (μ) was calculated as

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

where N_o is the initial rotifer density, N_t is the rotifer density on day t of culture (rotifer mL^{-1}), and t is the duration in days.

Data analysis

Parametric assumptions were evaluated using Shapiro–Wilk's test for normality and Levene's test for homogeneity of variances. The data of luminescence induction of reporter strain were compared between treatments, using one-way ANOVA, followed by the Tukey test. Dunnett's T3 test was used for the sets of data that did not conform to the parametric assumptions. For the *in vivo* experiment, the *Brachionus* growth rates on day 4 were compared between pairs of treatments using an independent samples *t*-test. The difference between pairs of treatments was considered to be significant if the *P*-value was below 0.01. All the tests were performed using the SPSS program version 11.5.

Results

Growth of ECs

During the isolation process, the densities of shrimp microbial communities in AHL medium increased from 10^6 CFU mL^{-1} at the start of each cycle to *c.* 10^8 CFU mL^{-1} at the end of each cycle, as determined by $OD_{550\,nm}$ measurement).

AHL degradation by the ECs

The ability of AHL degradation by the ECs was assessed qualitatively in a 96-well microplate. The ECs were grown in semisolid LB agar supplemented with 1 mg L^{-1} of HHL. This concentration was determined previously (data not shown) to be the detection limit of HHL by the reporter strain CV026 for this type of assay. All the three ECs could degrade HHL below the detection limit after 24 h of incubation.

The AHL degradation kinetics were evaluated in another assay. A standard curve was determined in advance, with the following equation: diameter of purple-pigmented halo = 7.9655 ln(HHL) + 11.425 (regression coefficient R^2 = 0.9744). According to Fig. 1, no chemical degradation was noticed in the negative control treatment, as the pH of LB medium was buffered at 6.5–7.0 during the assay. EC5's degradation pattern was almost the same as that of the positive control strain, and these cultures could degrade HHL after 24 h to below 0.1 mg L^{-1} (which is the detection limit by the reporter strain CV026). EC4 had a slow

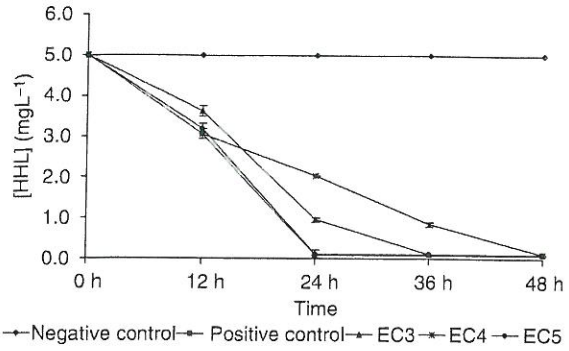

Fig. 1. HHL degradation curves of control cultures and ECs (EC3, EC4, EC5), as indicated by the residual HHL concentration in the medium over 48 h. The error bars represent the SD of three replicates. Blank LB medium was used as a negative control. Strain P3/pME6863 was inoculated in the positive control.

degradation rate, as the residual HHL concentration only declined below the detection limit after 48 h.

Degradation of *V. harveyi* HAI-1 autoinducer by the ECs

The capability of ECs to degrade the HAI-1 autoinducer was investigated by re-suspending the ECs' resting cells in the cell-free washwater obtained from the BB120 and JMH606 strains. As BB120 is a wild-type strain, its washwater should contain all the three autoinducers, namely HAI-1, AI-2 and CAI-1. JMH606's washwater should contain only HAI-1. The levels of luminescence induction were decreased dramatically in the presence of ECs, in both washwaters from BB120 and JMH606 cultures, compared with the control treatments ($P < 0.001$) (Table 3), indicating that all the three ECs were able to degrade the *V. harveyi* HAI-1 autoinducer under the experimental conditions.

