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In vitro quenching of fish pathogen *Edwardsiella tarda* AHL production using marine bacterium *Tenacibaculum* sp. strain 20J cell extracts

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ABSTRACT: Quorum quenching (QQ) has become an interesting alternative for solving the problem of bacterial antibiotic resistance, especially in the aquaculture industry, since many species of fish-pathogenic bacteria control their virulence factors through quorum sensing (QS) systems mediated by *N*-acylhomoserine lactones (AHLs). In a screening for bacterial strains with QQ activity in different marine environments, *Tenacibaculum* sp. strain 20J was identified and selected for its high degradation activity against a wide range of AHLs. In this study, the QQ activity of live cells and crude cell extracts (CCEs) of strain 20J was characterized and the possibilities of the use of CCEs of this strain to quench the production of AHLs in cultures of the fish pathogen *Edwardsiella tarda* ACC35.1 was explored. *E. tarda* ACC35.1 produces *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-oxohexanoyl-L-homoserine lactone (OC6-HSL). This differs from profiles registered for other *E. tarda* strains and indicates an important intra-specific variability in AHL production in this species. The CCEs of strain 20J presented a wide-spectrum QQ activity and, unlike *Bacillus thuringiensis* serovar Berliner ATCC10792 CCEs, were effective in eliminating the AHLs produced in *E. tarda* ACC35.1 cultures. The fast and wide-spectrum AHL-degradation activity shown by this member of the *Cytophaga–Flexibacter–Bacteroidetes* group consolidates this strain as a promising candidate for the control of AHL-based QS pathogens, especially in the marine fish farming industry.

KEY WORDS: *Edwardsiella tarda* · Quorum sensing · Quorum quenching · *N*-acylhomoserine lactone · AHL · Lactonase · *Tenacibaculum* · *Cytophaga–Flexibacter–Bacteroidetes* · CFB

INTRODUCTION

In recent decades, the intensive farming of marine fish has registered a strong growth; however, infectious diseases continue to be a threat for this industry (FAO 2010). Due to the limitations of the use of antibiotics in aquaculture, in the search for alternative disease-control strategies, the efforts of researchers have been focused especially on the fields of new vaccines (Adams & Thompson 2006), probiotics (Tinh et al. 2008a, Wang et al. 2008) and enzybiotics (Hermoso et al. 2007). Since pathogenic bacteria coordinate the expression of many important genes, including their virulence factors, in a cell density-dependent manner through the production, liberation and sensing of signal molecules called autoinducers, the interception of this type of bacterial communication or 'quorum sensing' (QS) has also been proposed as a promising method to control pathogenic bacteria (Dong & Zhang 2005, Dong et al. 2007), especially in the field of aquaculture (Defoirdt et al. 2004). When the bacterial communication system is intercepted,

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the virulence is seriously hampered in many of these pathogens, facilitating the elimination of the pathogen by the immune system and/or the action of antibiotics. This new strategy for the treatment of pathogens, that has been generally termed as 'quorum quenching' (QQ), does not affect pathogen survival and therefore it is not expected to generate resistances.

