N-acylhomoserine lactone-degrading bacteria isolated from hatchery bivalve larval cultures

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Abstract

Quorum sensing (QS) systems, which depend on N-acylhomoserine lactone (AHL) signal molecules, mediate the production of virulence factors in many pathogenic microorganisms. One hundred and forty-six bacterial strains, isolated from a bivalve hatchery, were screened for their capacity to degrade five synthetic AHLs [N-butyryl-dl-homoserine lactone (C_4-HSL), N-hexanoyl-dl-homoserine lactone (C_6-HSL), N-octanoyl-dl-homoserine lactone (C_8-HSL), N-decanoyl-dl-homoserine lactone (C_{10}-HSL) and N-dodecanoyl-dl-homoserine lactone (C_{12}-HSL)] using well diffusion agar-plate assays with three biosensors, Chromobacterium violaceum CV026, C. violaceum VIR07 and Agrobacterium tumefaciens NTL4 (pZLR4). The results of these assays led to our choosing four strains (PP2-67, PP2-459, PP2-644 and PP2-663) that were able to degrade all five synthetic AHLs, thus showing a wide spectrum of quorum quenching (QQ) activity. We subsequently confirmed and measured the QQ activity of the four strains by high-performance liquid chromatography plus mass-spectrometry analysis (HPLC–MS). One of the strains which showed the highest AHL-degrading activity, PP2-459, identified as being a member of the genus Thalassomonas was chosen for further study. Finally, using thin-layer chromatography (TLC), we went on to confirm this strain's capacity to degrade the AHLs produced by other non-pathogenic and pathogenic bacteria not taxonomically related.

1. Introduction

Bacterial diseases in bivalve hatcheries are a considerable limiting factor in commercial aquaculture and result in substantial economic losses. For many years treatment with antibiotics has been the only viable strategy to tackle the problem. Nevertheless, their use does nothing to guarantee the ultimate success of larval cultures and their overuse has resulted in the development of resistance. The seriousness of the situation has recently promoted the exploration of novel strategies to control marine pathogens, but only some of them, such as the use of probiotics, are applicable to bivalve aquaculture (Defoirdt et al. 2007a, 2011a; Prado et al. 2010; Verschuere et al. 2000). One of the most promising alternatives is based on the inhibition of the expression of virulence genes, regulated in many aquaculture pathogens by bacterial cell-to-cell signalling, known as quorum sensing (QS) (Bjarnsholt et al. 2011; Dong et al. 2007; González and Keshavan 2006; Natrah et al. 2011). QS is a population-density-dependent gene-expression mechanism which involves the production of signal molecules known as autoinducers, a ubiquitous phenomenon in bacteria (González and Marketon 2003). The most thoroughly characterized Gram-negative, bacterial intraspecific autoinducers are N-acylhomoserine lactones (AHLs), which have been reported to accumulate in the culture medium as the density of the population increases, and on reaching a critical threshold concentration bind to an AHL-receptor protein belonging to the LuxR family of transcriptional regulators. The activated LuxR/AHL complex then binds specific DNA sequences, resulting in the activation or repression of target genes, including in many cases the activation of important virulence phenotypes (Eberhard et al. 1991; Fuqua et al. 1994; Natrah et al. 2011).

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Table 1
Marine bacteria showing QQ activity against all synthetic AHLs tested.

Origin	No. of strains tested	Isolates showing QQ activity	C_4-HSL ($10\,\mu mol\,l^{-1}$)	C_6-HSL ($10\,\mu mol\,l^{-1}$)	C_8-HSL ($10\,\mu mol\,l^{-1}$)	C_{10}-HSL ($10\,\mu mol\,l^{-1}$)	C_{12}-HSL ($10\,\mu mol\,l^{-1}$)	Phylogenetically related species	% identity
Pod razor clam (*Ensis siliqua*)	15	PP2-67	+	+	+	+	+	*Alteromonas marina*	99.44
Carpet-shell clam (*Venerupis decussata*)	61	PP2-459	+	+	+	+	+	*Thalassomonas agariperforans*	96.65
		PP2-644	+	+	+	+	+	*Alteromonas stellipolaris*	99.93
Manila clam (*V. philippinarum*)	36	PP2-663	+	+	+	+	+	*Paracoccus homiensis*	97.43
Pullet carpet shell (*V. corrugata*)	27	–							
Groover razor clam (*Solen marginatus*)	7	–							

It has been reported that some aquatic organisms such as micro-algae, macro-algae, invertebrates and also other bacteria have the potential to disrupt QS by means of various different mechanisms (Natrah et al. 2011). One such mechanism involves the production of compounds that interfere with the detection of signal molecules; these compounds, known as quorum sensing inhibitors (QSIs), were first described in the red marine alga Delisea pulchra. This alga synthesizes halogenated furanones, which protect both fish and shrimp from vibriosis (Givskov et al. 1996; Rasch et al. 2004). The same mechanism that inhibits QS in aquaculture pathogens has been described in the marine bacterium Halobacillus salinus (Teasdale et al. 2008). A second strategy, known as quorum quenching (QQ), is the enzymatic inactivation of AHLs by the production of acylases or lactonases, as described elsewhere with reference to the fish pathogen Tenacibaculum maritimum (Defoird et al. 2011b; Romero et al. 2010, 2011; Tait et al. 2005). Thus, the use of AHL-degrading bacteria have obtained success when cultures from the intestinal tract of healthy shrimp and fish enriched in AHL-degrading enzymes increase the survival rate of turbot larvae (Scophthalmus maximus) (Tinh et al. 2008) and of giant freshwater prawns (Macrobrachium rosenbergii) (Nhan et al. 2010).

