

# Acylhomoserine lactone production and degradation by the fish pathogen *Tenacibaculum maritimum*, a member of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group

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## Keywords

quorum sensing; quorum quenching; acylhomoserine lactone; *Cytophaga-Flavobacterium-Bacteroides*.

## Abstract

*Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) is a filamentous, biofilm-forming member of the *Cytophaga-Flavobacterium-Bacteroides* group (or *Bacteroidetes*), which causes the widely distributed marine fish disease tenacibaculosis. A search for *N*-acylhomoserine lactones (AHLs) quorum-sensing (QS) signals in the culture media of nine representative strains of this species using different biosensor strains revealed the presence of short-type AHL activity in all of them. *N*-butyryl-L-homoserine lactone (C4-HSL) was identified in *T. maritimum* NCIMB2154<sup>T</sup> by LC-MS. A degradation activity for long-acyl AHLs (C10-HSL) was subsequently demonstrated in *T. maritimum* NCIMB2154<sup>T</sup>. The acidification of the culture medium after degradation did not allow the recovery of C10-HSL, which indicates a possible acylase-type degradation activity. Even though the physiological processes under the control of AHL-mediated QS in *T. maritimum* need to be further characterized, this discovery extends the paradigm of AHL-mediated QS signalling beyond the *Proteobacteria* and reinforces its ecological significance.

## Introduction

Many bacterial species coordinate responses to environmental changes using complex cell–cell communication mechanisms in a cell-density-dependent manner. This phenomenon is known as quorum sensing (QS) and relies on the accumulation of signal molecules in the surrounding environment to threshold concentrations at which target structural genes are activated (Williams *et al.*, 2007). Several communication systems exist that use different signal molecules, also known as autoinducers (Waters & Bassler, 2005; Williams *et al.*, 2007). In Gram-negative bacteria, the most intensively studied QS systems rely on the use of *N*-acylhomoserine lactones (AHLs), a family of signal molecules differing in the length and substituents of the acyl chain. The use of these molecules as QS signals has been well

established, and their role in the control of important physiological processes such as bioluminescence, biofilm formation, plasmid conjugation, production of exoenzymes and virulence factors, swarming, etc., has been shown in a number of *Proteobacteria*, including several important animal and plant pathogens (Williams *et al.*, 2007). The production of AHLs has so far been limited to a few genera within the *Proteobacteria* (Williams *et al.*, 2007), which has raised questions with regard to the ecological significance of these molecules (Manefield & Turner, 2002). Outside *Proteobacteria*, the production of AHLs has been recently demonstrated for the colonial cyanobacterium *Gloeotheca* PCC6909 and for several strains of *Bacteroidetes* isolated from marine biofilms, although the physiological processes under the control of the QS system were not completely elucidated (Sharif *et al.*, 2008; Huang *et al.*, 2009). AHL-like

activity was also found in the haloalkalophilic archaeon *Natronococcus occultus* (Paggi *et al.*, 2003), but the biochemical nature of the signal has not been confirmed. Short-chain AHL-type activity was also found in *Flavobacterium* sp., a member of the *Cytophaga–Flavobacterium–Bacteroides* (CFB) cluster, but the presence of AHL could not be confirmed by GC-MS (Wagner-Do¨bler *et al.*, 2005).

QS seems to be of special significance in the marine environment. AHL signal molecules are produced by more than half of the marine *Alphaproteobacteria* isolated from various marine habitats (Wagner-Do¨bler *et al.*, 2005). Moreover, the production of AHLs is common among marine and fish pathogenic *Proteobacteria* (Bruhn *et al.*, 2005; Wagner-Do¨bler *et al.*, 2005), controlling the expression of key virulence factors (Defoirdt *et al.*, 2005). Because of the prevalence of QS systems among these pathogens, the inhibition of these processes has been proposed as an alternative to the use of antibiotics in aquaculture (Defoirdt *et al.*, 2004). The inhibition of AHL-mediated QS processes was first described in the marine alga *Delisea pulchra* (Givskov *et al.*, 1996) and has now been described in several eukaryotes and bacteria of terrestrial origin (reviewed by Dong & Zhang, 2005). The isolation and characterization of marine bacterial strains capable of inhibiting QS, a process known as quorum quenching (QQ), either enzymatically or through the production of inhibitors or antagonists may help to develop new biotechnological tools. Several results indicate the viability of this approach for the treatment of infections in aquaculture (Rasch *et al.*, 2004).

The marine bacteria *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) (Suzuki *et al.*, 2001) is a filamentous member of the CFB group causing the fish ‘gliding bacterial disease’ or tenacibaculosis/flexibacteriosis (Avenidaño-Herrera *et al.*, 2004). *Tenacibaculum maritimum* belongs to the CFB cluster, which is also known as *Bacteroidetes* (Ludwig &

Klenk, 2001), and constitutes one of the dominant heterotrophic bacterial groups in aquatic habitats. The fact that *T. maritimum* shifts abruptly from a biofilm to a planktonic mode of growth, a characteristic that could be related to a QS-controlled process (Rice *et al.*, 2005; Wagner-Do¨bler *et al.*, 2005), led us to investigate the possible production and degradation of AHLs by this fish pathogen.