The most studied QS system is the one based on *N*-acylhomoserine lactone (AHL) autoinducers employed by Gram-negative bacteria. AHLs are small molecules composed of a lactone ring connected through an amide bond to a lateral chain that usually contains between 4 and 14 carbons, with or without saturation or C3 hydroxy or oxo substitutions, and which is the part of the molecule that confers specificity to the signal (Whitehead et al. 2001). Approximately half of the marine *Alphaproteobacteria* isolated from various marine habitats produce AHL signal molecules (Wagner-Döbler et al. 2005) and important pathogens in aquaculture such as *Aeromonas hydrophila, A. salmonicida*, *Yersinia ruckeri*, *Vibrio anguillarum, V. harveyi, V. salmonicida*, *V. vulnificus*, *Edwardsiella tarda* and *Tenacibaculum maritimum* produce these autoinducers (Freeman & Bassler 1999, Swift et al. 1999, Croxatto et al. 2002, Buch et al. 2003, Kim et al. 2003, Bruhn et al. 2005, Han et al. 2010, Romero et al. 2010). Several studies support the feasibility of the application of QQ strategies in the field of aquaculture. The use of the furanone C-30 in non-toxic concentrations significantly reduced the pathogenicity of *V. anguillarum* in rainbow trout (Rasch et al. 2004). Furanones also inhibit the pathogenicity of *V. harveyi*, *V. campbellii* and *V. parahaemolyticus* against *Artemia* sp. (Defoirdt et al. 2006), and neutralize the negative effect of *V. harveyi* on rotifers (Tinh et al. 2007a). Unfortunately, furanones are toxic for artemias and rotifers, which limits the use of these natural compounds. Bacterial consortia with AHL degradation activity have been demonstrated to be able to protect *Artemia* sp., rotifers and turbot and prawn larvae from infection (Tinh et al. 2007b, 2008b, Cam et al. 2009, Nhan et al. 2010). Degradation of AHLs by the *Bacillus* QQ enzyme AiiA also increased the survival of carp when co-injected with *Aeromonas hydrophila* (Chen et al. 2010). Moreover, the inclusion of a thermostable AiiA from *Bacillus* sp. strain AI96 in fish feed significantly attenuated *Aeromonas hydrophila* infection in zebrafish (Cao et al. 2012).

Edwardsiella tarda is an important fish pathogen causing the septicaemia edwardsiellosis, which affects a wide variety of fish, including the economically important species *Psetta maxima* and *Solea senegalensis* (Nougayrede et al. 1994, Castro et al. 2012). The presence of QS systems in this species, including LuxI and LuxR homologues and AHL production, has been reported. AHL detection has been controversial, however, because while Morohoshi et al. (2004) described the presence of C6-HSL and C7-HSL in culture media of *E. tarda* NUF251, Han et al. (2010) encountered C4-HSL, C6-HSL, OC6-HSL and an uncharacterized AHL in *E. tarda* LTB-4. The *E. tarda* QS system also employs LuxS to synthesize AI-2 molecules. This QS system was found to positively regulate expression of virulence genes in *E. tarda*, as small peptides that interfere with LuxS could alter biofilm production and the expression of the type III secretion system genes *esrA* and *orf26* (Zhang et al. 2009).

In a screening for bacterial strains with QQ activity in different marine environments (Romero et al. 2011), *Tenacibaculum* sp. strain 20J was identified and selected for its high degradation activity against a wide range of AHLs. In this study, the QQ activity of live cells and crude cell extracts (CCEs) of strain 20J was characterized and the possibilities of the use of CCEs of this strain to quench the production of AHLs in cultures of the fish pathogen *Edwardsiella tarda* ACC35.1 was explored. The activity of strain 20J was compared with the activity of *Bacillus thuringiensis* cell extracts, for which the QQ activity has been widely characterized (Dong et al. 2002, Lee et al. 2002).

MATERIALS AND METHODS

Strains and culture conditions

The *Tenacibaculum* sp. strain 20J CECT7426, isolated in our laboratory from a marine sample (Romero et al. 2011), was routinely cultured at 22°C on marine agar/broth pH 7.0 (MA/MB, Difco). Strain 20J has a 99% 16S rRNA gene sequence identity with *T. discolor* DSM18842, but was isolated in tryptone soya agar (TSA) with 1% NaCl (wt/vol), which excludes its classification within this species. Moreover, the pathogenic activity of strain 20J could not be demonstrated in an injection challenge with turbot juveniles (12 g, n = 20 fish in 25 l tanks) in which up to 10^9 CFUs were injected into fish (data not shown). The AHL biosensor strain *Chromobacterium violaceum* CV026 CECT5999 (McClean et al. 1997) was used for the AHL-degradation assays in solid plates as explained below. This strain was routinely

cultured on Luria-Bertani (LB) medium supplemented with kanamycin (50 µg ml^{-1}) at 30°C. The fish pathogen *Edwardsiella tarda* ACC35.1 (Castro et al. 2006) was cultured at 25°C in TSA or tryptone soya broth (TSB) pH 7.0. *Bacillus thuringiensis* serovar Berliner ATCC10792 was cultured in LB at 22°C.