Within this context we have studied the capacity of a number of marine bacteria isolated from a bivalve hatchery to disrupt AHL mediated QS systems in bivalve pathogens.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The 146 strains tested in this study were isolated from successful larval cultures of different bivalve species at a bivalve hatchery in Galicia (NW Spain) and belong to the culture collection of the research group of the University of Santiago de Compostela involved in this work (Table 1). They are all Gram-negative rods with respiratory metabolism and grow best at about 2.5–3% NaCl (w/v), classified according to Kushner and Kamekura (1988) as marine bacteria.

These 146 marine bacteria and Vibrio anguillarum ATCC 19264^T were routinely cultured at 20◦C in marine broth (MB, Difco). Halomonas anticariensis FP35^T (CECT 5854^T) (Martínez-Cánovas et al. 2004) was cultured at 32◦C in MY medium (3 g malt extract, 3 g yeast extract, 10 g glucose and 5 g peptone per litre) (Haynes et al. 1955; Moraine and Rogovin 1966) modified with a balanced mixture of sea salts (Rodríguez-Valera et al. 1981). Agrobacterium tumefaciens NTL4 (pZLR4) (Shaw et al. 1997; Chilton et al. 1974) was cultured at 32◦C in Luria Bertani (LB) medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre) supplemented with 2.5 mmol l^{-1} CaCl and 2.5 mmol l^{-1} MgSO (LB/MC), in MGM minimal medium (11 g Na_2HPO_4, 3 g KH_2PO_4, 0.5 g NaCl, 1 g glutamate, 10 g mannitol, 1 mg biotin, 27.8 mg $CaCl_2$ and 246 mg $MgSO_4$ per litre) or AB medium (3 g K_2HPO_4, 1 g $Na_2H_2PO_4$, 1 g NH_4Cl, 0.3 g $MgSO_4{\cdot}7H_2O$, 0.15 g KCl, 0.01 g $CaCl_2$, 0.0025 g $FeSO_4{\cdot}7H_2O$ and 5 g glucose per litre) containing 50 g gentamycin ml^{-1}. Chromobacterium violaceum CV026 (McClean et al. 1997) and C. violaceum VIR07 (Morohoshi et al. 2008) were grown at 32◦C in LB medium supplemented with kanamycin (50 g ml^{-1}).

2.2. Screening for AHL-degradation activity using synthetic AHLs

The potential QQ activity of the 146 isolates was tested by well diffusion agar-plate assays as described elsewhere (Romero et al. 2011). Briefly, synthetic AHLs were added at 10 mol l^{-1} to 500 l of an overnight culture of each marine bacterial strain in MB medium and incubated for 24 h at 20◦C and 150 rpm rotary shaking. The same quantities of AHLs were added to 500 l of cell free MB media and incubated for 24 h at 20◦C as negative controls. In parallel, each marine bacterial strain was also cultivated in MB medium with no exogenous AHLs added for 24 h at 20◦C to detect its own production of AHLs. The AHLs in each overnight culture were detected on agar plates covered with 5 ml of semi-solid LB agar containing 500 l of overnight cultures of indicator strains.

