Quorum quenching in cultivable bacteria from dense marine coastal microbial communities

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Abstract

Acylhomoserine lactone (AHLs)-mediated quorum-sensing (QS) processes seem to be common in the marine environment and among marine pathogenic bacteria, but no data are available on the prevalence of bacteria capable of interfering with QS in the sea, a process that has been generally termed 'quorum quenching' (QQ). One hundred and sixty-six strains isolated from different marine dense microbial communities were screened for their ability to interfere with AHL activity. Twenty-four strains (14.4%) were able to eliminate or significantly reduce *N*-hexanoyl-L-homoserine lactone activity as detected by the biosensor strain *Chromobacterium violaceum* CV026, a much higher percentage than that reported for soil isolates, which reinforces the ecological role of QS and QQ in the marine environment. Among these, 15 strains were also able to inhibit *N*-decanoyl-L-homoserine lactone activity and all of them were confirmed to enzymatically inactivate the AHL signals by HPLC-MS. Active isolates belonged to nine different genera of prevalently or exclusively marine origin, including members of the *Alpha*- and *Gammaproteobacteria* (8), *Actinobacteria* (2), *Firmicutes* (4) and *Bacteroidetes* (1). Whether the high frequency and diversity of cultivable bacteria with QQ activity found in near-shore marine isolates reflects their prevalence among pelagic marine bacterial communities deserves further investigation in order to understand the ecological importance of AHL-mediated QS and QQ processes in the marine environment.

Introduction

Quorum sensing (QS) constitutes a bacterial communication system based on the production and secretion of small signal molecules called autoinducers that accumulate in the extracellular environment when high cell densities are reached (Fuqua *et al*., 1994). Once a threshold intracellular concentration is attained, the signalling molecule triggers synchronous expression of multiple genes in the population, regulating important biological functions such as plasmid conjugal transfer, motility, swarming, aggregation, luminescence, antibiotic biosynthesis, virulence, symbiosis, siderophore production or biofilm maintenance and differentiation (Swift *et al*., 2001; Waters & Bassler, 2005; Williams *et al*., 2007). The best characterized QS signalling system involves *N*-acylhomoserine lactones (AHLs), a family of molecules consisting of a homoserine lactone (HSL) ring which is *N*-acylated with a fatty acyl group at the a-position. These signals have been initially described as being exclusively produced by a relatively small number of *Proteobacteria* (Fuqua & Greenberg, 2002; Williams *et al*., 2007), but recently the production of these signals has been also reported for the colonial cyanobacterium *Gloeothece* (Sharif *et al*., 2008) and different marine *Bacteroidetes* (Huang *et al*., 2008; Romero *et al*., 2010) which may reinforce the role of QS systems in natural populations in the environment.

The mechanisms causing the inactivation of QS communication systems have been generally termed as 'quorum quenching' (QQ) (Dong *et al*., 2001, 2007), although some authors prefer to restrict this term to the enzymatic degradation of the AHL signals (Kjelleberg *et al*., 2008). QQ mechanisms described so far in nature include the production of inhibitors or antagonists of signal reception by marine algae and invertebrates (Givskov *et al*., 1996; Kim

et al., 2007; Skindersoe *et al*., 2008), terrestrial plants (Gao *et al*., 2003) and bacteria (Teasdale *et al*., 2009) and the enzymatic inactivation of signals found in mammalian cells (Xu *et al*., 2003; Chun *et al*., 2004), plants (Delalande *et al*., 2005) and in different bacteria (Dong *et al*., 2007; Uroz *et al*., 2009). Two groups of AHL degradation enzymes have been identified so far. The acylases that cleave the AHL amide bond generating the corresponding free fatty acid and HSL ring (Romero *et al*., 2008), and the lactonases that hydrolyse the HSL ring of the AHL molecule to produce corresponding acyl homoserines (Dong *et al*., 2007). An additional mechanism of inactivation of AHLs based on the enzymatic production of the oxidized halogen HOBr, that reacts specifically with 3-oxo-acyl HSLs, has been described for the brown alga *Laminaria digitata* (Borchardt *et al*., 2001). QQ enzymes have been described to be able to catabolize other important biological compounds (Park *et al*., 2005; Khan & Farrand, 2009) which may increase their ecological significance.

Although QS and QQ processes were both discovered in marine organisms (Nealson *et al*., 1970; Givskov *et al*., 1996) little attention has been paid so far to their ecological significance in the marine environment. Evidence is beginning to accumulate on the importance of AHL-mediated QS processes in the sea. Almost 60% of the *Alphaproteobacteria* isolated from different marine samples, including free-living and algae-associated strains, were able to activate AHL sensor strains, although the presence of AHLs could not be confirmed in all of them (Wagner-Dobler *et al*., 2005). A high percentage of AHL-producing strains was also reported for isolates from subtidal biofilms (Huang *et al*., 2008) and sponges (Mohamed *et al*., 2008). The low bacterial population encountered in the open sea and the low chemical stability of AHLs in seawater have lead to the suggestion that the AHL-mediated QS activity may be concentrated in specific microhabitats such as biofilms, marine snow and eukaryotic niches (Cicirelli *et al*., 2008; Hmelo & Van Mooy, 2009). AHLs also seem to have an important role in the eukaryotic–prokaryotic interactions in the marine environment (Tait *et al*., 2005, 2009; Huang *et al*., 2007; Weinberger *et al*., 2007). Moreover, the production of AHLs is common among marine fish pathogenic bacteria, controlling the expression of important virulence factors (Defoirdt *et al*., 2007).