Localization of AHL-degradation activity

To determine the cellular localization of the AHL-degradation activity, supernatant and CCE were obtained from a 24 h 15 ml MB culture of *Tenacibaculum* sp. strain 20J. The culture was centrifuged for 5 min at 2000 × *g* in order to separate the biomass from the culture media. The supernatant was filtered through a 0.22 µm filter and stored at 4°C prior to use. The pellet was washed with 15 ml of phosphate buffered saline (PBS) pH 6.5, resuspended in another 5 ml of the same buffer, sonicated for 5 min on ice and centrifuged at 16 000 × *g* for 30 min at 4°C. The CCE obtained in this way was filtered through a 0.22 µm filter and stored at 4°C. Total protein concentration in the CCE was estimated by Lowry's method (Lowry et al. 1951). Usually, a 15 ml MB culture rendered a CCE equivalent to 2 mg of protein. To determine if the QQ activity of 20J was associated with the soluble or particulate fraction of the CCEs, the extracts were centrifuged at 300 000 × *g* for 1 h at 4°C and the supernatant was filtered (0.22 µm) and stored at 4°C prior to use. The same methodology was used to prepare crude cell extracts from *Bacillus thuringiensis* serovar Berliner ATCC10792.

The AHL degradation activity in the cell-free culture medium and the CCEs of 20J was tested by incubating 500 µl of supernatant and CCEs with 50 µM AHL for 24 h at 22°C and 200 rpm, and the remaining AHL was detected using bioassays and high pressure liquid chromatography-mass spectrometry (HPLC-MS) methods. In the bioassay, the capacity to degrade 10 µM *N*-hexanoyl-L-homoserine lactone (C6-HSL) was tested. After 24 h of degradation, 100 µl of the mixture were placed in wells made on an LB plate overlaid with a 1/100 dilution of an overnight culture of *Chromobacterium violaceum* CV026 in soft LB (8% agar). The plates were incubated for 24 h at 30°C and the production of violacein was observed. As controls, wells were filled with 100 µl sterile MB or PBS plus 10 µM C6-HSL and treated as the samples.

The capacity of CCEs to degrade 50 µM *N*-butanoyl-L-homoserine lactone (C4-HSL) or *N*-dodecanoyl-L-homoserine lactone (C12-HSL) was also tested, and the activity was measured by HPLC-MS. AHLs were incubated for 24 h with 20J CCEs at 22°C and 200 rpm. Then the reaction mixtures were extracted with or without acidification to pH 2.0 (24 h at 22°C, 200 rpm). The remaining AHLs were extracted 3 times with the same volume of ethyl acetate and evaporated to dryness under nitrogen flux. Dried extracts were then resuspended in 200 µl acetonitrile and AHLs quantified by HPLC-MS. AHL degradation was compared with that obtained in PBS and with live 20J cells from a 24 h MB culture, washed and resuspended in PBS.

Specificity of the QQ activity

In order to determine the specificity of the QQ activity of *Tenacibaculum* sp. strain 20J, AHLs with different side chain lengths were exposed to 20J CCEs as explained above, and then extracted and quantified by HPLC-MS. Control samples were established with PBS plus 50 µM AHL and extracted the same way. The AHLs used in these assays were C4-HSL, C6-HSL, *N*-oxohexanoyl-L-homoserine lactone (OC6-HSL), *N*-heptanoyl-L-homoserine lactone (C7-HSL), *N*-octanoyl-L-homoserine lactone (C8-HSL), *N*-decanoyl-L-homoserine lactone (C10-HSL), C12-HSL, *N*-oxododecanoyl-L-homoserine lactone (OC12-HSL) and *N*-tetradecanoyl-L-homoserine lactone (C14-HSL).

HPLC-MS methodology

The HPLC system used was an Agilent 1100 series (Agilent Technologies). The column was a Zorbax Eclipse XDB-C18, 150 × 4.6 mm (5 µm particle size). The mobile phase was 0.1% formic acid in water (A), and methanol (B) and the flow rate was 0.4 ml min^{-1}. The gradient profile was as follows: 50% B over 0 to 10 min, a linear gradient from 50 to 90% B over 15 min, followed by 90% B for 25 min. The column was re-equilibrated for a total of 4 min. Samples (20 µl) were diluted in 0.1% formic acid in acetonitrile and injected onto the column.