Effect of EC5 in *B. plicatilis* cultures

The effect of one of the EC5, was evaluated in *B. plicatilis* cultures that were challenged with different *V. harveyi* mutants. All the *V. harveyi* mutants used in this study (except the double mutant MM77) have previously shown a negative effect on *Brachionus* growth rate (Tinh *et al.*, 2007). According to the results shown in Table 4, the EC5-EC could only exert a neutralizing effect against the MM30 and BB886 mutants, which are dysfunctional in the AI-2-mediated quorum sensing system, in both experiments ($P < 0.01$). In another series of experiments, *Brachionus* were challenged with the double-mutant MM77, while the washwater of the MM30 culture was added as an exogenous source of the HAI-1 and CAI-1 molecules (Table 5). It is obvious that the EC5 was also able to neutralize the negative effect of MM77 strain in this case ($P < 0.01$).

Table 3. Induction of luminescence (mean ± SD, $n = 3$) in the reporter strain by cell-free washwater from the *Vibrio harveyi* strains BB120 and JMH606, in the absence and presence of enrichment cultures

Washwater	Treatment	Induction of luminescence in reporter strain (log RLU)
BB120	Control, at 0 h	5.63 ± 0.08
	Control, after 24 h	5.62 ± 0.10[b]
	EC3, after 24 h	2.13 ± 0.07[a]
	EC4, after 24 h	1.86 ± 0.09[a]
	EC5, after 24 h	1.64 ± 0.03[a]
BB120	Control, after 48 h	5.62 ± 0.15[b]
	EC3, after 48 h	1.94 ± 0.16[a]
	EC4, after 48 h	1.87 ± 0.06[a]
	EC5, after 48 h	1.63 ± 0.05[a]
JMH606	Control, at 0 h	5.66 ± 0.05
	Control, after 24 h	5.63 ± 0.03[b]
	EC3, after 24 h	2.16 ± 0.04[a]
	EC4, after 24 h	2.45 ± 0.06[a]
	EC5, after 24 h	2.52 ± 0.09[a]
JMH606	Control, after 48 h	5.62 ± 0.07[b]
	EC3, after 48 h	2.25 ± 0.06[a]
	EC4, after 48 h	2.13 ± 0.10[a]
	EC5, after 48 h	2.01 ± 0.05[a]

Treatments with different superscripts, for the same strain and at the same sampling time, are significantly different from each other ($P < 0.001$, Tukey test).

Table 4. Growth rate of *Brachionus plicatilis* (mean ± SD, $n = 4$) over 72 h: effect of challenge with *Vibrio harveyi* single mutants, in the absence and presence of the enrichment culture EC5

Treatment	Mutation in	Experiment 1	Experiment 2
Control	–	0.40 ± 0.096	0.19 ± 0.05
EC5		0.48 ± 0.02	0.26 ± 0.03
BB120	–	0.22 ± 0.06	0.08 ± 0.06
EC5+BB120		0.25 ± 0.07	0.09 ± 0.07
MM30	AI-2 synthase	0.15 ± 0.03*	0.08 ± 0.03*
EC5+MM30		0.34 ± 0.16*	0.26 ± 0.09*
BB152	HAI-1 synthase	0.20 ± 0.08	0.09 ± 0.04
EC5+BB152		0.23 ± 0.16	0.10 ± 0.04
BB886	AI-2 receptor	0.16 ± 0.05*	0.07 ± 0.05*
EC5+BB886		0.36 ± 0.12*	0.22 ± 0.04*
BB170	HAI-1 receptor	0.13 ± 0.06	0.06 ± 0.03
EC5+BB170		0.22 ± 0.08	0.10 ± 0.08

*Significant difference in growth rate between the paired treatments ($P < 0.01$, *t*-test).

All the strains were added at 5×10^6 CFU mL^{-1}. *Vibrio harveyi* strains were added 3 h after the addition of EC5. Rotifers were fed with axenic yeast twice, at the start of experiment and 24 h after the challenge.

Sequence analysis of ECs

The DGGE pattern of the ECs (Fig. 2) shows that the three ECs are clearly different. The 16S rRNA gene sequences of the dominant bands (rectangles) were compared with those in the rRNA database. Most of the matched genera are

Table 5. Growth rate of *Brachionus plicatilis* (mean ± SD, $n = 4$) over 72 h: effect of challenge with the MM77 strain (HAI-1 and AI-2 synthase mutant), with and without the addition of the MM30 (AI-2 synthase mutant) washwater, in the absence and presence of the EC5

Treatment	Experiment 1	Experiment 2
Control	0.31 ± 0.04	0.54 ± 0.04
EC5	0.42 ± 0.06	0.60 ± 0.03
MM77	0.40 ± 0.04	0.50 ± 0.04
EC5+MM77	0.43 ± 0.04	0.53 ± 0.04
MM77+MM30 washwater	0.15 ± 0.02*	0.21 ± 0.06*
EC5+MM77+MM30 washwater	0.30 ± 0.10*	0.61 ± 0.02*

*Significant difference in growth rate between the paired treatments ($P < 0.01$, *t*-test).