On the contrary, information on QQ processes in the marine environment is still scarce; although some indirect evidence exists that indicate that these phenomena could be frequent. AHL signals degrade more rapidly in natural seawater than in artificial seawater, an observation that has been related to the presence of QQ enzyme activity (Hmelo & Van Mooy, 2009). Moreover, the addition of AHLs to the culture media enhanced the cultivation efficiency in marine natural populations, which may indicate that AHLs are readily available in the marine environment (Bruns *et al*., 2002). Despite this, no studies have been done either on the presence of QQ activity among marine bacteria nor on its ecological significance. In contrast, many studies describe the presence of QQ activity in soil samples, including soil metagenome screenings (Williamson *et al*., 2005; Riaz *et al*., 2008; Schipper *et al*., 2009) that have allowed the isolation of several strains/genes capable to degrade AHLs and the establishment of the prevalence of QQ processes in soil samples (Leadbetter & Greenberg, 2000; Park *et al*., 2003; Uroz *et al*., 2003; Wang & Leadbetter, 2005). Recently, Tinh *et al*. (2007) have successfully applied AHL enrichment cultures to demonstrate the capability of bacterial communities from the gut of farmed shrimp to degrade the signals, although neither the marine bacteria responsible for that activity have been identified nor the type of activity characterized.

The aim of this work was to study the presence and prevalence of marine cultivable bacteria capable of interfering with AHL-mediated QS systems in different marine samples. In a first approach, we studied the presence of QQ activity among isolates from dense coastal bacterial communities. The analysis was carried out with isolates from three different marine samples characterized by their high organic and microbiological load: the marine algae *Fucus vesiculosus*, a diatom-dominated loosely attached biofilm from a filtered seawater reservoir tank and the sediment from an inland fish culture tank. The enrichment culture technique in which AHLs are the only source of carbon and/or nitrogen used in many studies (Leadbetter & Greenberg, 2000; Park *et al*., 2003; Uroz *et al*., 2003; Tinh *et al*., 2007) was avoided, because it does not allow the establishment of the prevalence of QQ activity in a particular environment.

Materials and methods

Bacterial quantification and strain isolation

Three different samples were taken with sterile laboratory material from different marine origins in June 2007 for bacterial isolation and screening for QQ activity against AHLs. Sample sites were selected for its high microbial density. Two of the samples were obtained in a natural coastal environment in Illa de Arousa (421.33′.45.32″N 81.53′.08.23″W Spain): one from the diatom-dominated biofilm loosely attached to the wall of an outdoor concrete tank (25 000 L), that was used as reservoir of filtered seawater for an aquaculture facility and the other from the brown seaweed *F. vesiculosus* harvested from the rocks of the intertidal zone close to it. The third sample was obtained from the sedimentation tank of a closed marine fish culture circuit at the University of Santiago (Spain).

The integrity of QS systems has been demonstrated to be more important in nutrient-deficient conditions (Diggle *et al*., 2007) and therefore we expected that QQ activity would be higher among bacteria being able to grow in poor nutrient culture media. On the basis of that hypothesis both rich and oligotrophic culture media were used for the bacterial isolation. Rich media included tryptone soy agar 1% NaCl (TSA-I) and marine agar (MA) suitable for eutrophic bacteria and low organic formulations included MA diluted 1/100 with seawater (MA 1/100), filtered auto-claved seawater medium (FAS) supplemented with 1 g L^{-1} casaminoacids (FAS-CAS) (Schut *et al*., 1993) and FAS medium supplemented with 0.5 g L^{-1} polymers: agarose, chitin and starch (FAS-POL) (Bruns *et al*., 2002). Different 10-fold dilutions were prepared in sterilized seawater for each of the samples and plated in the above mentioned culture media. Sediment and biofilm samples were collected below the water level using a 50-mL sterile pipette and therefore contained a significant amount of water. Samples were vigorously vortexed in order to obtain a homogeneous suspension for dilutions. One gram of strained and sliced *F. vesiculosus* was added to 10 mL of sterilized seawater, vigor-ously vortexed and used for dilutions. Plates were incubated at 15 and 22 ºC for 15 days. For the estimation of CFUs plates with 30–300 colonies were selected. A total of 166 colonies were picked up and isolated on the basis of its different colour and morphology and used for QQ screen-ing. The 166 isolates obtained were able to grown on MA at 22 ºC and therefore these culture conditions were selected as standard for laboratory manipulation.

QQ screening

The 166 isolated strains were tested for their QQ activity in solid plate assays carried out with the AHL biosensor *Chromobacterium violaceum* CV026 (McClean *et al*., 1997). This strain responds to AHLs with acyl chains from four to eight carbons. The marine strains isolated were grown in 1 mL of marine broth (MB) at 22 ºC and 200 r.p.m. After 24 h, 40 mL of a stock solution (50 mg mL^{-1} in water) of *N*-hexanoyl-L-homoserine lactone (C6-HSL, Sigma-Aldrich) were added to achieve a final concentration of 2 mg mL^{-1} (10 mM), and incubated for further 24 h. In order to detect the inhibition of C6-HSL activity, 50 mL of the supernatants were spotted in duplicate in wells made in Luria–Bertani (LB) plates overlaid with 5 mL of a 1/100 dilution of an overnight culture of *C. violaceum* CV026 in soft LB (0.8% agar). Another 50 mL of sterile distilled water were added to wells. Sterile MB and MB plus the same amount of C6-HSL were used as controls in all plates. To check the spectrum of substrates of the QQ activity, positive strains were further analysed for their capacity to eliminate *N*-decanoyl-L-homoserine lactone (C10-HSL, Sigma-Aldrich) activity from the culture medium using the biosensor strain *C. violaceum* VIR07 (Morohoshi *et al*., 2008a) and the same methodology. The biosensor strains were maintained in LB plates supplemented with kanamycin (25 mg mL^{-1}). The production of detectable amounts of AHLs that could interfere with the *C. violaceum* QQ plate bioassay was tested for the positive strains by assaying the capacity to induce violacein production of 50 mL of spent culture media from 48 h cultures in plate bioassays for both biosensors. The capacity to interfere with *N*-oxododecanoyl-L-homoserine lactone (OC12-HSL) was tested with the lux-based biosen-sor strain *Escherichia coli* JM109 pSB1075 (Winson *et al*., 1998). OC12-HSL was added to 1 mL of an overnight culture of the marine QQ strains in MB (final concentration 2 mg mL^{-1}) and incubated for 24 h at 22 ºC. The remaining AHL activity in the culture media was evaluated in LB plates overlaid with 5 mL of semi-solid LB agar seeded with 50 mL of an overnight culture at 37 ºC 200 r.p.m. of *E. coli* JM109 pSB1075. Fifty microlitres of culture supernatants were loaded in wells. Sterile MB and MB plus OC12-HSL were set as controls. Plates were incubated for 3 h at 37 ºC and the production of light derived from AHL activity was exam- ined using a light camera (E.G. & G. Berthold).