The MS experiments were conducted on an API4000 triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems) used in positive ion electrospray mode.

Synthetic AHLs with acyl side chain lengths of 4 to 14 carbons were used as standards. The AHLs were quantified by comparison with a calibration curve constructed for molecular ion abundance, using each

of the appropriate AHL synthetic standards (Milton et al. 2001).

Characterization of AHL-degradation activity

To determine the minimal active concentration (MAC) of the 20J CCEs, signal C10-HSL at 50 µM was exposed to different dilutions of CCE in autoclaved marine water (pH 6.5) with protein concentrations ranging from 1000 to 0.1 µg ml^{-1}. The mixture was incubated for 24 h at 22°C and 200 rpm prior to organic extraction in order to quantify the remaining AHLs by HPLC-MS methodology.

To define the AHL-degradation kinetics of CCEs from *Tenacibaculum* sp. strain 20J, two 24 h cultures in 200 ml of MB were used to obtain 2 CCEs in autoclaved marine water pH 6.5 or PBS as described before, and the protein concentrations of the CCEs were adjusted to the MAC established in the previous experiment (10 µg ml^{-1}). Both CCEs were then inoculated with 50 µM C12-HSL and incubated at 22°C and 200 rpm for 2 h. Aliquots (500 µl) were extracted from the reaction mixtures at different times from time 0 h to quantify remaining C12-HSL by HPLC-MS. The AHL-degradation kinetics of CCEs was compared to that of marine water, PBS and live 20J cells resuspended in PBS.

The activity of 20J CCEs on different sized AHLs was also analysed. CCEs (10× MAC) obtained in PBS were exposed to 50 µM C6-HSL and C12-HSL for 12 h and the remaining signal was measured at different time points by HPLC-MS and compared to that of controls in PBS. Cultures were carried out in triplicate. AHL-degradation units were defined as the amount of protein in the CCE (µg ml^{-1}) that degrades 1 nM of AHL per minute.

Production and degradation of AHLs in cultures of the fish pathogen *Edwardsiella tarda* ACC35.1

The AHL production of *Edwardsiella tarda* ACC35.1 was studied in 100 ml cultures with or without CCEs from *Tenacibaculum* sp. strain 20J at a concentration of 10× MAC. A previous experiment was carried out to establish that this dose was sufficient to completely eliminate the AHLs in 24 h. Cultures were inoculated 1/100 with an overnight culture of the pathogen and incubated at 25°C and 200 rpm for 48 h. At different times from time 0 h, 15 ml samples were taken and centrifuged (2000 × *g*, 5 min) to obtain the supernatants. Remaining AHLs in supernatants were extracted twice with 15 ml of dichloromethane, and solvent was evaporated to dryness in a rotavapor system at 40°C. AHLs produced by *E. tarda* ACC35.1 were reconstituted in 1 ml acetonitrile and quantified by HPLC-MS methodology. To compare the activity of 20J CCEs with that of a known AHL-QQ bacteria, *E. tarda* ACC35.1 cultures were also supplemented with CCEs from *Bacillus thuringiensis* serovar Berliner ATCC10792 obtained the same way. Cultures were carried out in triplicate.

RESULTS

Characterization of QQ activity in *Tenacibaculum* sp. strain 20J

Among the marine bacterial strains isolated and identified in a previous study (Romero et al. 2011), *Tenacibaculum* sp. strain 20J was selected for a more detailed study of its AHL-QQ activity due to its ability to degrade long and short chain signals. Bioassays revealed that QQ activity against C6-HSL was present in the CCEs but not in cell-free culture medium of 20J (Fig. 1A, as revealed by biosensor CV026). Moreover, the QQ activity of the CCEs was present in the soluble fraction of the extracts obtained after ultracentrifugation, which indicates that the QQ activity should be derived from a soluble enzyme (data not shown). The HPLC-MS analysis confirmed that C4-HSL and C12-HSL were degraded by live cells and CCEs of *Tenacibaculum* sp. strain 20J. In the case of C4-HSL, acidification produced a recovery of the molecule, especially in CCE-treated samples, indicating the presence of a possible lactonase enzyme (Yates et al. 2002). This was also supported by detection of open lactone rings in the products of C6-HSL degradation by live cells and CCEs from 20J (data not shown). For C12-HSL, a long chain AHL, there was only a faint recovery of AHL after acidification, indicating that these AHLs could be degraded by a non-lactonase enzyme or be further degraded by a different enzyme after the action of lactonase, precluding its detection (Fig. 1B). CCEs from *Tenacibaculum* sp. strain 20J were also assayed for their AHL degradation specificity and showed 100% degradation activity against all the AHLs tested within 24 h (data not shown).