All the strains were added at 5×10^6 CFU mL^{-1}. The MM77 strain and the MM30 washwater were added 3 h after the addition of EC5. Two milliliters of MM30 washwater was added to 18 mL of rotifer culture water. Rotifers were fed with axenic yeast twice, at the start of experiment and 24 h after the challenge.

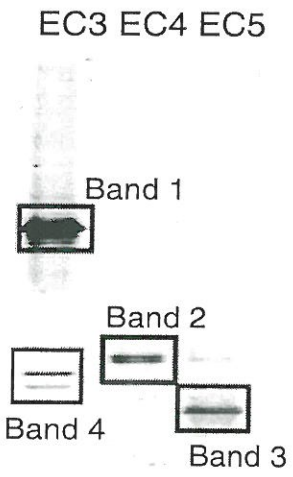

Fig. 2. DGGE pattern of the ECs. Rectangles indicate the bands that are sequenced. The species to which the sequences have high homology are indicated in the text.

Gram-negative, aerobic rod-shaped bacteria, which are widely distributed in oligotrophic environments. The most dominant species of EC3 (band 1) has 95.7% similarity to *Pseudomonas* sp., while the less dominant species (band 4) has 99% similarity to *Achromobacter xylosoxidans*. The dominant species of EC4 (band 2) resembles *Sphyngomonas* sp. and *Sphingopyxis* sp. (97.1% similarity). The matched genera for EC5 (band 3) are *Rhizobium*, *Ensifer*, *Sinorhizobium* and *Aminobacter* (96.2% similarity).

Discussion

To the authors' knowledge, this is the first time that AHL-degrading bacteria have been isolated from aquatic animals. The enrichment procedure was based on the ability of a microbial community collected from shrimp gut to grow in a minimal medium containing only a mixture of AHL molecules as carbon and nitrogen sources. This approach is similar to that used for enrichment of AHL-degrading bacteria from a tobacco rhizosphere, where a minimal medium supplemented with ammonium sulfate as a nitrogen source and *N*-hexanoyl homoserine lactone as a carbon source was used by Uroz *et al.* (2003). The HHL-degrading isolates isolated by the latter authors were identified as members of the genera *Pseudomonas*, *Comamonas*, *Variovorax* and *Rhodococcus*.

As determined by 16S rRNA gene sequence, the ECs obtained in this study are composed of bacteria that inhabit oligotrophic environments. The dominant species of EC3 is similar to *Pseudomonas* sp., which metabolize divergent nutrients (Vamsee-Krishna *et al.*, 2006) and often serve as biocontrol agents (Kamilova *et al.*, 2006). The less dominant species of EC3 matches *Achromobacter xylosoxidans*, which degrades monoaromatic hydrocarbons (Nielsen *et al.*, 2006). Representatives of *Sphingomonas* and *Sphingopyxis*, genera with similarity to the dominant species of EC4, are widely distributed in terrestrial and aquatic habitats. Several *Sphingopyxis* species have recently been isolated from seawater of the Yellow Sea (Yoon & Oh, 2005; Yoon *et al.*, 2005), and members of the *Sphingopyxis* genus possess biodegradative capabilities (Godoy *et al.*, 2003; Sohn *et al.*, 2004). The dominant group of EC5 closely matched *Rhizobium* and *Aminobacter*. An isolate of the *Rhizobium*, *Agrobacterium tumefaciens*, was among the bacteria that expressed AHL degradation enzymes in biofilms formed in a water reclamation system (Hu *et al.*, 2003). Several *Aminobacter* isolates capable of degrading insecticides and herbicides have been isolated from agricultural soil in Northern Ireland and Canada (McDonald *et al.*, 2005). Molecular analysis of the ECs characterized in this study shows that the ability to degrade AHL is probably common in different bacterial groups.