Statistical analysis for of the effect of temperature, culture media and origin of the sample on the number of QQ active strains recovered was carried out using Fisher's exact and Pearson's w^2 tests at significance level a = 0.05, with SPSS statistics V17.0 (SPSS Inc.) program.

flonfirmation of AHL degradation activity by HPLfl-MS

To determine whether the 15 strains presenting QS inhibi-tion activity against both C6- and C10-HSL in the plate bioassays were producing an AHL inhibitor/antagonist or were enzymatically degrading the signal, the final concentra-tion of AHL in the culture media was evaluated using HPLC-MS. A shorter and longer AHL were selected for the HPLC-MS analysis in order to check the spectrum of activity. *N*-butyryl-L-homoserine lactone (C4-HSL) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) were added to 1 mL of 24 h cultures of the selected strains at a final concentration of 50 mM and incubated for another 24 h at 22 ºC and 200 r.p.m. Five hundred microlitres of spent culture media obtained after centrifugation (2000 g, 5 min) were directly extracted while another 500 mL were acidified with HCl to a pH of 2 and incubated for 24 h at 25 ºC before extraction in order to facilitate the recovery of the AHL activity derived from the hydrolysis of the lactone ring derived from the action of lactonases (Yates *et al*., 2002). Culture supernatants were extracted three times with an equal volume of ethyl-acetate, evaporated under flux of nitrogen and resuspended in 200 mL of acetonitrile for

HPLC-MS analysis and quantification. MB samples supplemented with the same amount of C4- or C12-HSL were processed and extracted in the same way and used as controls.

Analysis were carried out with a HPLC 1100 series (Agilent) equipped with a C8 precolumn (2.1 × 12.5 mm, 5 mm particle size) and a ZORBAX Eclipse XDB-C18 2.1 × 150 mm (5 mm particle size) column that was maintained at 45 ºC. The mobile phase was built by 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) (Ortori *et al*., 2007). The flow rate was 0.22 mL min^{-1}. The elution conditions were as follows: 0 min 35% B, linear gradient to 60% B in 10 min and then a linear gradient from 60% to 95% B over 5 min, then 5 min 95% B and then ramped back to starting conditions in 9 min. The column was re-equilibrated for a total of 5 min. A 2 mL volume was injected onto the column. The MS experiments shown were conducted on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystem, CA) equipped with a TurboIon 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).

16S-based bacterial identification and phylogenetic analysis

The identification of the strains was carried out by amplifying and partial sequencing of the 16S rRNA gene (approximate length of the amplicons of 1300 bp). Genomic DNA from the different isolates was extracted (Puregene Tissue Core Kit B) and bacterial 16S rRNA gene was amplified using the universal primers ANT1 (forward, position 8–27) (5'-AGAGTTTGATCATGGCTCAG) and S (reverse, position 1491–1509) (5'-GGTTACCTTGTTACGACTT) (Martinez-Murcia & Rodriguez-Valera, 1994). PCRs were carried out under the following standard conditions: 35 cycles (denaturation at 94 ºC for 15 s, annealing at 50 ºC for 30 s, extension at 72 ºC for 2 min) preceded by 2 min denaturation at 94 ºC and followed by 7 min extension at 72 ºC. PCR products were partially sequenced, revised and corrected with BIOEDIT Sequence Alignment Editor program (v. 7.0.9.0, http://www.mbio.ncsu.edu/BioEdit/bioedit.html). A total of fifteen 16S rRNA gene sequences were identified and compared with 16S rRNA gene sequences available in the GenBank using NCBI BLAST (Altschul *et al*., 1997) and the Ribosomal Database Project (http://rdp.cme.msu.edu/). 16S rRNA gene sequences were aligned using MUSCLE (Edgar, 2004) with their closest relatives in databases as identified by BLAST and those isolates with an acylase or lactonase previously identified or with known QQ activity (Park *et al*., 2003; Uroz *et al*., 2005, 2008; Morohoshi *et al*., 2008a; Czajkowski & Jafra, 2009; Tait *et al*., 2009). In order to eliminate gaps and unambiguously aligned positions, Gblocks was used (Castresana, 2000) yielding 649 positions available for constructing the tree. Phylogenetic analysis was then performed using the MEGA4 phylogenetic tool software package (Zmasek & Eddy, 2001; Kumar *et al*., 2004) using the default parameters.

The sequences have been deposited in GenBank under the accession numbers: HQ441213–HQ441227.

Results

Bacterial growth and isolation

Very different results in the number of CFUs were obtained depending on the culture medium and growth temperature for each of the samples (Fig. 1). The sample with a highest bacteria density was that obtained from the sediment from the fish culture tank, reaching 3 × 10^6 CFUs mL^{-1}. Highest CFUs obtained from the water tank biofilm were one order of magnitude lower. Although not directly comparable, the number of viable bacteria isolated from *F. vesiculosus* was similar, on a weight basis, to the bacterial density in the