As all AHLs tested were degraded, the middle-sized signal C10-HSL was chosen to determine the MAC of 20J CCEs. C10-HSL at 50 µM was exposed to different dilutions of CCE in marine water for 24 h and remaining AHL was measured by HPLC-MS. The MAC was 10 µg CCE protein ml^{-1}.

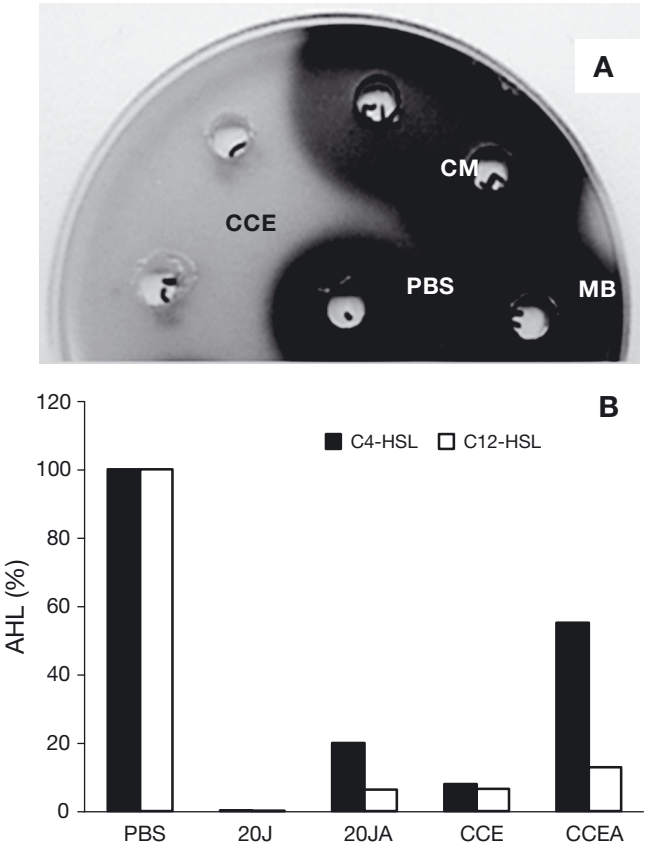

Fig. 1. *Tenacibaculum* sp. (A) Bioassay with *Chromobacterium violaceum* CV026 to detect degradation of *N*-hexanoyl-L-homoserine lactone (C6-HSL) by *Tenacibaculum* sp. strain 20J spent culture media (CM) and crude cell extract (CCE) in relation to controls of marine broth (MB) and phosphate buffered saline (PBS). (B) Percentage of *N*-acylhomoserine lactone (AHL) degradation activities, as measured by HPLC-MS, shown by the whole culture (20J) and CCEs after 24 h, with or without further acidification to pH 2.0 (20JA and CCEA), in relation to controls of 50 µM AHL in PBS. *N*-butanoyl-L-homoserine lactone (C4-HSL) (black bars) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) (white bars). Data are means of 2 independent experiments