## Materials and methods

### Strains and culture conditions

The *T. maritimum* strains NCIMB2154<sup>T</sup>, NCIMB2153 and NCIMB2158 were obtained from The National Collections of Industrial, Food and Marine Bacteria Ltd (Aberdeen, UK). In addition, six strains isolated in our laboratory from fish farm disease outbreaks from Spain and Portugal were used. These strains belong to the main serotypes and clonal lineages described within this pathogen (Table 1) (Avenidaño-Herrera *et al.*, 2004, 2006), and were confirmed as *T. maritimum* by PCR-based analysis (Toyama *et al.*, 1996). The strains were routinely cultured at 20 °C on *F. maritimus* medium (FMM) agar or broth (Pazos *et al.*, 1996) and on marine broth (MB, Difco) for some of the experiments. Liquid cultures were inoculated with a 10% volume of a 24-h liquid culture and maintained in a shaker at 100 r.p.m. Cultures were double-checked for purity on Marine Agar (Difco) and FMM before and after each experiment.

Three *lux*-based *Escherichia coli* JM109 AHL biosensor strains that respond to AHLs with different side chain lengths were used for the detection of AHL production (Swift *et al.*, 1997; Winson *et al.*, 1998). The biosensor strains were grown at 37 °C in Luria–Bertani (LB) broth or agar supplemented with the adequate antibiotics.

Table 1. AHL activity in acidified culture media extracted with dichloromethane (1 : 1 v/v) of nine different isolates of *Tenacibaculum maritimum* as detected by TLC using the *lux*-based reporter strain *Escherichia coli* JM109 pSB536 (specific for short-length AHLs)

Bacterial isolate	Host species	Origin	Serotype <sup>x</sup>	AHL activity (sensor <i>Escherichia coli</i> pSB536)	
				24 h	48 h
NCIMB2154 <sup>T</sup>	<i>Pagrus major</i>	Japan	O1/O2 (O2)	+	++
NCIMB2158	<i>Solea solea</i>	UK	O2	++	+
ACR104.1	<i>Scophthalmus maximus</i>	Spain	O2	+++	+
PC424.1	<i>Scophthalmus maximus</i>	Spain	O2	+	++
NCIMB2153	<i>Acanthopagrus schlegeli</i>	Japan	O1/O2 (O1)	++	+
PC503.1	<i>Solea senegalensis</i>	Spain	O1	++	+++
PC538.1	<i>Sparus aurata</i>	Spain	O1	++	+
LgH35-O3	<i>Solea senegalensis</i>	Spain	O3	—	+++
ACC6.1	<i>Scophthalmus maximus</i>	Portugal	O3	+	++

Samples were extracted 24 and 48 h after inoculation. Activity was evaluated in comparison with the intensity of the spots obtained for the type strain *Tenacibaculum maritimum* NCIMB2154<sup>T</sup> (Fig. 1).

<sup>x</sup>Serotypes follow the typing schema of Avenidaño-Herrera *et al.* (2006).

Additionally, the AHL biosensor strains *Chromobacterium violaceum* CV026 (McClellan *et al.*, 1997) and *C. violaceum* VIR07 (Morohoshi *et al.*, 2008) were used for the AHL-degradation assays in solid plates as explained below (McClellan *et al.*, 1997). These strains were routinely cultured on LB medium supplemented with kanamycin (50 mg mL<sup>-1</sup>) at 30 °C.

### Sampling and AHL extraction

Samples (100 mL) from cultures of nine different strains of *T. maritimum* grown in liquid FMM were obtained 24 and 48 h after inoculation, acidified to pH 2 with HCl 1 M in a shaker at 200 r.p.m. for 12 h at 20 °C, to ensure the absence of any AHL lactonolysis products, and extracted with dichloromethane as described previously (Yates *et al.*, 2002). Dried extracts were reconstituted in 1 mL ethyl acetate and stored at -20 °C until further analysis.

### Thin-layer chromatography (TLf) detection assays of extracted samples

Two microlitres of the ethyl acetate extracts obtained from the spent culture media were run on reversed-phase hydrocarbon-impregnated C18 silica gel plates using methanol : water 60 : 40 v/v as the mobile phase (Shaw *et al.*, 1997). Two microlitres of synthetic AHLs (Sigma, stock concentration 50 mg mL<sup>-1</sup>) were run as controls: *N*-octanoyl-L-homoserine lactone (C8-HSL) for *E. coli* JM109 pSB401, *N*-butyryl-L-homoserine lactone (C4-HSL) for *E. coli* JM109 pSB536 and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) for *E. coli* JM109 pSB1075 (Winson *et al.*, 1998). Plates were dried and overlaid with 3 mL of semi-solid LB medium (8% agar) inoculated with 30 mL of an overnight culture of the corresponding sensor strain. Plates were incubated at 37 °C and every hour, radiographic plates were laid over them to detect the emission of bioluminescence.