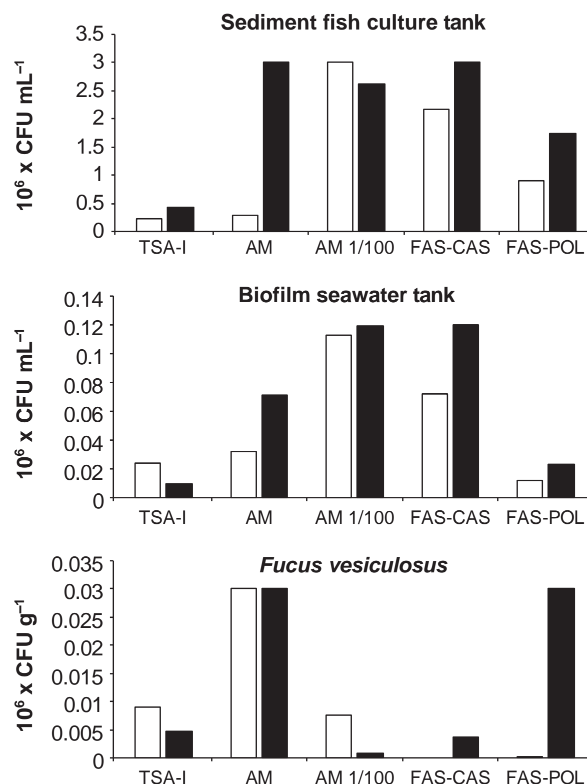


Fig. 1. CFU mL^{-1} or CFU g^{-1} obtained for each of the culture media used for marine strains isolation in the three marine samples selected at 15 ºC (white bars) and 22 ºC (black bars).

water tank biofilm, reaching 3×10^5 CFUs g^{-1} at best. Growth temperature and more importantly the culture media used for isolation also had a great influence on the number of viable bacteria isolated. Higher temperature (22 ºC) allowed the growth of a higher number of colonies in almost all cases. Diluted MA (MA 1/100) and FAS-CAS were most effective for CFU recovery in the sediment and biofilm samples, while in the *F. vesiculosus* sample, MA and FAS-POL were equally effective at high temperature, yielding three times more CFUs than the other culture media (Fig. 1).

A total of 166 isolates were obtained from the selected marine environments for QQ screening on the basis of distinct colony morphology, 85 strains from the tank sediment sample, 48 from tank biofilm and 33 from *F. vesiculosus* (Table 1). Despite the higher CFUmL^{-1} obtained with oligotrophic culture media in the first two samples (Fig. 1), around half of the isolates used for QQ screening were obtained from richer media, TSA 1% NaCl and MA, due to the higher variability of colonies observed. In the case of the sediment sample, more than 70% of the isolates came from these rich culture media while in the biofilm sample most strains were isolated from the FAS culture media (Table 1). The number of strains isolated at the two different temperatures used was very similar: 91 strains were isolated from plates maintained at 22 ºC and 75 strains were isolated from plates maintained at 15 ºC (data not shown).

Table 1. Summary of isolated strains (morphological types) from different samples and culture media, showing number and per cent of strains with QS inhibition activity against C6-HSL obtained using the solid plate *Chromobacterium violaceum* CV026 assay

	No. of isolated strains	QQ strains	% QQ
Fish tank sediment			
TSA–1% NaCl	30	2	6.7
MA	31	4	12.9
MA 1/100	9	1	11.1
FAS-CAS	7	0	0
FAS-POL	8	1	12.5
Total	85	8	9.4
Water tank biofilm			
TSA–1% NaCl	9	0	0
MA	5	0	0
MA 1/100	7	0	0
FAS-CAS	17	2	11.8
FAS-POL	10	1	10.0
Total	48	3	6.3
Fucus vesiculosus			
TSA–1% NaCl	9	3	33.3
MA	9	2	22.2
MA 1/100	5	3	60.0
FAS-CAS	7	3	42.9
FAS-POL	3	2	66.7
Total	33	13	39.4
All samples			
TSA–1%NaCl	48	5	10.4
MA	45	6	13.3
MA 1/100	21	4	19.0
FAS-CAS	31	5	16.1
FAS-POL	21	4	19.0
Total	166	24	14.4

Bioassay-based detection of QS inhibition activity

A solid plate assay was then carried out using the reporter strain *C. violaceum* CV026 that produces violacein in response to the presence of short-chain AHLs, therefore allowing the detection of the inhibition of C6-HSL activity added externally (Fig. 2). This assay enabled us to directly differentiate the strains presenting growth-inhibition activity from those with a real QQ activity. The *C. violaceum* CV026 solid assay permitted the identification of 24 strains with QQ activity against C6-HSL, which represents a 14.4% of the isolated strains (Table 1). Against the initial hypothesis of a higher probability of isolating QQ active strains using oligotrophic culture media, no statistically significant effect of the culture media used for the isolation was found on the percentage of strains with QQ activity (Table 1, w^2 test, *P* 4 0.05). Fifty per cent of the active strains were isolated at 22 ºC and therefore the effect of isolation temperature was not statistically significant (Fisher's exact test, *P* 4 0.05). On the contrary, an important effect of the origin of the sample on the percentage of strains with QQ activity was found (Table 1, w^2 test, *P* O 0.05). While strains isolated from the tanks presented a percentage of QQ activity between 6% and 9% (Table 1), almost 40% of the strains isolated from the *F. vesiculosus* were active against C6-HSL (Table 1).

The capacity to interfere with C10-HSL and OC12-HSL of the 24 strains being able to inactivate C6-HSL was further tested using the biosensor strains *C. violaceum* VIR07 and *E. coli* JM109 pSB1075, respectively (Fig. 2, Table 2). Among them only 15 were able to completely eliminate the activity of C10-HSL as detected by *C. violaceum* VIR07 within 24 h (Fig. 2) and therefore these strains were selected for further characterization of QQ activity and identification. These strains were isolated from *F. vesiculosus* (7), fish tank sediment (7) and water tank biofilm (1) (Table 2). Ten out of these 15 strains were also able to completely suppress the OC12-HSL activity detectable to the biosensor (Table 2), indicating that a wide range of AHLs can be inactivated by these strains.

fIharacterization of QQ activity by HPLfI-MS analysis

All 15 strains being able to eliminate the activity of C6- and C10-HSL in the plate bioassays were able to significantly

