Quorum quenching in cultivable bacteria from dense marine coastal microbial communities

Manuel Romero1, Ana-Belen Martin-Cuadrado2, Arturo Roca-Rivada1, Ana María Cabello1 & Ana Otero1

1Departamento de Microbiología y Parasitología, Facultad de Biología-CIBUS, Universidad de Santiago de Compostela, Santiago, Spain; and
2Evolutionary Genomics Group, Departamento Producción Vegetal y Microbiología, Universidad Miguel Hernández, San Juan de Alicante, Spain

Correspondence: Ana Otero, Departamento de Microbiología y Parasitología, Facultad de Biología-CIBUS, Universidad de Santiago de Compostela, 15782 Santiago, Spain. Tel.: +34 881 816 913; fax: +34 981 528 006; e-mail: anamaria.otero@usc.es

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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 α-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 (42°33'45.32"N 8°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 autoclaved 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, vigorously vortexed and used for dilutions. Plates were incubated at 15 and 22 °C 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 their different colour and morphology and used for QQ screening. The 166 isolates obtained were able to grow on MA at 22 °C 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 were isolated grown in 1 mL of marine broth (MB) at 22 °C and 200 r.p.m. After 24 h, 40 μL of a stock solution (50 μg mL\(^{-1}\) in water) of N-hexanoyl-L-homoserine lactone (C6-HSL, Sigma-Aldrich) were added to achieve a final concentration of 2 μg mL\(^{-1}\) (10 μM), and incubated for further 24 h. In order to detect the inhibition of C6-HSL activity, 50 μL 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 μL 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 μg 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 μL 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 biosensor 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 μg mL\(^{-1}\)) and incubated for 24 h at 22 °C. 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 μL of an overnight culture at 37 °C 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 °C and the production of light derived from AHL activity was examined 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 \(\chi^2\) tests at significance level \(\alpha = 0.05\), with SPSS statistics V17.0 (SPSS Inc.) program.

**Confirmation of AHL degradation activity by HPLC-MS**

To determine whether the 15 strains presenting QS inhibition 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 concentration 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 μM and incubated for another 24 h at 22 °C 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 μL were acidified with HCl to a pH of 2 and incubated for 24 h at 25 °C 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 μL 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 \times 12.5 \text{ mm}, 5 \mu \text{m particle size}) and a ZORBAX Eclipse XDB-C18 2.1 \times 150 \text{ mm} (5 \mu \text{m particle size}) column that was maintained at 45 °C. 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 \mu L 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) (Martínez-Murcia & Rodriguez-Valera, 1994). PCRs were carried out under the following standard conditions: 35 cycles (denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, extension at 72 °C for 2 min) preceded by 2 min denaturation at 94 °C and followed by 7 min extension at 72 °C. 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 × 10⁶ 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
water tank biofilm, reaching $3 \times 10^5$ 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°C) 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 CFU mL$^{-1}$ 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°C and 75 strains were isolated from plates maintained at 15°C (data not shown).

### 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, $\chi^2$ test, $P > 0.05$). Fifty per cent of the active strains were isolated at 22°C and therefore the effect of isolation temperature was not statistically significant (Fisher’s exact test, $P > 0.05$). On the contrary, an important effect of the origin of the sample on the percentage of strains with QQ activity was found (Table 1, $\chi^2$ test, $P < 0.05$). 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.

### Characterization of QQ activity by HPLC-MS analysis

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

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

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>No. of isolated strains</th>
<th>QQ strains</th>
<th>% QQ</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fish tank sediment</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA–1% NaCl</td>
<td>30</td>
<td>2</td>
<td>6.7</td>
</tr>
<tr>
<td>MA</td>
<td>31</td>
<td>4</td>
<td>12.9</td>
</tr>
<tr>
<td>MA 1/100</td>
<td>9</td>
<td>1</td>
<td>11.1</td>
</tr>
<tr>
<td>FAS-CAS</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FAS-POL</td>
<td>8</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td>Total</td>
<td>85</td>
<td>8</td>
<td>9.4</td>
</tr>
<tr>
<td><strong>Water tank biofilm</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TSA–1% NaCl</td>
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<td>0</td>
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<tr>
<td>MA</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MA 1/100</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FAS-CAS</td>
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<td>11.8</td>
</tr>
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<tr>
<td>Total</td>
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<td>6.3</td>
</tr>
<tr>
<td><strong>Fucus vesiculosus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA–1% NaCl</td>
<td>9</td>
<td>3</td>
<td>33.3</td>
</tr>
<tr>
<td>MA</td>
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<td>2</td>
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</tr>
<tr>
<td><strong>All samples</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TSA–1% NaCl</td>
<td>48</td>
<td>5</td>
<td>10.4</td>
</tr>
<tr>
<td>MA</td>
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<tr>
<td>Total</td>
<td>166</td>
<td>24</td>
<td>14.4</td>
</tr>
</tbody>
</table>
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 168, identified as Hyphomonas sp.; strain 24, identified as Bacillus circulans; strain 30 and 33, identified as Oceanobacillus sp.; strain 176, identified as Stappia sp.; and strain 172, identified as Alteromonas sp., produced an almost complete degradation of both AHLs that could not be significantly recovered by acidification, indicating an enzymatic activity different from lactonase. Among the 15 characterized isolates, only three, strain 24 (B. circulans 98% identity) and strains 30 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%) 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 the 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 30 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%)
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

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**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 µM. Spent culture media were acidified to pH 2 in order to allow the recovery of the lactone ring after lactonolysis.
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 (Poinderux, 2006), while Alteromonas sp. (strain 168) is a genus of marine Gammaproteobacteria frequently isolated from diverse marine environments, including algae (Gauthier & Breitmayer, 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 ‘giding 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...
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

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**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.
nine different genera belonging to the Alpha- and Gamma-proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes (Fig. 4) were isolated from our marine samples and a novel species related to \textit{Phaeobacter} has been identified (strain 177). Almost all the active isolates belong to genera prev

...not allow the recovery of any of the C12-HSL activity and only \textit{B. circulans} isolate did. The acidification of the media after AHL degradation in our \textit{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 \textit{Bacillus megaterium} (Chowdhary et al., 2007; Cirou et al., 2009) is responsible for the QQ capacity of our marine \textit{B. circulans} isolate (strain 24).

\textit{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 different enzymatic mechanisms: a lactonase, an amidohydrolase – or acylase – and an oxidoreductase activity that reduces 3-oxo-AHLs to their hydroxylated equivalents (Park et al., 2006; Uroz et al., 2008). This unique combination could not be found in other terrestrial \textit{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 \textit{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 \textit{R. erythropolis} W2 is proposed for these marine strains, that may deserve further characterization.

...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 \textit{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 \textit{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 \textit{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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