### Lfl-MS AHL identification

LC-MS analyses were carried out simultaneously in the laboratories in Nottingham and Santiago using different equipment and slightly different conditions to confirm the presence of AHLs unequivocally. In Nottingham, a Shimadzu series 10AD VP equipped with binary pumps, a vacuum degasser and an SIL-HTc autosampler and column oven (Shimadzu, River Drive, MD) was used as the LC system. As column a Phenomenex Gemini C18, 150 × 2 mm (5 mm particle size), at 45 °C was used. The mobile phase was built by 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.45 mL min<sup>-1</sup>. The elution conditions were as follows: 1 min 0% B, linear gradient to 50% B for 0.5 min and then a linear gradient from 50% to 90% B over 4 min, then 2.5 min 99% B over

2 min, then ramped back to the starting conditions in 0.2 min. The column was re-equilibrated for a total of 4 min. Samples were redissolved in 50 mL acetonitrile before use and a 10-mL volume was injected onto the column (Ortori *et al.*, 2007). Parallel analyses were carried out using an HPLC 1100 series (Agilent, Santa Clara, CA) 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. Temperature and mobile phases were the same as above, but the flow rate was set at 0.22 mL min<sup>-1</sup>. In this equipment, 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 in 1 min, ramped back to the 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 in Santiago on an API 4000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, 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 and to trigger subsequent quality product ion spectra (product ion PI, MS2). The conditions for the generation of the MRM-triggered spectra were as follows: DP ramped from 35 to 57, CE 14-28, CXP 8. Analyses were confirmed on a 4000 QTRAP hybrid triple-quadrupole linear ion trap mass spectrometer (Applied Biosystems) equipped with a TurboIon source used in positive ion electrospray, MRM mode. In this case, the conditions for the generation of the MRM-triggered spectra were as follows: DP ramped from 25 to 50, CE 15-45, CXP 12.

AHL with or without a 3-oxo or a 3-hydroxy substitution and with an acyl side chain length of 4 (C4-HSL, 3-oxo-C4-HSL and 3-hydroxy-C4-HSL), 6 (C6-HSL, 3-oxo-C6-HSL and 3-176 hydroxy-C6-HSL), 7 (C7-HSL), 8 (C8-HSL, 3-oxo-C8-HSL and 3-hydroxy-C8-HSL), 10 (C10-HSL, 3-oxo-C10-HSL and 3-hydroxy-C10-HSL), 12 (C12-HSL, 3-oxo-C12-HSL and 3-hydroxy-C12-HSL), 13 (C13-HSL, 3-oxo-C13-HSL and 3-hydroxy-C13-HSL) or 14 (C14-HSL, 3-oxo-C14-HSL and 3-hydroxy-C14-HSL) were used as standards. Acyl-HSLs were identified and confirmed by comparing both the elution time and the spectra from any peaks obtained with those of the standards.

### AHL-degradation activity assay

*Chromobacterium violaceum*-based solid plate assays (McClellan *et al.*, 1997) were carried out to detect AHL degradation activity in *T. maritimum* NCIMB2154<sup>T</sup>. Two different sensor strains were used to detect AHL degradation. *Chromobacterium violaceum* CV026 (McClellan *et al.*,

1997) was used to measure the degradation of C6-HSL and *C. violaceum* VIR07 was used to measure the degradation of C10-HSL (Morohoshi *et al.*, 2008). Twenty microlitres of stock solutions of C6-HSL or C10-HSL were added to 500 mL of an overnight culture of *T. maritimum* NCIMB2154<sup>T</sup> in MB (final concentration 2 mg mL<sup>-1</sup>) and incubated for 24 h at 20 °C. The same amount of AHL was added to 500 mL of spent culture medium obtained from a 24-h-old culture by filtration through 0.22 mm. The amount of remaining AHL in the culture media of *T. maritimum* was evaluated in LB plates overlaid with 5 mL of semi-solid LB agar seeded with 500 mL of overnight cultures of *C. violaceum* CV026 for C6-HSL or *C. violaceum* VIR07 for C10-HSL. Fifty microlitres of culture supernatants were loaded in wells and adjusted to 100 mL with distilled water. Sterile MB and MB plus C4 or C10-SHLs were set as controls. The same experiment was carried out in FMM broth (data not shown). Plates were incubated for 12–24 h and the production of violacein was examined.

To evaluate the possible type of AHL degradation activity, two flasks with 15 mL of FMM were supplemented with C10-HSL to a final concentration of 2 mg mL<sup>-1</sup>. One of them was inoculated with 1 mL of a 48-h culture of *T. maritimum* NCIMB2154<sup>T</sup> and the other was maintained as control. Flasks were incubated in a shaker at 22 °C under soft agitation (110 r.p.m.). After 24 h, 500 mL of normal and acidified culture media were extracted three times with ethyl acetate, dehumidified onto MgSO<sub>4</sub>, evaporated under nitrogen flux and resuspended in acetonitrile for LC-MS analysis as described above. Before inoculation, 500 mL of FMM+C10-HSL were also extracted and the value of C10-HSL obtained was used to calculate the percentage of degradation.