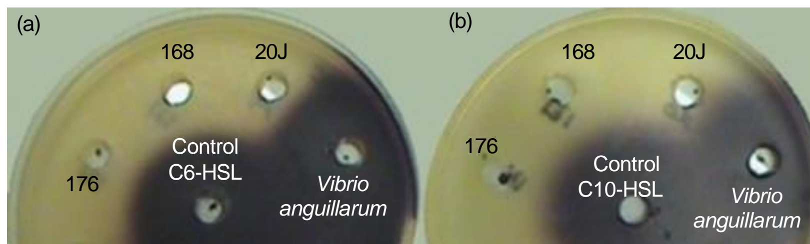


Fig. 2. Picture of the solid plate assay to detect marine QQ strains with the AHL biosensors *Chromobacterium violaceum* CV026 (a) and *C. violaceum* VIR07 (b). Positive QQ strains degraded both AHLs (10 mM) after 24 h, eliminating the violacein production compared with control (central well). A strain of *Vibrio anguillarum* was also used as negative control.

Table 2. Identification of the 15 strains with QQ activity on the basis of 16S rRNA gene sequence

Origin	Strain	Closest cultivated bacteria	% ID at 16S rRNA gene locus	OC12-HSL	Putative sequences
Fucus vesiculosus	2	*Hyphomonas* sp. DG895	99	+++	Acylase/lactonase
	5	*Stappia* sp.	98	+	Lactonase
	168	*Alteromonas* sp. BCw156	99	+++	Acylase
	172	*Oceanobacillus* sp. YIM DH3	99	—	Acylase
	173	*Rhodococcus erythropolis* PR4	100	+++	Lactonase
	176	*Stappia* sp.	98	+++	Lactonase
	177	*Phaeobacter* sp. NH52F	96	+++	
Fish tank sediment	20J	*Tenacibaculum discolor* DSM 18842	99	+++	
	24	*Bacillus circulans* strain X3	98	+++	Lactonase[×]
	30	*Oceanobacillus* sp. YIM DH3	99	—	Acylase
	33	*Halomonas taeanensis* strain BH539	99	—	
	50	*Rhodococcus erythropolis* MM30	99	+++	Lactonase
	97-1	*Stappia* sp.	98	+++	Lactonase
	97-2	*Oceanobacillus* sp. YIM DH3	99	—	Acylase
Water tank biofilm	61	*Roseovarius aestuarii* SMK-122	99	+++	

Capacity to degrade OC12-HSL is shown, as well as the presence of putative sequences homologous to known acylases and lactonases in the available sequenced genomes.
[×]Based on the common presence of putative lactonases in the available genomes of species of the genus *Bacillus*.

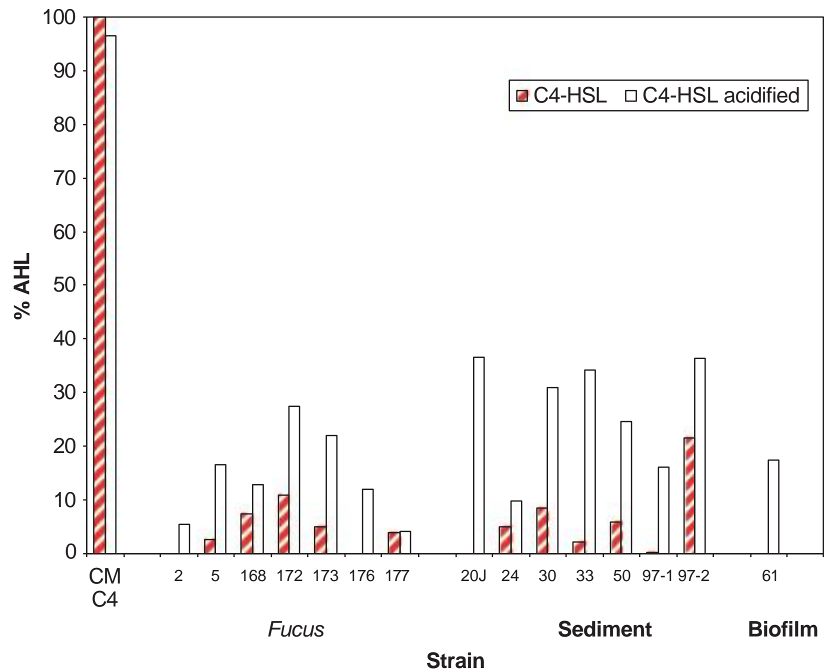
reduce the concentration of C4- and C12-HSL as measured by HPLC-MS (Fig. 3). The final pH of cultures after the 24 h degradation assay was lower than 7 in all cases and therefore the spontaneous lactonization of the AHLs due to high pH values can be disregarded (Yates *et al*., 2002). This result indicated the presence of enzymatic degradation activity in all the strains.

The recovery of AHL concentration derived from acidification of the spent culture media to pH 2, that drives spontaneous reformation of the lactone ring opened by lactonase activity, was more frequent for C4-HSL (Fig. 3). Only strain 177, a novel species of *Alphaproteobacteria* related to *Phaeobacter*; strain 2, identified as *Hyphomonas* sp.; strain 168, identified as *Alteromonas* sp.; and strain 24, identified as *Bacillus circulans* produced an almost complete degradation of both AHLs that could not be significantly recovered by acidification, indicating an enzymatic activity different from lactonase. Several strains such as strain 20J (identified as a *Tenacibaculum discolor*) present a different degradation profile for short and long AHLs, indicating more than one type of enzymatic activity, while others belonging to the genera *Stappia* (strains 5, 176 and 97-1), *Oceanobacillus* (strains 172, 30 and 97-2) and *Halomonas* (strain 33) seem to present a wide-spectrum lactonase activity, because the amount of AHL is partially recovered after acidification of the spent culture media (Fig. 3).