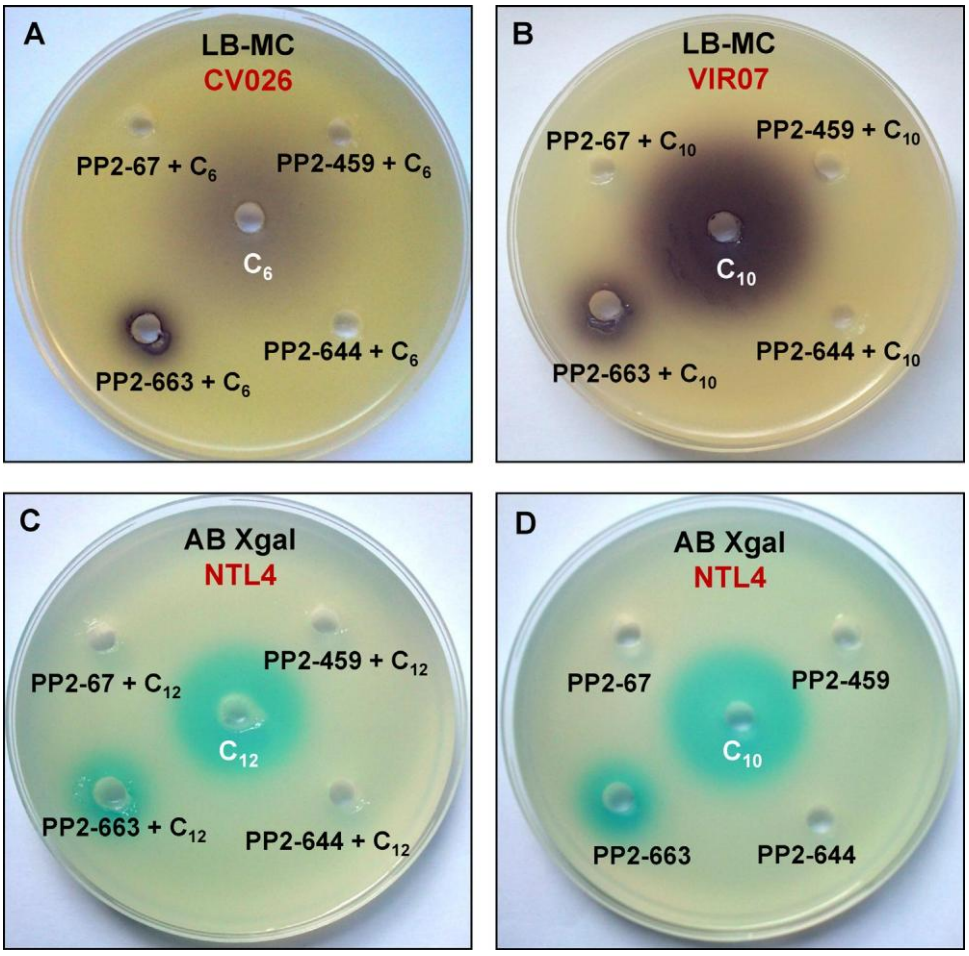


Fig. 1. Well diffusion agar-plate assay to detect AHL using indicator strains. Degradation of synthetic AHLs (10moll-[1]) after 24h incubation was revealed by the suppression of violacein production in Chromobacterium violaceum CV026 (A) and C. violaceum VIR07 (B) and absence of blue colour in Agrobacterium tumefaciens NTL4 (pZLR4) (C) compared to the synthetic AHL compounds C_6-HSL (10moll-[1]), C_{10}-HSL (10moll-[1]) and C_{12}-HSL (10moll-[1]) used as negative controls. Detection of the production of AHLs in the four AHL-degrading strains using Agrobacterium tumefaciens NTL4 (pZLR4) as biosensor (D).

LB/MC agar plates were used for C. violaceum CV026 and C. violaceum VIR07 indicator strains and AB agar plates supplemented with 80 g of 5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside (X-Gal) per ml were used for A. tumefaciens NTL4 (pZLR4). Once the plates have solidified, 100 l aliquots of culture supernatants were put in wells hollowed into the surface of the medium. The plates were then incubated at 32◦C for 24 h to check for the appearance of a coloured halo around the wells. The strains that showed positive activity against all the AHLs tested were those that did not activate the indicator strains and were selected for further studies. We referred them as AHL-degrading strains.

The synthetic AHLs tested were the following: C_4-HSL (N-butyryl-dl-homoserine lactone), C_6-HSL (N-hexanoyl-dlhomoserine lactone), C_8-HSL (N-octanoyl-dl-homoserine lactone), C_{10}-HSL (N-decanoyl-dl-homoserine lactone) and C_{12}-HSL (N-dodecanoyl-dl-homoserine lactone) (Sigma®).

2.3. Confirmation of AHL degradation activity by HPLC–MS

To confirm the quorum quenching activity revealed in the well diffusion agar-plate assays by the AHL-degrading strains we used high-performance liquid chromatography plus mass spectrometry analysis (HPLC–MS) (Romero et al. 2011). C_4-HSL, C_6-HSL, C_8-HSL or C_{10}-HSL were added to 500 l samples of overnight cultures of AHL-degrading strains in MB medium to a final concentration of 10moll-[1] and incubated for a further 24 h at 20◦C and 150 rpm rotary shaking. The cultures were then centrifuged at 2000×g for 5 min, extracted twice with an equal volume of ethyl-acetate, evaporated under nitrogen flux at 50◦C and resuspended in 400 l of acetonitrile for HPLC–MS analysis and quantification. As negative controls, AHLs (10moll-[1]) were added to fresh MB medium and processed and extracted in the same way.

Analyses were carried out with a HPLC 1100 series (Agilent) equipped with a C8 pre-column (2.1 mm×12.5 mm, 5 m particle size) and a ZORBAX Eclipse XDB-C18 2.1 mm × 150 mm column (5 m particle size) kept at 45◦C. The mobile phase was composed of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B) (Ortori et al. 2007). The elution conditions are described by Romero et al. (2011). MS experiments were conducted on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, CA) equipped with a Turbolon source using positive ion electrospray, multiple-reaction monitoring (MRM) mode. The MRM signals were used to generate relative quantification information by comparison with a calibration curve constructed for molecular-ion abundance, using each of the appropriate AHL synthetic standards (Milton et al. 2001).

2.4. Identification of the AHL degradation enzymatic activity

To determine whether the QQ activity was related or not to hydrolysis of the lactone ring caused by lactonases (Yates et al. 2002; Romero et al. 2011) parallel extractions of

AHL were carried out under acidic conditions following the same procedure described above. In this case we chose two AHLs with medium and long fatty-acid chains (C_6-HSL or C_{10}-HSL), which were added to 500 l of overnight cultures of the marine strains in MB medium to a final concentration of 10 mol l^{-1} and incubated for a fur-

ther 24 h at 20 °C and 150 rpm rotary shaking. The cultures were then centrifuged at 2000 × g for 5 min. Then, the supernatant was acidified with HCl to pH 2 and incubated for 24 h at 20 °C and 200 rpm rotary shaking before extracting the AHLs. Finally, the remaining AHLs were subjected to analyze and quantify by HPLC–MS.