## Results and discussion

The presence of AHLs was investigated in ethyl-acetate extracts of acidified culture media of the type strain *T. maritimum* NCIMB2154<sup>T</sup> obtained at 24 and 48 h using the three *E. coli* JM109 *lux*-based biosensor strains carrying pSB536, pSB401 or pSB1075 to detect a wide range of AHLs differing in the length of their acyl chain. TLC analysis revealed the presence of short-chain AHLs using the *E. coli* JM109 pSB536 biosensor (Fig. 1). A search for AHL-type QS signals in extracts obtained from the culture media of another eight representative isolates of *T. maritimum* using the same technique revealed the presence of short-chain AHL activity in all of them, although differences were recorded in relation to their peak in activity (Table 1). LC-MS analysis confirmed the presence of *N*-butyryl-L-homoserine lactone (C4-HSL) in the culture media of *T. maritimum* NCIMB2154<sup>T</sup> grown in both FMM (Fig. 2) and MB (data not shown). This AHL was unequivocally identified by

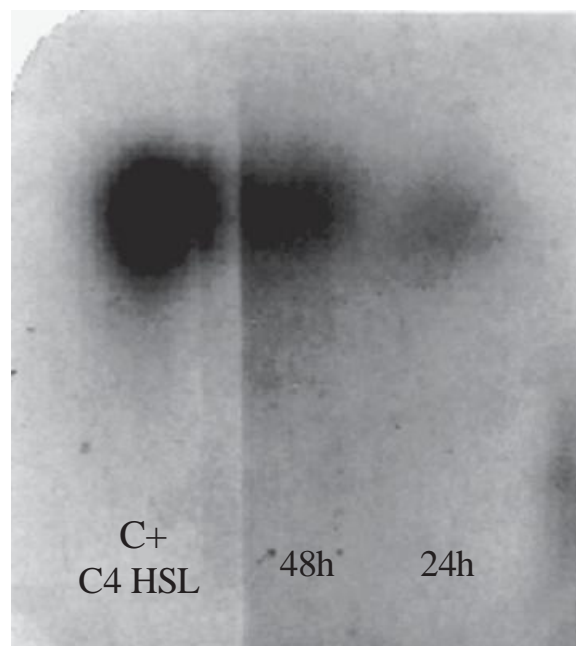


Fig. 1. Detection of AHL activity in the culture media of *Tenacibaculum maritimum* NCIMB2154<sup>T</sup> with the TLC assay. TLCs of ethyl acetate extracts of acidified culture media obtained after 24 and 48 h were covered with the *lux*-based sensor strains, followed by exposure to X-ray films. Positive results were obtained with the sensor strain *Escherichia coli* JM109 pSB536, specific for the detection of short-length AHLs. Control (C+): C4-HSL.

comparison of its mass spectra with those of pure standards (Fig. 3). As this is the first description of the production of AHLs by a pathogenic member of the CFB cluster, the analyses were carried out simultaneously in both laboratories using different chromatographic conditions. The results confirmed unequivocally the presence of the C4-HSL.

So far, no physiological role other than as QS signals has been assigned to AHLs, except as a chelator, for tetramic acid (a derivative of 3-oxo-C12) or antibiotic activity for both 3-oxo-C12-HSL and tetramic acid (Kaufmann *et al.*, 2005; Schertzer *et al.*, 2009). In addition, a role as biosurfactant has been attributed to long-chain AHLs (Daniels *et al.*, 2006). Therefore, even though the physiological features under the control of these molecules in *T. maritimum* remain to be investigated, the production of C4-HSL by *T. maritimum* strains extends the paradigm of AHL-mediated QS beyond the *Proteobacteria*. As the physiological processes under the control of AHL-mediated QS have so far been described for a limited number of genera of the *Alpha*-, *Beta*- and *Gammaproteobacteria*, many of them human or plant pathogens (Williams *et al.*, 2007), the ecological significance of AHL-mediated QS has been questioned as a key switch controlling gene expression within bacterial populations in nature (Manefield & Turner, 2002). The fact