Bacteria identification and database search

The sequences of the 16S rRNA gene of the 15 selected isolates were obtained and used for a BLAST search against sequences in GenBank in order to assess their taxonomic affiliation. The nearest isolate identification is shown in Table 2. Of the 15 isolates, two of them belonged to *Gammaproteobacteria* (33 and 168), six to the *Alphaproteobacteria* (5, 97-1, 176, 2, 61 and 177), six to *Firmicutes* (24, 30, 97-2 and 172), two to the *Actinobacteria* (50 and 173) and one to the *Bacteroidetes* (20J).

Among the 15 characterized isolates, only three, strain 24 (*B. circulans* 98% identity) and strains 50 and 173 (*Rhodococcus erythropolis*, 99% and 100% identity, respectively) belong to genera in which terrestrial isolates had been previously described to have QQ activity (Dong *et al*., 2002; Uroz *et al*., 2003). Another one, strain 20J (*T. discolor* 99%)



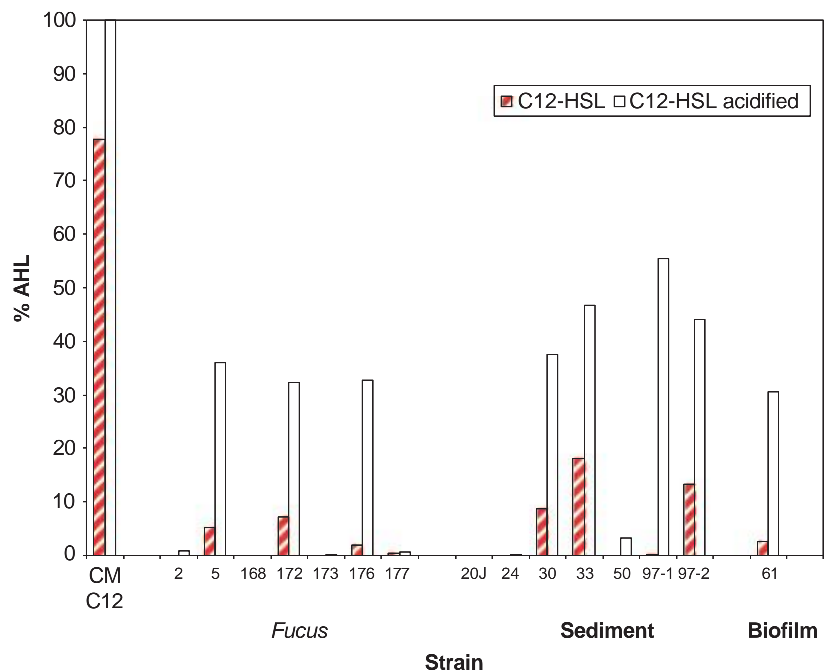


Fig. 3. HPLC-MS analysis of C4-HSL and C12-HSL in the culture media of the 15 positive QQ isolates after 24 h. Initial AHL concentration was 50 mM. Spent culture media were acidified to pH 2 in order to allow the recovery of the lactone ring after lactonolysis.

belongs to a marine genus in which QQ activity has been recently described for the pathogenic species *Tenacibaculum maritimum* (Romero *et al*., 2010), although the latter is not able to degrade short-chain AHLs. The presence of *Bacillus* species is not uncommon in marine samples (Ivanova *et al*., 1999), but strain 24 was isolated from the sediment of an inland fish culture system and therefore a terrestrial origin cannot be excluded. Two isolates obtained from different sources, strain 50 from fish tank sediment and strain 173 from *F. vesiculosus*, were identified as *R. erythropolis* (Table 2). The genus *Rhodococcus* is widely distributed in aquatic and terrestrial habitats and several species of this genus are able to degrade AHLs, all of them of terrestrial origin (Uroz *et al*., 2008).

All the new isolates presenting QQ activity belong to genera typical of marine environments. The *Alphaproteobacteria Stappia* sp. (strains 5, 176 and 97-1), a genus that comprises several marine species formerly classified as

belonging to *Agrobacterium*, and the firmicute *Oceanobacillus* sp. (strains 172, 30 and 97-2), a genus comprising many facultative alkaliphilic and marine species, seem to be ubiquitous because representatives from both genera could be isolated several times from samples of very different origin (*F. vesiculosus* and fish tank sediment). Among the strains isolated from *F. vesiculosus*, *Hyphomonas* sp. (strain 2) belongs to the group of marine prosthecate bacteria that are typical algal epibionts (Poindexter, 2006), while *Alteromonas* sp. (strain 168) is a genus of marine *Gammaproteobacteria* frequently isolated from diverse marine environments, including algae (Gauthier & Breittmayer, 1992). Finally, strain 177 represents a new species of *Alphaproteobacteria* that is related (ID 96%) to *Phaeobacter* sp., a genus of marine bacteria proximate to the *Roseobacter* clade (Martens *et al*., 2006; Fig. 4), although more close relatives have been described among noncultivable bacteria (Jones *et al*., 2007).

Among the isolates obtained from the fish tank sediment, strain 20J presented a 99% identity with the fish pathogen *T. discolor*. The genus *Tenacibaculum* belongs to the *Cytophaga–Flavobacterium–Bacteroides* cluster, also known as *Bacteroidetes*, which constitutes one of the dominant heterotrophic bacterial groups in aquatic habitats and includes several species causing the marine fish 'gliding bacterial disease' or tenacibaculosis/flexibacteriosis. Surprisingly, strain 20J was isolated in TSA–NaCl 1%, a characteristic that would exclude its classification as a member of this species (Piñeiro-Vidal *et al*., 2008). Strain 33 was identified as a member of the genus *Halomonas* (ID 99%), a group of mainly marine *Gammaproteobacteria* that includes several moderately halophilic strains.

The only active strain isolated from the surface biofilm of the water tank – strain 61 – was identified as *Roseovarius aesturarii* (ID 99%), a genus of strictly marine *Alphaproteobacteria* (Labrenz *et al*., 1999). This species belongs to the *Roseobacter* lineage that is estimated to comprise 20–30% of the 16S rRNA gene sequences in the photic zone of marine environments (Wagner-Dobler & Biebl, 2006).