2.5. Study of QQ activity upon crude AHL extracts from non-pathogenic and pathogenic bacteria

AHL molecules were collected in crude extracts from 5 ml of cultures of non-pathogenic (H. anticariensis FP35^T) and pathogenic

(V. anguillarum ATCC 19264^T) bacteria following the technique

described in previous studies (Llamas et al. 2005; Marketon et al. 2002). Briefly, 5 l of each crude extracts were added to 5000 l of overnight cultures diluted 1:100 of an AHL-degrading strain in MB medium and incubated for 24 h at 20 °C and 150 rpm rotary shaking. The same quantity of crude extracts were added to 5000 l of cell-free MB medium and incubated for 24 h at 20 °C as negative controls. The remaining AHLs were extracted twice with equal volumes of dichloromethane, dried and finally suspended in 20 l of 70% (v/v) methanol. To detect AHLs an overnight culture of one of the AHL indicator strain A. tumefaciens NTL4 (pZLR4) was diluted 1:100 in 5 ml of the corresponding medium and poured onto AB medium supplemented with 80 g X-Gal per ml. Once the plates were dry, paper disks 5 mm in diameter were placed onto them and the 20 l of the AHL samples applied. The assay plates were incubated overnight at 32 °C to allow the indicator organisms to grow and surround the paper disks with blue haloes (Tahrioui et al. 2013). Then, to characterize the patterns of the remaining AHLs of each culture the samples (20 l) were subjected to analytical and preparative thin-layer chromatography (TLC) following the procedure described below (Llamas et al. 2005; Marketon et al. 2002).

2.6. AHL analysis of the AHL-degrading strains by thin-layer chromatogragphy

To characterize the AHLs the samples were subjected to analytical and preparative thin-layer chromatography (TLC) as described in our previous studies (Marketon et al. 2002; Llamas et al. 2005). Briefly, 5 ml cultures were grown until the early stationary phase (optical density of approximately 2.8 at 600 nm) and then extracted twice with equal volumes of dichloromethane. The extracts were dried and suspended in 20 l of 70% (v/v) methanol. AHL samples and standards were spotted onto a TLC plate and developed with 70% (v/v) methanol in water. The plate was air-dried and overlaid with top agar containing the A. tumefaciens NTL4 (pZLR4) indicator strain before being incubated at 32 °C. For the A. tumefaciens NTL4 (pZLR4) overlay, a 6–8 h culture in MGM medium was mixed with an equal volume of fresh medium, 1.5% (w/v) Bacto Agar and 80 g of X-Gal per ml.

2.7. Identification of the marine strains

Genomic DNA was extracted as described elsewhere (Prado et al. 2005). A >1400 bp fragment of the 16S rRNA gene was amplified from the extracted DNA using bacterial universal primers specific to the 16S rRNA gene (27F and 1510R) (Lane 1991). The 16S rRNA genes were amplified using Ready To Go PCR beads (Amersham Pharmacia Biotech). The resulting sequences were analyzed with the DNAstar Lasergene Seqman program (Madison, WI, USA). The comparative analysis was carried out in the EzTaxon-e Server (http://eztaxon-e.ezbiocloud.net/; Kim et al. 2012) and BLAST (Altschul et al. 1997). Neighbour joining (NJ) (Saitou and Nei 1987) was done using MEGA4 software (Tamura et al. 2007) using the Kimura-2-parameters model using 1000 bootstrap replicates in both cases.

3. Results

3.1. Choice of AHL-degrading strains

The 146 strains studied in this work were chosen from a collection of isolates commonly associated with bivalve larval cultures with high survival rates (Table 1). Moreover, all of these marine bacteria have respiratory metabolism to rule out the most frequent pathogens associated with aquaculture, which are mainly fermentative bacteria belonging to the genus Vibrio.

We undertook an initial screening of the 146 isolates by well diffusion agar-plate assays in the presence of five commercial AHLs with fatty-acid chains of different lengths (C_4-HSL C_6-HSL, C_8-HSL, C_{10}-HSL and C_{12}-HSL) (see Section 2). The AHLs remaining in the media were detected using the indicator strains C. violaceum CV026 (Fig. 1A) which produces violacein in the presence of AHLs with short and medium fatty-acid chains, C. violaceum VIR07 (Fig. 1B) which originates a violacein pigment in the presence of C_{10}-HSL and A. tumefaciens NTL4 (pZRL4), which turns blue in the presence of AHLs with medium and long fatty-acid chains (Fig. 1C). We found that four out of the 146 strains tested (PP2-67, PP2-459, PP2-644 and PP2-663) were able to degrade all the AHLs tested; suppressing the activation of the three indicator strains. These experiments have been carried out three times and the same results have been obtained. The four bacteria were chosen as AHL-degrading strains for subsequent studies (Table 1).

A parallel analysis to determine the production of AHLs of the four AHL-degrading strains was done using the well diffusion agar plate assay (see Section 2). Our results indicated that only the PP2-663 strain synthesized AHLs which activated the A. tumefaciens NTL4 (pZLR4) (Fig. 1D) and the C. violaceum indicator strains (data not shown).