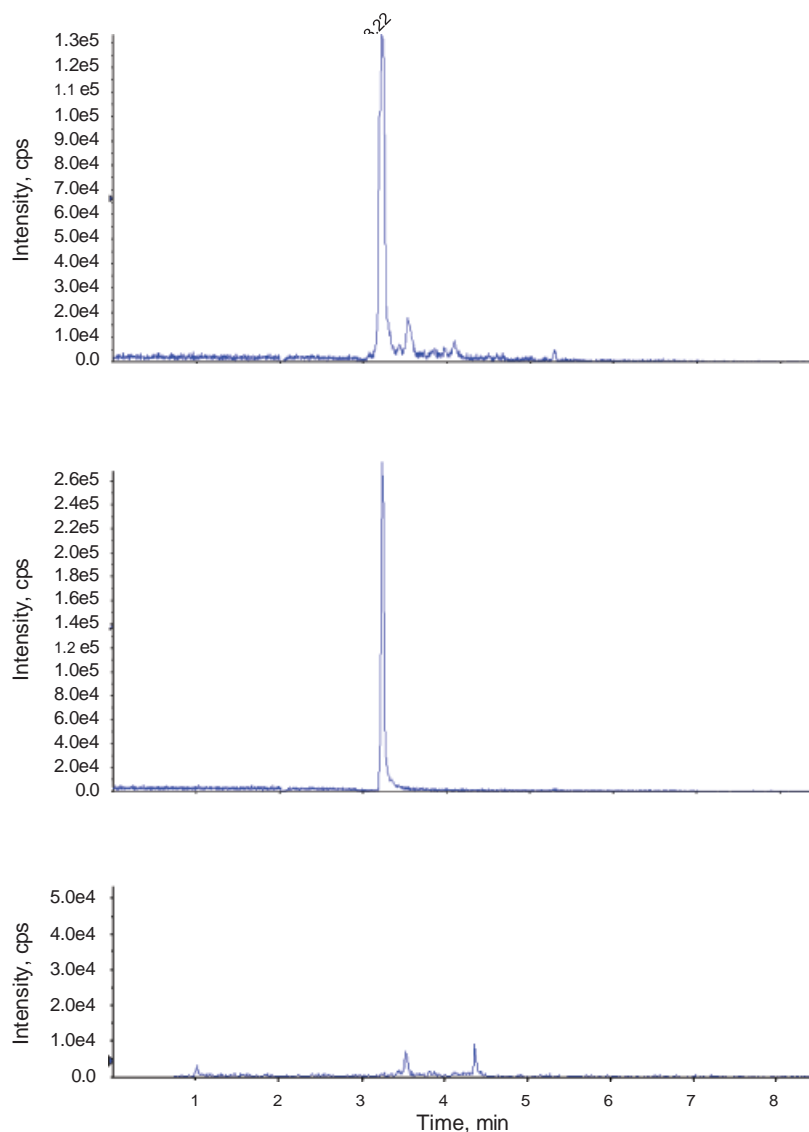


Fig. 2. Extraction ion chromatograms of the MRM transition 172.1 > 102.1 (C4 HSL) of extracts of acidified culture media of *Tenacibaculum maritimum* NCIMB2154<sup>T</sup> (48-h sample), C4-HSL standard and FMM broth.

that genera outside the *Proteobacteria* produce the same signal molecules, and that AHL-degrading activity has been found in Gram-positive, Gram-negative and *Cyanobacteria* (Dong & Zhang, 2005; Romero *et al.*, 2008) and in mammalian cells (Chun *et al.*, 2004), reinforces the ecological significance of AHL-mediated QS processes. The presence of AHL-mediated QS beyond the *Proteobacteria* is not surprising, as a phylogenetic study based on the LuxI/LuxR genes suggested that QS mechanisms were established very early in the evolution of bacteria, although horizontal transfer may have also played an important role in the distribution of QS genes, at least within this group (Lerat & Moran, 2004). The cloning of genes responsible for the synthesis and detection of AHLs in *T. maritimum* will undoubtedly provide new insights into the evolutionary history of QS.

The production of AHLs was demonstrated for all isolates of *T. maritimum* analysed (Table 1), therefore being a conserved trait within this species, which is not the case in some other marine pathogens such as *Aeromonas salmonicida* (Bruhn *et al.*, 2005). Some contradictory results have been published previously regarding the production of AHLs by the genus *Flavobacterium* belonging to the *Bacteroidetes* group. While AHL-like activity was detected in a planktonic isolate of *Flavobacterium* sp. using *E. coli* MT102 carrying the biosensor plasmid pJBA132 based on the luxR gene from *Vibrio fischeri*, the presence of AHLs could not be demonstrated by GC-MS (Wagner-Do"bler *et al.*, 2005). Furthermore, no AHL activity was found in different pathogenic strains of *Flavobacterium psychrophilum* using the sensor strains *C. violaceum* CV026 and *Agrobacterium tumefaciens* NT1 (Bruhn *et al.*, 2005). The differences in the

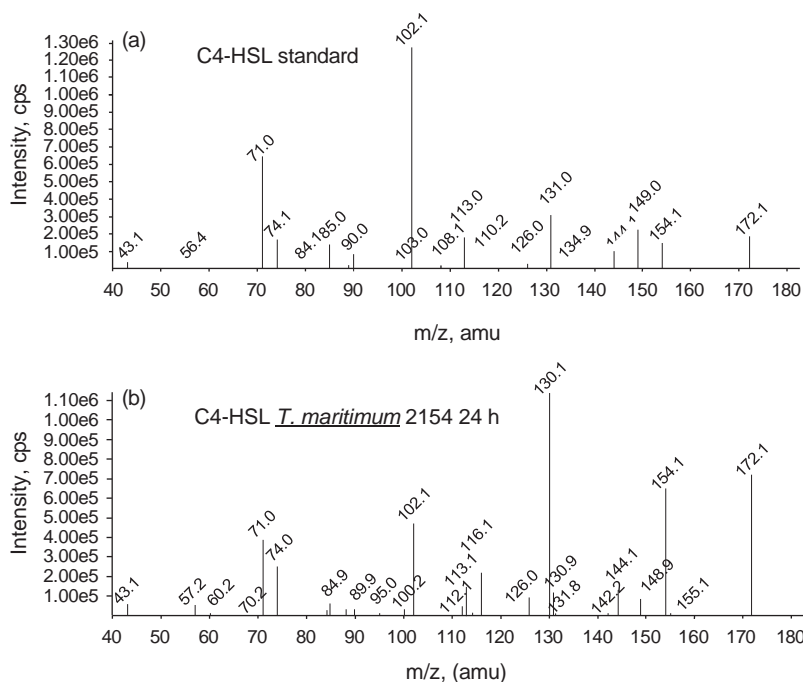


Fig. 3. Product ion spectrum of synthetic C4-HSL (a) and the corresponding peak (b) in the chromatograms of extracts of spent culture medium of *Tenacibaculum maritimum* NCIMB2154<sup>T</sup>.