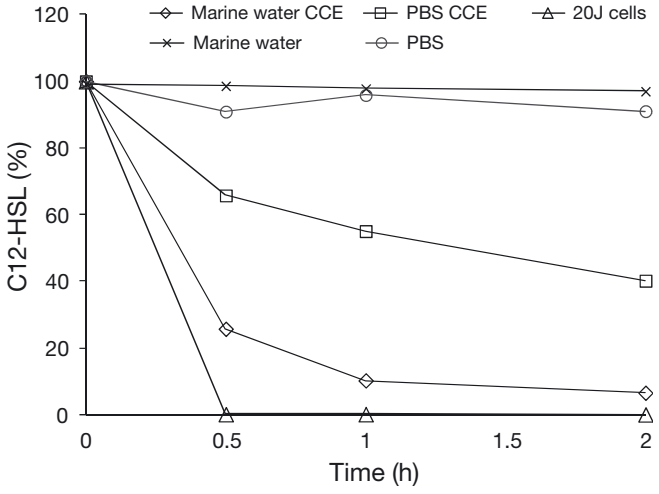

Fig. 2. *Tenacibaculum* sp. Kinetics of 50 µM *N*-dodecanoyl-L-homoserine lactone (C12-HSL) degradation by crude cell extracts (CCEs) from *Tenacibaculum* sp. strain 20J obtained in marine water or PBS, compared with that of living 20J cells. Controls were set with marine water and PBS

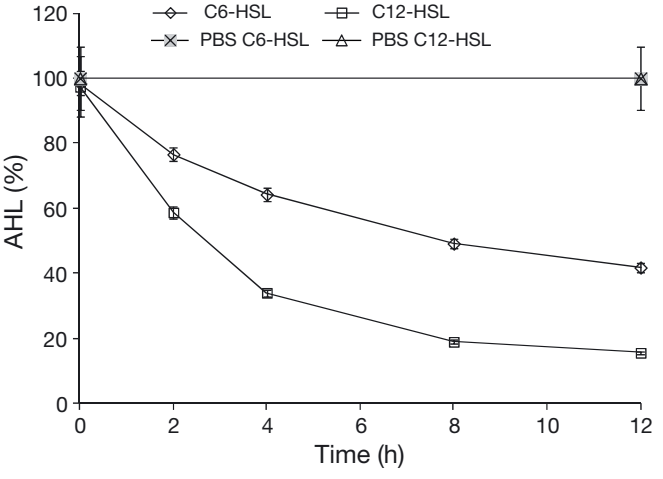

Fig. 3. *Tenacibaculum* sp. Kinetics of 50 µM *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) degradation by crude cell extracts (CCEs) from *Tenacibaculum* sp. strain 20J obtained in PBS. Controls were set with the signals in PBS. Data are means ± SD of 3 independent degradation reactions

The C12-HSL degradation kinetics of 20J CCEs obtained in PBS or marine water were investigated by taking samples at different times: 0, 30, 60 and 120 min. The activity was higher when the CCE was prepared in marine water, since after 2 h of assay, 95% of the C12-HSL was degraded by 10 µg CCE protein ml^{-1}, but a CCE of the same concentration in PBS could only degrade 60% of the signal (Fig. 2). Living *Tenacibaculum* sp. strain 20J cells showed a strong degradation activity towards C12-HSL, since 100% of the AHL was degraded after 30 min (Fig. 2). C6-HSL and C12-HSL degradation kinetics of 20J CCEs obtained in PBS were investigated by taking samples at different times: 0, 2, 4, 8 and 12 h. Results showed that AHL-degradation activity was higher for the longer signal (C12-HSL), since after 12 h of assay, 85% of the C12-HSL was degraded compared to only 60% of the C6-HSL (Fig. 3). C6-HSL and C12-HSL degradation units, defined as the amount of protein in CCEs (µg ml^{-1}) that degrade 1 nM of AHL per minute, were 0.48 and 0.57 U ml^{-1}, respectively.

Production and degradation of AHLs in cultures of the fish pathogen *Edwardsiella tarda* ACC35.1

The AHLs produced by *Edwardsiella tarda* strain ACC35.1, studied in this work, were C6-HSL and OC6-HSL. The maximum concentrations of these signals in the media were detected after 24 h of culture and approximately 2 times more OC6-HSL was produced than C6-HSL (Fig. 4A).