To confirm and characterize the nature of the AHLs produced by PP2-663 strain, we analyzed the culture extracts from 5 ml of an overnight culture using TLC in combination with the biosensor A. tumefaciens NTL4 (pZLR4). The AHL pattern from strain PP2-663 contains 2 spots with mobilities similar to those of the C_6-HSL and C_{10}-HSL standards (Fig. 2B, lane 3). This analysis confirmed the production of AHLs in the AHL-degrading strain PP2-663 indicating that it has also a quorum sensing system AHL dependent.

3.2. Confirmation of AHL-degradation activity by HPLC–MS

We applied HPLC–MS analyses to further confirm the degradation of AHLs by the four selected AHL-degrading strains. All four strains were able to reduce significantly the concentration of all the AHLs tested, showing more than 60% of QQ activity in all of cases. This capacity was higher against C_8-HSL and C_{10}-HSL (Fig. 3) for all the strains. To ascertain whether QQ activity was due to a lactonase enzyme we acidified the supernatants of the two cultures containing C_6-HSL and C_{10}-HSL (samples referred as C_6-HSL Ac and C_{10}-HSL Ac in the Fig. 3). This acidification allows the lactone ring to restructure itself in the event of its having been previously opened by a lactonase (Yates et al. 2002). In neither case did the AHL concentrations recover to any significant extent in comparison to the negative control (referred as MB red bar in the Fig. 3), indicating that their degradation was due to the activity of some other enzyme.

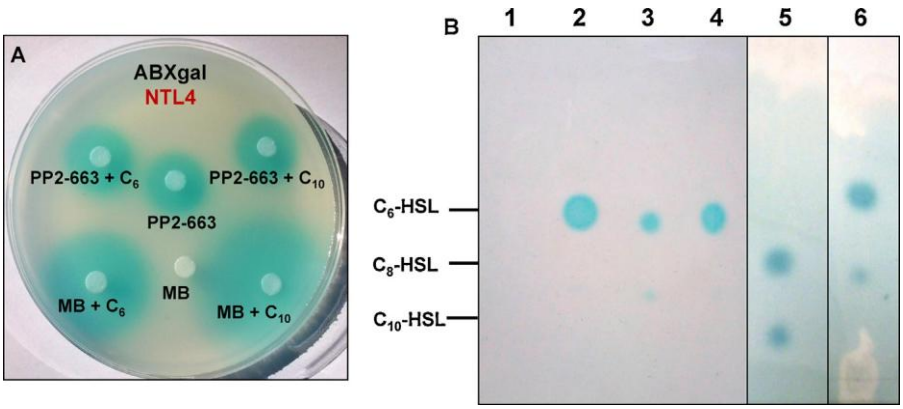


Fig. 2. AHL production and AHL-degrading activity by strain PP2-663. (A) AHLs extracted from 5 ml cultures of strain PP2-663 with or without AHLs added were visualized on an agar plate assay by means of the indicator strain A. tumefaciens NTL4 (pZLR4). Cell-free MB medium samples under similar culture conditions were used as controls. (B) TLC analysis of AHLs in crude extracts from 5 ml cultures of strain PP2-663 (lane 3) and strain PP2-663 with C_6-HSL (lane 4) and C_8-HSL + C_{10}-HSL (lane 6) after 24 h exposure. The AHLs were visualized by TLC overlaid with the Agrobacterium tumefaciens NTL4 (pZLR4) indicator strain. AHLs extracted from 5 ml cell-free MB medium (lane 1) and cell-free medium with C_6-HSL (lane 2) and C_8-HSL + C_{10}-HSL (lane 5) were used as negative controls. Synthetic AHL compounds used as references: C_6-HSL (804 pmol);
C_8-HSL (31.6 pmol); C_{10}-HSL (2 pmol).

Eventually we chose strain PP2-459 for further studies because it showed the strongest QQ activity against all of the AHLs tested. As it is shown in Fig. 3, the strain PP2-459 eliminated the AHLs assayed showing an activity close to 100% in all assays carried out. Moreover, this strain does not produce any detectable AHLs signal molecules (Fig. 1D) which could interfere in the quorum quenching results.

Regarding to the strain PP2-663, it may have also a high QQ activity as PP2-459 because the remaining AHLs showed in their samples (Fig. 3) could be associated to its own AHLs (Figs. 1D and 2B, lane 3). To determine whether strain PP2-663 degraded its own AHLs in the quorum quenching assays, AHLs were detected from 5 ml cultures with and without exogenous AHLs added (see Section 2). Cell-free MB medium with and without AHLs added were used as