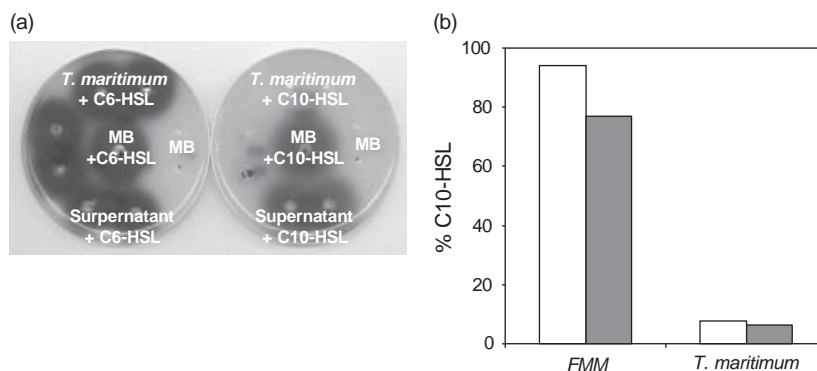


Fig. 4. Agar plate assay for the detection of AHL degradation activity in *Tenacibaculum maritimum* NCIMB2154<sup>T</sup>. (a) Complete degradation of C10-HSL (right) and no degradation of C6-HSL (left) was found after 24 h of exposure. The degradation of C10-HSL was monitored using *Chromobacterium violaceum* VIR07 (right), and the degradation of C6-HSL was monitored using the biosensor strain *C. violaceum* CV026 (left). As negative controls, AHL incubated in culture media were used (central wells). (b) HPLC quantitation of C10-HSL after 24 h in FMM and FMM inoculated with *T. maritimum* NCIMB2154<sup>T</sup>. Spent culture media (open bars) and acidified spent culture media (grey bars) were extracted. Data are expressed as the percentage of the initial C10-HSL concentration ( $2 \text{ mg mL}^{-1}$ ).

AHL activity described probably depend on the assay conditions and the sensor strain utilized. In our experience, data based on the direct evaluation of culture media of marine bacteria, usually MB, should be interpreted with caution, as the media composition could result in inhibition of growth or bioluminescence production by the sensor strain (unpublished data). On the other hand, due to the ability of different compounds to activate the AHL biosensors (Holden *et al.*, 1999), the results should be viewed with caution unless the presence of AHLs can be confirmed by analytical chemical methods. On the basis of our results and as the

detection of the QS activity is strongly dependent on the biosensor strain used and on the culture conditions, it is possible that AHL-based QS systems are more widespread than described so far (Wagner-Do"bler *et al.*, 2005).

An *in vivo* degradation assay was carried out using two biosensor strains of *C. violaceum*. *Chromobacterium violaceum* CV026 was used to detect degradation of short AHLs (C6-HSL), and *C. violaceum* VIR07 was used to detect degradation of long AHLs (C10-HSL). Complete degradation of C10-HSL was observed after 24 h, but no changes in C6-HSL activity were observed (Fig. 4a). The activity should

be cell bound, as no significant degradation was obtained when the C10-HSL was added to cell-free spent culture medium (Fig. 2a). HPLC analysis of the degradation of C10-HSL revealed that 90% of the AHL was degraded after 24 h of exposition to *T. maritimum* cultures, and no recovery of the AHL could be achieved by medium acidification, which may discard a lactonase-type degrading enzyme (Fig. 4b). Further analyses are required to confirm an acylase-type activity. The presence of AHL degradation enzymes has been described in Gram-negative bacteria, possibly as a mechanism to outcompete Gram-positive neighbours (Roche *et al.*, 2004). In Gram negatives, these enzymes can be used for the self-modulation of the level of the signals as already found in *A. tumefaciens* (Zhang *et al.*, 2002). In the case of the bacteroidete *T. maritimum*, the presence of a QQ enzyme for long AHLs may represent an exclusion mechanism to interfere with the QS systems of competitors (Dong & Zhang, 2005).

Evidence is beginning to accumulate indicating that QS and QS inhibition processes, including enzymatic degradation of the signal or QQ, are important in the marine environment. Besides the well-characterized phenomenon of the production of furanones by the red alga *D. pulchra* to avoid surface colonization by Gram-negative biofilm formers (Givskov *et al.*, 1996), QS systems mediated by AHLs have been found in many species of marine pathogenic bacteria (Bruhn *et al.*, 2005). AHLs also seem to play an important role in the eukaryotic–prokaryotic interactions in the marine environment, as demonstrated by the importance of the production of AHLs by marine biofilms for the surface selection and permanent attachment of zoospores of the green alga *Ulva* (Tait *et al.*, 2005), for spore release of the red alga *Acrochaetium* sp. (Weinberger *et al.*, 2007), and for some initial larval settlement behaviours in the polychaete *Hydroides elegans* (Huang *et al.*, 2007). As most of the isolates involved in algal morphogenesis belong to the CFB group (Hanzawa *et al.*, 1998; Matsuo *et al.*, 2003), the discovery of the production and degradation of AHLs by members of this group provides the possibility of new interactions between bacteria and eukaryotes in the marine environment.