The relationships among the 15 strains sequenced and other 16S rRNA gene sequences from other isolates with QQ activity (Czajkowski & Jafra, 2009; Tait *et al*., 2009; Uroz *et al*., 2009; Nithya *et al*., 2010) are shown in the dendrogram of Fig. 4. *Delftia* sp. A317 and *Ochrobactrum* sp. A44, which have been described to have QQ activity (Jafra *et al*., 2006), were not included in the analysis due to the short read 16S sequence.

Discussion

This study reveals that QQ is a common feature among cultivable bacteria isolated from dense marine bacterial communities. The percentage of isolated strains being able to eliminate AHL activity obtained in this study, 14.4% (Table 1), is much higher than the percentages obtained so far in soil and plant isolates. Moreover, this percentage could be underestimated because strains were first selected for their capacity to interfere with C6-HSL activity, while later analysis revealed that the capacity to interfere with long-chain AHLs is much more common among marine isolates (data not shown).

In the pioneer study that enabled the cloning of the first lactonase from the genus *Bacillus* (Dong *et al*., 2000), only 24 out of 500 strains (4.8%) isolated from soil were active against AHLs. The percentages of active strains obtained in later studies for soil samples were even lower, being only slightly higher than 2% (Dong *et al*., 2002; D'Angelo-Picard *et al*., 2005). A screening of more than 10 000 clones of a soil metagenomic library produced a single clone being able to degrade AHLs (Riaz *et al*., 2008), while the screening of more than 7000 soil metagenomic clones allowed the identification of three clones with lactonase activity (Schipper *et al*., 2009). These low percentages of activity may not be representative of the real QQ activity present in soil due to the intrinsic difficulties of recovering enzymatic activity from fosmid-based metagenomic libraries. On the contrary, two out of 16 isolates of a biofilm from a water-treatment system presented QQ activity (Lin *et al*., 2003), indicating already the high activity present in high organic content aquatic environments.

The QQ screening procedure used in this work avoided the use of enrichment cultures based on the ability to grow with AHLs as sole source of carbon and nitrogen that have been used in many studies (Leadbetter & Greenberg, 2000; Park *et al*., 2003, 2006; Uroz *et al*., 2003), in order to obtain a wide picture of the prevalence and ecological significance of QQ activities in these marine coastal samples. One of the more striking results is that all the isolates actively degraded the AHLs even in highly organic media, in which other carbon sources are more readily available. Therefore, under the tested conditions, the capacity of AHL degradation cannot be regarded merely as a metabolic activity directed at obtaining energy.

Although a strong effect of the culture media used for isolation on the number of QQ active isolates was expected, such an effect could not be confirmed. On the contrary, a strong effect of the origin of the sample on the number of strains with QQ activity was found. Almost 40% of the strains isolated from *F. vesiculosus* were able to degrade AHLs. The *Fucus* and the water tank biofilm samples were exposed to the same coastal natural water; on the contrary, the fish tank sediment was obtained from an inland recirculating culture system and was exposed to a much higher organic load, as reflected by the high CFU values obtained for this sample. Therefore, no direct correlation between organic load and QQ activity can be concluded. The high