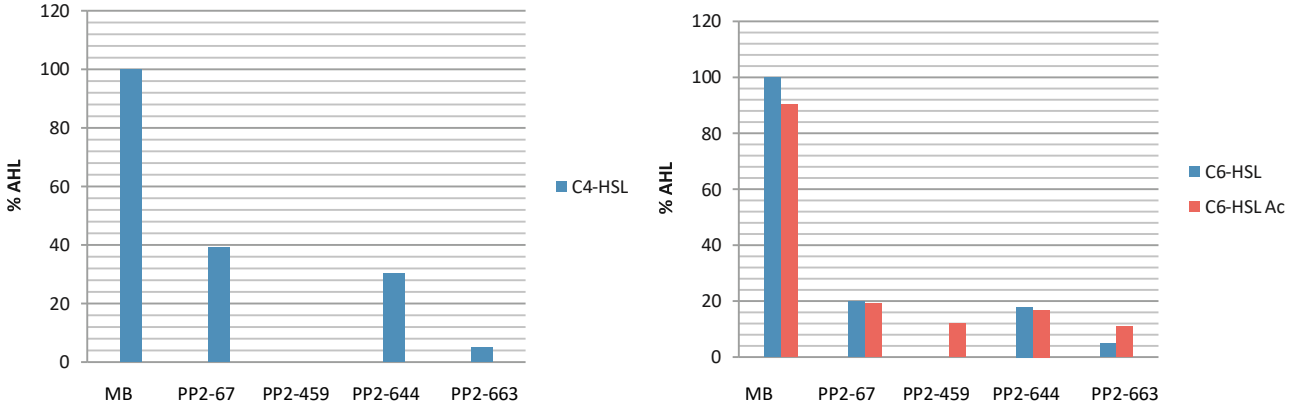
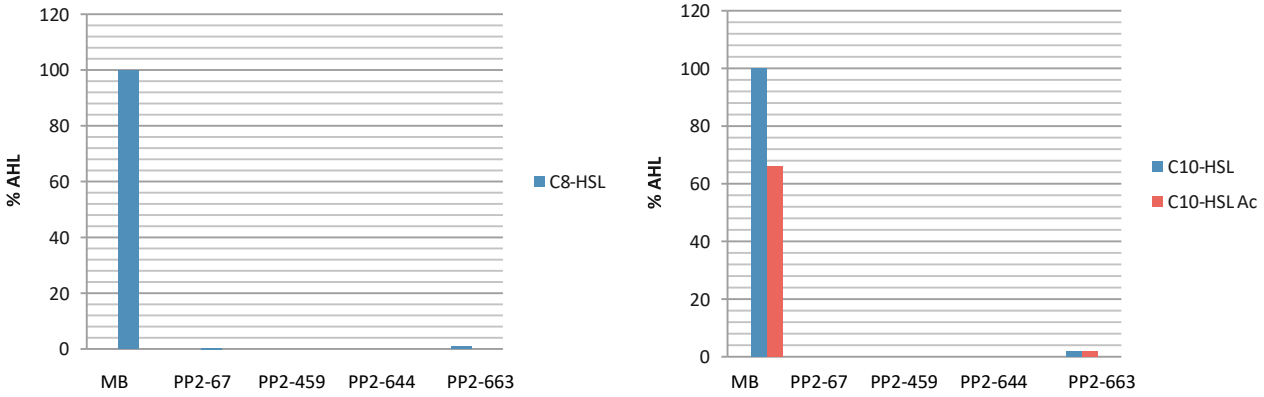


Fig. 3. HPLC–MS measurements of C_4-HSL, C_6-HSL, C_8-HSL and C_{10}-HSL in the cell-free culture media of 4 AHL-degrading strains after 24 h. Initial AHL concentration was 10 moll-^{1}. Spent culture media containing C -HSL and C -HSL were acidified to pH 2 in order to allow the recovery of the lactone ring after lactonolysis. C -HSL Ac and C_{10}-HSL Ac corresponding to samples in which the supernatants have been acidified.



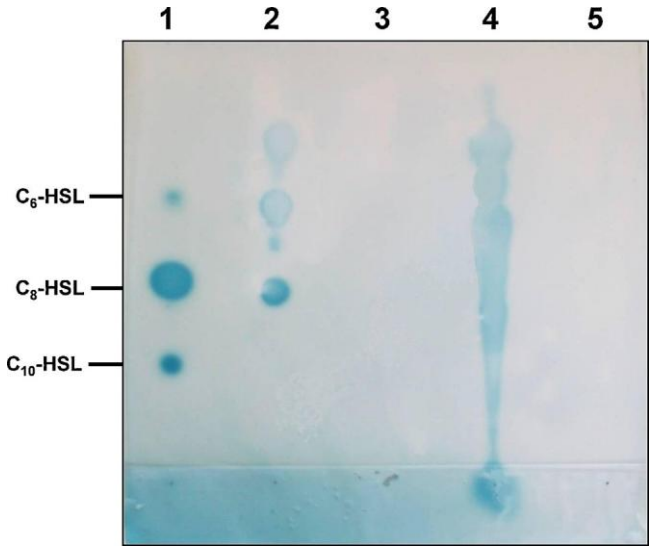


Fig. 4. Detection of AHL-degradation activity by strain PP2-459 in AHLs in crude extracts from cultures of H. anticariensis FP35^T (lane 3) and V. anguillarum ATCC 19264^T (lane 5) after 24 h exposure. The remaining AHLs were visualized by TLC overlaid with the Agrobacterium tumefaciens NTL4 (pZLR4) indicator strain. AHLs extracted from 5 ml of H. anticariensis FP35^T (lane 2) and V. anguillarum ATCC 19264^T (lane 4) cultures were used as negative controls. Lane 1, synthetic AHL compounds used as references: C_6-HSL (804 pmol); C_8-HSL (31.6 pmol); C_{10}-HSL (2 pmol).

controls (Fig. 2A). AHLs profiles of each sample were also analyzed and compared by TLC (Fig. 2B). The results indicated that strain PP2663 showed quorum quenching activity against the AHLs added to the medium (Fig. 2). Strain PP2-663 activated the indicator A. tumefaciens NTL4 (pZLR4) in similar extension in the presence of exogenous AHLs (Fig. 2A). Moreover, the AHL profile corresponding to culture of strain PP2-663 (Fig. 2B, lane 3) was similar to those from the cultures of strain PP2-663 with exogenous AHLs added (Fig. 2B, lanes 4 and 6). The TLC analysis of the different crude extracts has been carried out three times and the same results have been obtained.