For the first time, the production of AHL-type QS signals and QQ activity has been demonstrated simultaneously in a pathogenic member of the CFB group. Because of the ecological significance of the *Cytophaga–Flavobacterium* cluster, especially in the marine environment, the discovery of AHL-mediated QS processes among their members will advance our understanding of the microbial interactions in complex ecosystems. Moreover, cell-to-cell communication phenomena should be reconsidered in other habitats in which the *Bacteroidetes* play an important role, such as intestinal flora or dental plaque. As QS controls the expression of important virulence factors in many pathogenic

bacteria, the disruption of QS mechanisms in *T. maritimum* and other fish pathogenic bacteria may represent a new strategy for the treatment of infections in aquaculture.

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## References

- Avendan˜o-Herrera R, Magarin˜os B, Lo´pez-Romalde S, Romalde JL & Toranzo AE (2004) Phenotypic characterization and description of two major O-serotypes in *Tenacibaculum maritimum* strains from marine fishes. *Dis Aquat Organ* 58: 1–8.
- Avendan˜o-Herrera R, Toranzo AE & Magarin˜os B (2006) Tenacibaculosis infection in marine fish caused by *Tenacibaculum maritimum*: a review. *Dis Aquat Organ* 71: 255–266.
- Bruhn JB, Dalsgaard I, Nielsen KF, Buchholtz C, Larsen JL & Gram L (2005) Quorum sensing signal molecules (acylated homoserine lactones) in Gram-negative fish pathogenic bacteria. *Dis Aquat Organ* 65: 43–52.
- Chun CK, Ozer EA, Welsh MJ, Zabner J & Greenberg EP (2004) Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. *P Natl Acad Sci USA* 101: 3587–3590.
- Daniels R, Reynaert S, Hoekstra H *et al.* (2006) Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *P Natl Acad Sci USA* 103: 14965–14970.
- Defoirdt T, Boon N, Bossier P & Verstraete W (2004) Disruption of bacterial quorum sensing: an unexplored strategy to fight infections in aquaculture. *Aquaculture* 240: 69–88.
- Defoirdt T, Bossier P, Sorgeloos P & Verstraete W (2005) The impact of mutations in the quorum sensing systems of *Aeromonas hydrophila*, *Vibrio anguillarum* and *Vibrio harveyi* on their virulence towards gnotobiotically cultured *Artemia franciscana*. *Environ Microbiol* 7: 1239–1247.
- Dong YH & Zhang LH (2005) Quorum sensing and quorum-quenching enzymes. *J Microbiol* 43: 101–109.
- Givskov M, De Nys R, Manfield M, Gram L, Maximilien R, Eberl L, Molin S, Steinberg PD & Kjelleberg S (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* 178: 6618–6622.
- Hanzawa N, Nakanishi K, Nishijima M & Saga N (1998) 16S rDNA-based phylogenetic analysis of marine flavobacteria that induce algal morphogenesis. *J Mar Biotechnol* 6: 80–82.