Many soil/plant isolates can degrade AHL molecules secreted by pathogenic bacteria, and thus have potential as biocontrol agents in disease prevention. AHL-producing and AHL-degrading bacteria coexist in every ecosystem, possessing different strategies to gain competitive advantages. The bacterial biofilms developed in a water reclamation system exhibited two groups of bacteria producing AHL signals, while three isolates, namely *Agrobacterium*

tumefaciens, *Bacillus cereus* and *Ralstonia* sp., expressed AHL-degrading enzymes Hu *et al.* (2003). *Bacillus thuringiensis* suppressed the AHL-dependent virulence of a plant pathogen *Erwinia carotovora* by interfering with the accumulation of AHL signals (Dong *et al.*, 2004). An *Acinetobacter* sp. strain C1010, isolated from the rhizospheres of cucumbers degraded AHL molecules produced by a phytopathogenic bacterium *Burkholderia glumae* (Kang *et al.*, 2004), and a soil bacterium *Variovorax paradoxus* can grow on AHL signal molecules as the sole source of energy and nitrogen (Leadbetter & Greenberg, 2000). Several groups of AHL-degradation enzymes have been identified. These enzymes belong to either the acylase group, which breaks down the amide bond connecting the homoserine lactone ring to the acyl chain (Xu *et al.*, 2003), or the lactonase group, which hydrolyzes the lactone ring (Dong *et al.*, 2000, 2002; Lee *et al.*, 2002). The ECs characterized in this study grow on AHL molecules as the sole source of energy and nitrogen; thus, they will possess at least one type of AHL-degrading enzymes.

Microtiter and degradation kinetics assays were used to, respectively, characterize AHL degradation patterns either qualitatively or quantitatively. The latter assay reveals the change in degradation pattern with time, and HHL was used in these assays as a substrate because it is the single quorum-sensing molecule secreted by the wild-type *Chromobacterium violaceum* strain (McClean *et al.*, 1997). In this assay, the HHL degradation capacity of the ECs was tested against an LB medium background, which differed from the enrichment procedure. HHL degradation occurred during the first 24 h for most of the ECs, with the degradation rate subsequently reduced.

To verify the degradation of the HAI-1 autoinducer produced by *V. harveyi*, the resting cells of ECs were inoculated into the cell-free washwaters obtained from the strains BB120 and JMH606. The use of cell-free washwater is to assure the absence of further autoinducer production by the *V. harveyi* cells after a predetermined incubation period. Interestingly, the three ECs were capable of degrading the HAI-1 autoinducer from *V. harveyi*, although this molecule was not incorporated into the growth medium used to obtain these cultures.

One of the ECs, EC5, was evaluated for its effect *in vivo*, using gnotobiotically grown *Brachionus* as a test model. The autoinducers HAI-1 and AI-2 are responsible for the growth-retarding (GR) effect of *V. harveyi* strain BB120 towards *Brachionus* (Tinh *et al.*, 2007). EC5 could only neutralize the GR effect of the mutants MM30 and BB886 in which HAI-1 and CAI-1 are functional, but not that of the mutants BB152 and BB170 in which AI-2 and CAI-1 are functional. Hence, the results of both *in vitro* and *in vivo* experiments show that EC5 is capable of degrading the *V. harveyi* HAI-1 autoinducer.

In conclusion, the ECs isolated in this study effectively degraded HHL and *V. harveyi* HAI-1 signaling molecules, as revealed in the degradation kinetic assay and the analysis using *V. harveyi* washwater. Under *in vivo* conditions, it is likely that only the degradation of HAI-1 takes place. Consequently, AHL-degrading ECs may not be effective against pathogens that regulate their virulence via a multichannel quorum-sensing system, such as *V. harveyi*. In the future, these ECs should be tested for their positive effect on survival on fish larvae that are predating on *Brachionus*. The currently described MCs could be combined with AI-2-degrading bacteria, if such bacteria could be isolated. It is also suggested to characterize individual strains from the ECs (in relation to AHL degradation kinetics and enzymes involved). This could facilitate the reconstitution of microbial communities with optimal degradation kinetics but also with optimal additional characteristics such as the capacity to colonize the fish gut.

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