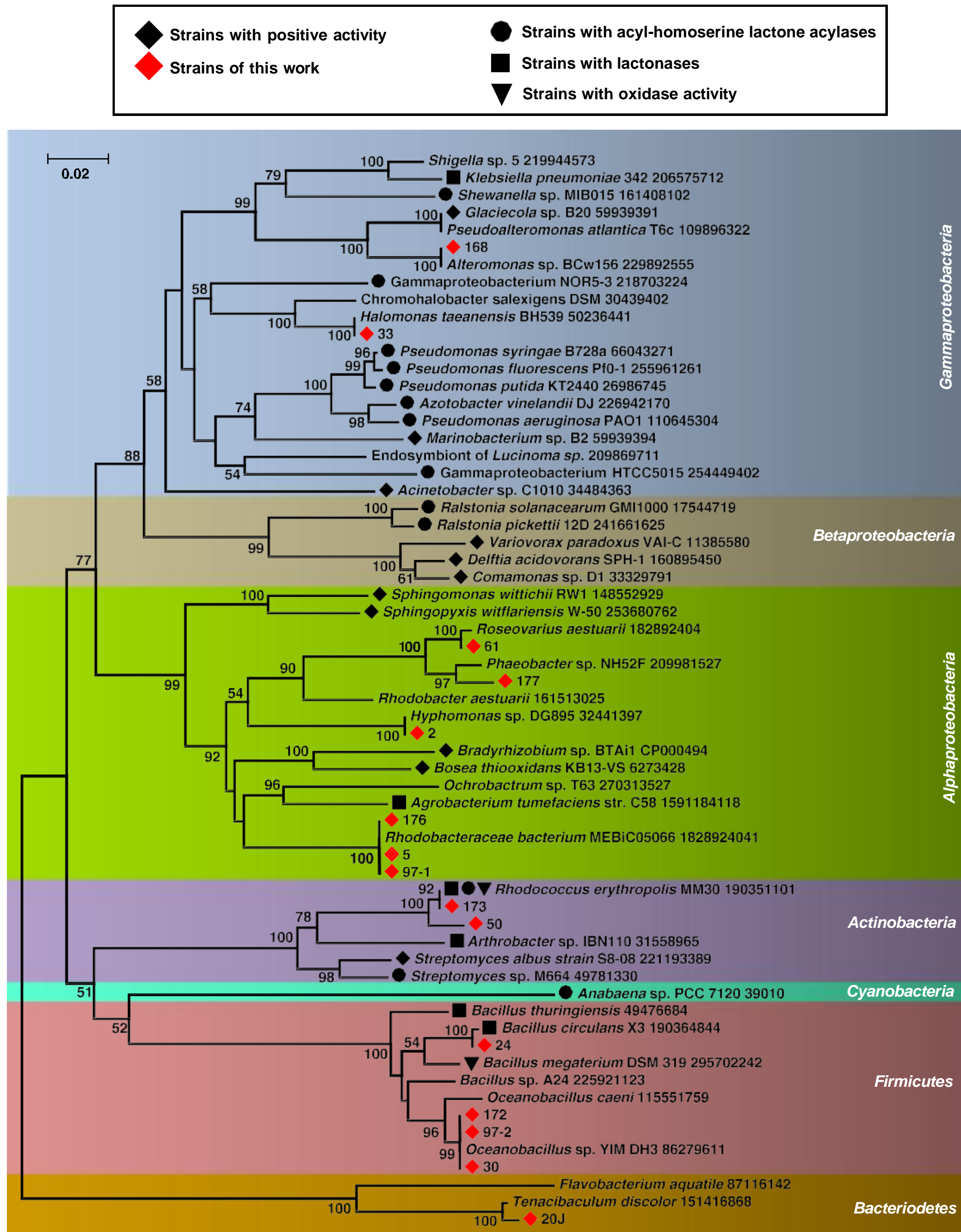


Fig. 4. Neighbour-joining tree based on the 16S rRNA gene, showing relationships among the marine isolates characterized in this study – in red – and species with known lactonases and acylases or known QQ activity. The number found after each taxon name is the accession number for the respective 16S rRNA gene sequence. Bootstrap values over 50% from the neighbour-joining analysis are shown. Scale bar, 0.02 substitutions per nucleotide position.

percentage of QQ activity obtained in *Fucus* isolates sustains the existence of strong microbial interactions in the eukaryotic–prokaryotic boundary that promote unique biological activities (Gao *et al*., 2003; Egan *et al*., 2008).

Besides the higher frequency with which QQ bacteria could be isolated, the diversity reported for AHL-degrading bacteria isolated from the marine samples is much higher than that reported from soil and plant isolates. Members of

nine different genera belonging to the *Alpha*- and *Gammaproteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* (Fig. 4) were isolated from our marine samples and a novel species related to *Phaeobacter* has been identified (strain 177). Almost all the active isolates belong to genera prevalently or exclusively marine, excluding their terrestrial origin despite the coastal character of the samples. Only three of the isolates belong to genera (*Bacillus* and *Rhodococcus*) in which terrestrial isolates had been previously described to have QQ activity (Dong *et al*., 2002; Uroz *et al*., 2003). In contrast, in a screening of 800 soil strains, all the characterized isolates with strong activity belonged to the genus *Bacillus* (Dong *et al*., 2002). In a separate study AHL degraders belonging to both *Bacillus* sp. and the *Alphaproteobacteria* could be isolated from tobacco rhizosphere, but again only *Bacillus* species could be isolated from the same soil (D'Angelo-Picard *et al*., 2005).

In order to elucidate whether the disruption of QS observed in the positive strains selected was due to enzymatic activity or the production of other AHLs or AHL-like substances, the production of AHLs was tested using the same plate bioassay and culture conditions and the enzymatic activity of the 15 selected strains was assessed using HPLC-MS techniques (Fig. 3). The plate bioassays using *C. violaceum* CV026 and VIR07, covering the whole range of AHLs, could not detect AHL production for any of the 15 strains (data not shown). The HPLC results demonstrated that all 15 selected strains actively degraded both C4- and C12-HSL. Lactonase activity seems to be widespread in the genus *Bacillus* (Dong *et al*., 2002) but the acidification of the media after AHL degradation in our *B. circulans* isolate did not allow the recovery of any of the C12-HSL activity and only a little of C4-HSL (Fig. 3), indicating a type of enzymatic activity different from the lactonase described so far for this genus. It remains to be confirmed if a possible AHL oxidase as that reported for *Bacillus megaterium* (Chowdhary *et al*., 2007; Cirou *et al*., 2009) is responsible for the QQ capacity of our marine *B. circulans* isolate (strain 24).

Rhodococcus erythropolis strain W2 is a peculiar strain because it has been demonstrated to be able to inactivate a wide range of AHLs using three diverse enzymatic mechanisms: a lactonase, an amidohydrolase – or acylase – and an oxidoreductase activity that reduces 3-oxo-*N*-AHLs to their hydroxylated equivalents (Park *et al*., 2006; Uroz *et al*., 2008). This unique combination could not be found in other terrestrial *R. erythropolis* strains, in which only lactonolysis seem to be active for degrading AHLs (Uroz *et al*., 2008). Although a more detailed study on the enzymatic activities present in our marine *R. erythropolis* isolates is required, the HPLC analysis of the degradation of C12-HSL demonstrated that the peak could not be recovered after acidification of the culture media (Fig. 3) and therefore an enzymatic activity other than lactonolysis should be active in both isolates. On the contrary, C4-HSL could be partially recovered after acidification (Fig. 3), and therefore a complex enzymatic activity as that reported for *R. erythropolis* W2 is proposed for these marine strains, that may deserve further characterization. The search in the available genomes of the QQ active species revealed the presence of QQ sequences in several of them (Table 2). In some cases, such as the *Stappia* strains, that present a clear lactonase activity, the retrieved sequence is consistent with the type of AHL degradation revealed by the preliminary HPLC-MS analysis. On the contrary, in the case of the *Oceanobacillus* spp., the HPLC analysis indicates a lactonase-like activity, while only an acylase sequence could be found in the genome with significant homology to known QQ enzymes. The cloning of the enzymes responsible for the QQ activity in these novel species will surely extend our knowledge on their variability and modes of action. Moreover, further characterization of these marine isolates and their activity may drive the development of biotechnological applications, especially in the field of aquaculture (Defoirdt *et al*., 2007).

The high percentage of QQ strains isolated in the present study seems to indicate that QQ is a usual strategy adopted in the marine media to achieve competitive advantages, at least in the nutrient-rich coastal environments. This was especially true for the bacteria isolated from *F. vesiculosus*, indicating strong bacterial interactions in the surface of algae. More detailed studies on the presence of QQ activity in cultivable bacteria in the open ocean would probably add useful information in order to elucidate the ecological importance of AHL-mediated QS and QQ processes in the marine environment.

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