- Holden MTG, Chhabra SR, De Nys R *et al.* (1999) Quorum sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other Gram-negative bacteria. *Mol Microbiol* 33: 1254–1266.
- Huang YL, Dobretsov S, Ki JS, Yang LH & Qian PY (2007) Presence of acyl-homoserine lactone in subtidal biofilm and the implication in larval behavioural response in the polychaete *Hydroides elegans*. *Microb Ecol* 54: 384–392.
- Huang YL, Ki J-S, Lee OO & Qian P-Y (2009) Evidence for the dynamics of acylhomoserine lactone and AHL-producing bacteria during subtidal biofilm formation. *ISME J* 3: 296–304.
- Kaufmann GF, Sartorio R, Lee SH, Rogers CJ, Meijler MM, Moss JA, Clapham B, Brogan AP, Dickerson TJ & Janda KD (2005) Revisiting quorum sensing: discovery of additional chemical and biological functions for 3-oxo-acylhomoserine lactones. *P Natl Acad Sci USA* 102: 309–314.
- Lerat E & Moran NA (2004) The evolutionary history of quorum-sensing systems in Bacteria. *Mol Biol Evol* 21: 903–913.
- Ludwig W & Klenk HP (2001) Overview: a phylogenetic backbone and taxonomic framework for prokaryotic systematics. *Bergey's Manual of Systematic Bacteriology* (Boone DR & Castenholz RW, eds), pp. 49–65. Springer-Verlag, Berlin.
- Manefield M & Turner SL (2002) Quorum sensing in context: out of molecular biology and into microbial ecology. *Microbiology* 148: 3762–3764.
- Matsuo Y, Suzuki M, Kasai H, Shizuri Y & Harayama S (2003) Isolation and phylogenetic characterization of bacteria capable of inducing differentiation in the green alga *Monostroma oxyspermum*. *Environ Microbiol* 5: 25–31.
- McClellan KH, Winson MK, Fish L *et al.* (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* 143: 3703–3711.
- Morohoshi T, Kato M, Fukamachi K, Kato N & Ikeda T (2008) *N*-acylhomoserine lactone regulates violacein production in *Chromobacterium violaceum* type strain ATCC 12472. *FEMS Microbiol Lett* 279: 124–130.
- Ortori CA, Atkinson S, Chhabra SR, Ca´mara M, Williams P & Barrett DA (2007) Comprehensive profiling of *N*-acylhomoserine lactones produced by *Yersinia pseudotuberculosis* using liquid chromatography coupled to hybrid quadrupole-linear ion trap mass spectrometry. *Anal Bioanal Chem* 387: 497–511.
- Paggi RA, Martone CB, Fuqua C & De Castro R (2003) Detection of quorum sensing signals in the haloalkalophilic archaeon *Natronococcus occultus*. *FEMS Microbiol Lett* 221: 49–52.
- Pazos F, Santos Y, Macias AR, Nu´nˆez S & Toranzo AE (1996) Evaluation of media for the successful culture of *Flexibacter maritimus*. *J Fish Dis* 19: 193–197.
- Rasch M, Buch C, Austin B *et al.* (2004) An inhibitor of bacterial quorum sensing reduces mortalities caused by vibriosis in rainbow trout (*Oncorhynchus mykiss*, Walbaum). *Syst Appl Microbiol* 27: 350–359.
- Rice SA, Koh KS, Queck SY, Labbate M, Lam KW & Kjelleberg S (2005) Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues. *J Bacteriol* 187: 3477–3485.
- Roche DM, Byers JT, Smith DS, Glansdorp FG, Spring DR & Welch M (2004) Communications blackout? Do *N*-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing? *Microbiology* 150: 2023–2028.
- Romero M, Diggie SP, Heeb S, Ca´mara M & Otero A (2008) Quorum quenching activity in *Anabaena* sp. PCC 7120: identification of AiiC, a novel AHL-acylase. *FEMS Microbiol Lett* 280: 73–80.
- Schertzer JW, Boulette ML & Whiteley M (2009) More than a signal: non-signaling properties of quorum sensing molecules. *Trends Microbiol* 17: 189–195.
- Sharif DI, Gallon J, Smith CJ & Dudley E (2008) Quorum sensing in cyanobacteria: *N*-octanoyl-homoserine lactone release and response, by the epilithic colonial cyanobacterium *Gloeothece* PCC6909. *ISME J* 2: 1171–1182.
- Shaw PD, Ping G, Daly SL, Cha C, Cronan JE, Rinehart KL & Farrant SK (1997) Detecting and characterizing *N*-acylhomoserine lactone signal molecules by thin-layer chromatography. *P Natl Acad Sci USA* 94: 6036–6041.
- Suzuki M, Nakagawa Y, Harayama S & Yamamoto S (2001) Phylogenetic analysis and taxonomic study of marine *Cytophaga*-like bacteria: proposal for *Tenacibaculum maritimum* comb. nov. and *Tenacibaculum ovolyticum* comb. nov., and description of *Tenacibaculum mesophilum* sp. nov. and *Tenacibaculum amyolyticum* sp. nov. *Int J Syst Evol Micr* 51: 1639–1652.
- Swift S, Karlyshev AV, Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S & Stewart GSAB (1997) Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules. *J Bacteriol* 179: 5271–5281.
- Tait K, Joint I, Daykin M, Milton DL, Williams P & Ca´mara M (2005) Disruption of quorum sensing in seawater abolishes attraction of zoospores of the green alga *Ulva* to bacterial biofilms. *Environ Microbiol* 7: 229–240.
- Toyama T, Kita-Tsakamoto K & Wakabayashi H (1996) Identification of *Flexibacter maritimus*, *Flavobacterium branchiophilum* and *Cytophaga columnaris* by PCR targeted 16S ribosomal DNA. *Fish Pathol* 31: 25–31.
- Wagner-Do¨bler I, Thiel V, Eberl L, Allgaier M, Bodor A, Meyer S, Ebner S, Henning A, Pukall R & Schulz S (2005) Discovery of complex mixtures of novel long-chain quorum sensing signals in free-living and host-associated marine *Alphaproteobacteria*. *ChemBioChem* 6: 2195–2206.
- Waters CM & Bassler BL (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Bi* 21: 319–346.
- Weinberger F, Beltran J, Correa JA, Lion U, Pohnert G, Kumar N, Steinberg P, Kloareg B & Potin P (2007) Spore release in *Acrochaetium* sp. (*Rhodophyta*) is bacterially controlled. *J Phycol* 43: 235–241.

Williams P, Winzer K, Chan WC & Cámara M (2007) Look who's talking: communication and quorum sensing in the bacterial world. *Philos T Roy Soc B* 362: 1119–1134.

Winson MK, Swift S, Fish L, Throup JP, Jørgensen F, Chhabra SR, Bycroft BW, Williams P & Stewart GSAB (1998) Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acylhomoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* 163: 185–192.

Yates EA, Philipp B, Buckley C *et al.* (2002) *N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*. *Infect Immun* 70: 5635–5646.

Zhang HB, Wang LH & Zhang LH (2002) Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*. *P Natl Acad Sci USA* 99: 4638–4643.