3.3. Study of QQ activity of strain PP2-459 upon crude AHL extracts from non-pathogenic and pathogenic bacteria

To ascertain whether strain PP2-459 has the future potential to be used in vivo to disrupt the QS systems of other bacteria we assayed its capacity to eliminate AHLs produced by other microorganisms in vitro. To this end we assessed the QQ activity of strain PP2-459 against crude extracts of H. anticariensis FP35^T, a moderately halophilic bacterium that produces C_4-HSL, C_6-HSL, C_8-HSL and C_{12}-HSL, and the aquaculture pathogen V. anguillarum ATCC 19264^T, which produces 3-oxo-C_{10}-HSL and C_{10}-HSL as dominant signals. The remaining quantities of AHLs were firstly tested by agar-plate assays and then analyzed by TLC (see Section 2). As indicator organisms we used A. tumefaciens NTL4 (pZLR4) to detect medium- and long-chain AHLs (Fig. 4) and C. violaceum CV026, which is activated in the presence of short- and medium-chain AHLs (data not shown). No detectable levels of any AHLs from H. anticariensis FP35^T or V. anguillarum ATCC 19264^T were found.

3.4. Identification of the marine strains

To identify the 4 selected AHL-degrading bacteria their 16S rRNA gene sequences were used in a BLAST search and compared to the reference 16S rRNA gene sequences available in the GenBank database. As shown in Table 2, the strains were closely related to species of the genera Alteromonas (2 strains), Thalassomonas (1 strain) and Paracoccus (1 strain). Strain PP2-459, belonging to the genus Thalassomonas, could in fact be a new species, due to its low percentage of identity with its most closely related species. Fig. 5 shows the results of our phylogenetic analysis of strain PP2459 and the related members of the Colwelliaceae family according to the neighbour-joining algorithm.

4. Discussion

Bacterial infections are one of the most important set-backs to occur in the aquaculture of bivalves, particularly in the hatchery. Severe economic loss can result from such diseases wiping out complete batches of shellfish. The routine use of antibiotics as a prophylactic measure has inevitably led to a loss of effectiveness on the part of these drugs and the appearance of resistance. Thus new strategies are currently being investigated to help in the fight against bacterial diseases. Some of them focus on eliminating the pathogenic bacteria themselves (Nakai and Park 2002; Vinod et al. 2006; Yamaichi et al. 2009) whilst others attempt to inhibit their growth rather than killing them (Defoirdt et al. 2006, 2007b). A recent novel approach to solving this problem is through the disruption of bacterial cell-to-cell communication, known as quorum sensing, which regulates the expression of virulence genes in many aquaculture pathogens (Bjarnsholt et al. 2011; Dong et al. 2000, 2007; Natrah et al. 2011). In accordance with this approach we have studied a collection of 146 isolates commonly associated with bivalve larval cultures with high survival rates. We chose four strains on the basis of their QQ activity against five synthetic AHLs, as demonstrated by well diffusion agar-plate assays with three biosensors (Fig. 1). We have successfully used this procedure in the past to isolate culturable bacteria with QQ activity from dense marine microbial communities (Romero et al. 2011). The percentage of isolates found in this work with wide-spectrum of QQ activity was high (2.7%) and related to those found in previous studies, in which it has also been demonstrated that AHL-degrading bacteria are more frequent in marine than terrestrial environments (Dong and Zhang 2005; Romero et al. 2008, 2010).

It has been reported that QS systems may be disrupted either by chemical or enzymatic signal degradation (Yates et al. 2002; Dong et al. 2007) or by releasing AHL mimic molecules that are capable of blocking the LuxR-type signal receptor (Manefield et al. 2002). To understand more about the workings of the QS inhibition mechanisms shown in the well diffusion agar-plate assays we confirmed and measured the degree of AHL degradation using HPLC–MS (Fig. 3). The four chosen bacteria had the capacity to eliminate all of the AHLs tested although their QQ activity was more effective against long- and medium-chain AHLs than to short chain AHLs. Strains PP2-459 and PP2-663 proved to have almost 100% QQ activity in all the assays conducted. The AHL remaining shown in the HPLC–MS analysis from the samples of the strain PP2-663 added of C_6-HSL and C_{10}-HSL (Fig. 3) could be corresponded to its own AHLs which have mobilities similar to those of the C_6-HSL and C_{10}-HSL standards by TLC analysis (Fig. 2B, lane 3). Thus, although PP2-663 had a important quorum quenching activity, it is seemed that its own AHLs were not degraded (Fig. 2). We preferred to choose PP2-459 strain and not PP2-663 strain for further studies to avoid the interference of its QS system in the QQ results.