In order to study the feasibility of the application of QQ enzymes to tackle the AHL-QS system of a fish pathogen, we added 20J CCEs to cultures of *Edwardsiella tarda* strain ACC35.1 at a concentration of 10× MAC (100 µg CCE protein per ml of culture), as well as CCEs from *Bacillus thuringiensis* serovar Berliner ATCC10792 at the same protein concentration. In the case of cultures supplemented with 20J CCEs, the accumulation of AHL was scarce after 24 h but surprisingly, *Bacillus* CCEs at the same concentration and obtained in the same way were unable to degrade the signals at any sampling point, indicating that a significantly higher concentration was needed to reduce the amount of AHLs produced by the pathogen (Fig. 4A).

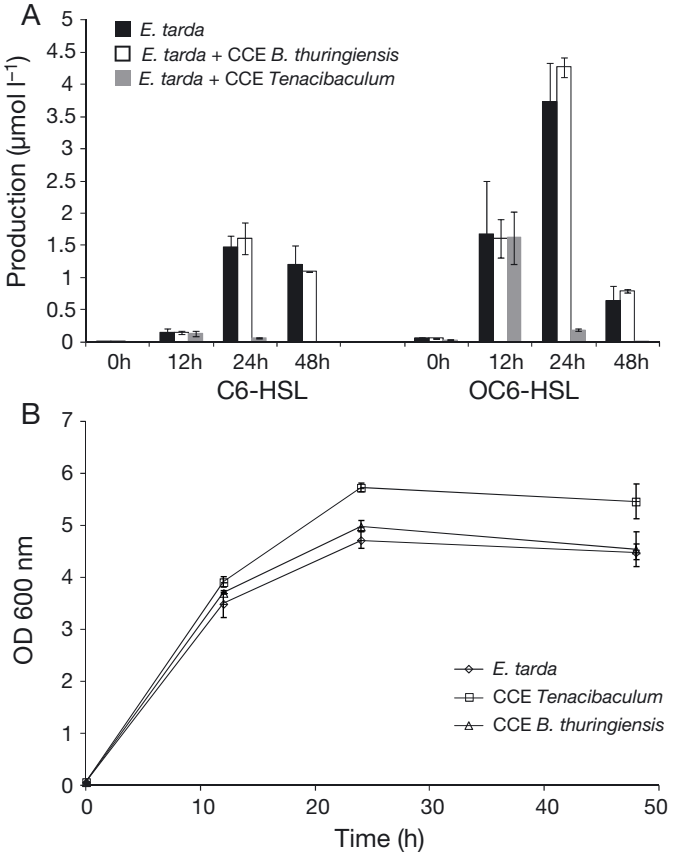

Fig. 4. *Edwardsiella tarda* ACC35.1. (A) Production of *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-oxohexanoyl-L-homoserine lactone (OC6-HSL) by cultures with or without addition of crude cell extracts (CCEs, 100 µg ml^{-1}) from *Tenacibaculum* sp. strain 20J or *Bacillus thuringiensis* serovar Berliner ATCC10792. (B) Growth curves with or without addition of CCEs. Data are means ± SD of 3 independent cultures

Interestingly, the cultures of *Edwardsiella tarda* ACC35.1 supplemented with 20J CCEs registered higher growth than control cultures or cultures supplemented with *Bacillus* CCEs (Fig. 4B), which could be related to a blockage of the expression of genes controlled by QS.

DISCUSSION

QQ activity in *Tenacibaculum* sp. strain 20J is cell-bound (Fig. 1) as are most of the known lactonases and acylases that have been described; exceptions are the acylases AhlM of *Streptomyces* sp., which is active in the spent culture media of the bacteria (Park et al. 2005), and AiiC of *Anabaena* sp. PCC7120, probably located in the periplasm of the cyanobacterium (Romero et al. 2008).

Tenacibaculum sp. strain 20J showed a highly unspecific degradation activity as 100% of the AHLs tested, ranging from C4-HSL to C14-HSL with or without oxo-substitutions, were degraded by 20J CCEs (data not shown). In the case of C12-HSL, degradation kinetics were also studied for live cells and CCEs of 20J (Fig. 2). The activity of 20J CCEs was significantly higher when prepared in marine water, which shows that the enzymatic activity of *Tenacibaculum* sp. strain 20J against AHL signals is improved by oligoelements present in marine water. A number of metal ions, including Na^+, K^+, Ca^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+} and Mn^{2+}, exhibited positive effects on the enzyme activity of the AHL-lactonase AiiA (Chen et al. 2010). These elements are present in sea water (Turekian 1968) which could explain the enhanced QQ activity of the CCEs. Although not directly comparable, enzymatic activity of live 20J cells against AHLs was significantly higher than the activity of bacterial consortia with AHL degradation activity, which needed 24 h to degrade C6-HSL and have been demonstrated to be able to protect *Artemia* sp. and rotifers from infection (Tinh et al. 2007b, Cam et al. 2009). This reinforces the potential of 20J as a QS blocker.