The lactone ring of AHL is unstable under alkaline conditions and temperatures above 37 °C, which result in lactonolysis (Yates et al. 2002). Since the recovery of the AHL concentration deriving from the acidification of the cultures was insignificant, the enzymatic degradation activity shown by our four AHL-degrading strains may well be due to an enzyme other than a lactonase. As far as this is concerned, two of the strains chosen belong to the

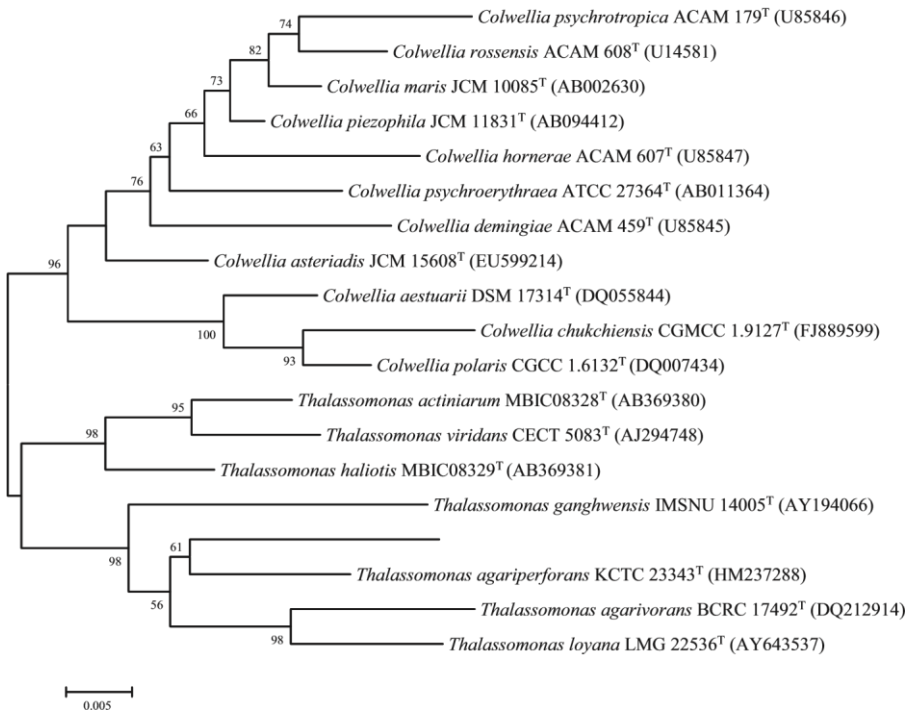


Fig. 5. Unrooted phylogenetic tree deriving from a neighbour-joining analysis of the 16S rRNA gene sequences of strain PP2-459 and related members of the Colwelliaceae family. GenBank accession numbers are given in parenthesis. Numbers above branch nodes are bootstrap values obtained with 1000 resamplings. Bar, 0.005 nucleotide substitutions per position.

genus Alteromonas, the genome of which has been found to contain a nucleotide sequence encoding a possible acylase (Romero et al. 2011). AHL-degrading enzymes are ubiquitous in both prokaryotes and eukaryotes and are highly specific against AHLs, in which they break down the signals and influence QS-regulated activity. Thus these bacteria may well produce these enzymes as a defence strategy against their competitors (Dong and Zhang 2005).

Since our efforts were directed towards disrupting QS systems in aquaculture pathogens, we tested the QQ activity of strain PP2459 against crude extracts of H. anticariensis FP35^T, a moderately

halophilic bacterium that produces a variety of AHLs such as C_4HSL, C_6-HSL, C_8-HSL and C_{12}-HSL (Llamas et al. 2005), and the aquaculture pathogen V. anguillarum ATCC 19264^T, which produces

3-oxo-C_{10}-HSL, C_{10}-HSL as dominant signals (Milton et al. 1997, 2001). In all cases AHL degradation was complete, indicating that strain PP2-459 can inactivate AHLs synthesized by other bacteria, including unrelated species.

A few studies related to the interference of QS systems in fish aquaculture, including turbot larvae (Tinh et al. 2008) and giant freshwater prawns (Nhan et al. 2010), have been undertaken with successful results. Nevertheless, no studies in bivalves appear to have been attempted yet. In order to take advantage of this novel strategy in bivalve aquaculture it is essential to have a better understanding of their pathogens' QS systems, and particularly to ascertain whether their virulence factors are QS-regulated.

As far as the bacterial strains chosen in this study are concerned, all of them belong to genera typical of marine environments. The predominant genus is Alteromonas, species of which have been reported to exert QQ activity in previous studies (Romero et al. 2011). Furthermore, the genome of Alteromonas sp. BCw156 contains a QQ sequence.

Among the active isolates only strain PP2-459 (Thalassomonas sp.) showed a low identity value with the other members of its genus (96.65%), suggesting that it may well be a new species. Interestingly, this strain showed the highest QQ activity in this study.

In conclusion, we have identified four marine bacteria that have the capacity to eliminate AHL signal molecules. According to our results, strain PP2-459 might make a good candidate to be used to attenuate the production of virulence factors by bivalve pathogens, although further assays in vivo are required to this end.

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