In this study, we also show that 20J CCEs can tackle the AHL-QS system of *Edwardsiella tarda* strain ACC35.1. *E. tarda* is an important fish pathogen causing the septicaemia edwardsiellosis, which

affects a wide variety of fish, including the economically important species *Psetta maxima* and *Solea senegalensis* (Nougayrede et al. 1994, Castro et al. 2012). The presence of QS systems in this species, including LuxI and LuxR homologues and AHL production, has been shown. However, AHL detection has been controversial because while Morohoshi et al. (2004) described the presence of C6-HSL and C7-HSL in culture media of *E. tarda* NUF251, Han et al. (2010) found C4-HSL, C6-HSL, OC6-HSL and an uncharacterized AHL in NUF251 and other strains of *E. tarda*. AHL identification in both studies was carried out by thin-layer chromatography combined with biosensors, which does not allow a definite identification of the molecules. The AHLs produced by *E. tarda* strain ACC35.1 studied in this work were C6-HSL and OC6-HSL, as detected by HPLC-MS methodology. Differences in AHL profiles among strains has been previously observed for other marine pathogens, such as *Aeromonas salmonicida*, where some strains have been described as AHL producers, while no signal could be detected in others strains of the same species (Bruhn et al. 2005). No accumulation of either of the 2 AHLs produced by *E. tarda* strain ACC35.1 could be detected after 24 h when 20J CCEs were added at a concentration of 100 µg CCE protein ml^{-1} (10× MAC) (Fig. 4A). It is worth noting that the culture media of *E. tarda* ACC35.1 (TSB) did not include marine salts and therefore lacked oligoelements that would enhance the activity of the CCEs. In contrast, the addition of the equivalent amount of *Bacillus thuringiensis* CCEs could not prevent the accumulation of either C6-HSL or OC6-HSL in the culture media (Fig. 4A). The lack of effectiveness of the *Bacillus* extract is probably derived from the lower activity of the *Bacillus* lactonase against short-chain AHLs (Momb et al. 2008, Liu et al. 2013). Although Wang et al. (2004) reported only small differences in AHL specificity for the *Bacillus* lactonase AiiA, our previous experiments demonstrated that at least 10 times more *B. thuringiensis* cell extract is necessary to degrade C6-HSL than the same molar amount of C10-HSL (data not shown). The low activity of the *Bacillus* lactonases towards the short-chain AHLs that are common in aquaculture pathogens (Bruhn et al. 2005) indicates that a much higher concentration of *Bacillus* extracts would be needed in comparison to those of 20J to counteract the QS systems of significant aquatic pathogens. Interestingly, the cultures of *E. tarda* ACC35.1 supplemented with CCEs registered higher growth (Fig. 4B), which could be related to a blockage of the expression of genes controlled by QS in rich media (Diggle et al. 2007). This hypothesis is supported by the lack of growth promotion in cultures to which CCEs from *Bacillus* were added.

Present results demonstrate the potential of live cells and CCEs from *Tenacibaculum* sp. strain 20J for use as quorum quenchers, with clear advantages in comparison with *Bacillus* species, mainly due to a higher activity against short-chain AHLs. The use of this strain as an antipathogenic could be an interesting, environmentally friendly solution for treating or preventing infections such as edwardsiellosis and other aquatic bacterial diseases, due to the wide spectrum of AHL-degradation activity presented. Certainly, the proximity of strain 20J to the pathogenic strain *T. discolor* limits its use as live cells, but the high activity found in the cell extracts, both in sea water and PBS, indicates that it could have a wide application as a quorum quencher.

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