Aii20J, a wide-spectrum thermostable *N*-acylhomoserine lactonase from the marine bacterium *Tenacibaculum* sp. 20J, can quench AHL-mediated acid resistance in *Escherichia coli*

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Abstract Acyl homoserine lactones (AHLs) are produced by many Gram-negative bacteria to coordinate gene expression in cellular density dependent mechanisms known as quorum sensing (QS). Since the disruption of the communication systems significantly reduces virulence, the inhibition of quorumsensing processes or quorum quenching (QQ) represents an interesting anti-pathogenic strategy to control bacterial infections. *Escherichia coli* does not produce AHLs but possesses an orphan AHL receptor, SdiA, which is thought to be able to sense the QS signals produced by other bacteria and controls important traits as the expression of glutamate-dependent acid resistance mechanism, therefore constituting a putative target for QQ. A novel AHL-lactonase, named Aii20J, has been identified, cloned and over expressed from the marine bacterium *Tenacibaculum* sp. strain 20 J presenting a wide-spectrum QQ activity. The enzyme, belonging to the metallo-β-lactamase family, shares less than 31 % identity with the lactonase AiiA from *Bacillus* spp. Aii20J presents a much higher specific activity than the *Bacillus* enzyme, maintains its activity after incubation at 100 ºC for 10 minutes, is resistant to protease K and α-chymotrypsin, and is unaffected by wide ranges of pH. The addition of Aii20J (20 μg/mL) to cultures of *E. coli* K-12 to which OC6-HSL was added resulted in a significant reduction in cell viability in comparison with the acidresistant cultures derived from the presence of the signal. Results confirm the interaction between AHLs and SdiA in *E. coli* for the expression of virulence-related genes and reveal the potential use of Aii20J as anti-virulence strategy against important bacterial pathogens and in other biotechnological applications.

Keywords Quorum sensing · Quorum-quenching acylhomoserine lactones · Lactonase · *E. coli* · SdiA

Introduction

Numerous human, animal, and plant pathogenic bacteria coordinate important biological functions, including virulence factors, antibiotic biosynthesis or biofilm formation and maturation, through a cell density-dependent gene regulation system known as quorum sensing (QS) (Swift et al. 2001; Waters & Bassler 2005; Williams et al. 2007). Bacteria produce, release, and sense extracellular small molecules called autoinducers (AIs), which are accumulated in the environment, to monitor their own population. When the concentration of AIs reaches a threshold level, the bacteria detect it and alter gene expression, approaching the behavior of a multicellular organism (Waters and Bassler 2005). Although different kinds of signal molecules have been described (LaSarre and Federle 2013) and different QS systems can appear overlapping in some pathogens, the *N*-acylhomoserine lactones (AHLs) are the best studied AIs, being employed by many Gram-negative bacterial species as signal molecules involved in a broad range of biological functions (Dong et al. 2001). AHLs are constituted by an homoserine lactone ring (HSL) lined by an amide bond to a fatty acid that generally contains between 4 and 14 carbons, with or without saturation or C3

hydroxy or oxo substitutions (Whitehead et al. 2001). This lateral fatty acid chain confers the specificity to the QS signal. The canonic AHL-mediated QS system was first identified in the marine bioluminescent bacterium *Vibrio fischeri* and relies on the presence of two genes: the signal synthase LuxI and the signal receptor LuxR (Ng and Bassler 2009).

Although the Gram-negative pathogens *E. coli* and *Salmonella* do not have a *luxI* gene and do not produce AHLs, both species possess an orphan receptor SdiA, a homologue of LuxR that could respond to AHLs released by other bacterial species (Dyszel et al. 2010; Michael et al. 2001) in what is supposed to be an adaptation to complex environment in the mammalian hosts. In *E. coli*, the orphan receptor SdiA has been proposed as a key factor for this pathogenic species to survive and colonize the gastrointestinal tract of cattle (Sharma and Bearson 2013; Smith et al. 2011; Sperandio 2010). A lower prevalence of an SdiA mutant of *E. coli* O157:H7 in feces of weaned calves in comparison with the wild-type strain has been reported, indicating a crucial role of this gene in the colonization of bovine intestine (Sharma and Bearson 2013). The relevant role of the *sdiA* gene in the regulation of the glutamate-dependent resistance acid system has been demonstrated for K-12 and EHEC strains (Dyszel et al. 2010), which could constitute one of the pathways through which SdiA influences gastrointestinal colonization. Although in the current model SdiA would be activated in the AHL-rich environment of bovine rumen, triggering the glutamate-dependent acid resistance system (Soares and Ahmer 2011; Sperandio 2010) and the addition of AHLs increases the expression of genes related to the glutamate-dependent acid resistance system (Dyszel et al. 2010), the addition of AHLs to the culture media did not result in a significant increase in the acid resistance of cultures, suggesting that SdiA could be activated by a different signal and opening the possibility that molecules different from AHLs being the cognate and/or synergistic inducers of SdiA activity in *E. coli*. The picture could be even more complicated since *E. coli* uses several QS systems to coordinate its gene expression through extracellular signals, including indole signaling, the *lux*S/AI-2, and the AI-3/epinephrine QS systems (Walters and Sperandio 2006), and therefore synergistic or interference effects could be present among the different QS channels, as reported for other pathogens with complex QS networks such as *Pseudomonas aeruginosa* or *Vibrio* spp. (Ng and Bassler 2009).

Since QS systems are a key in the expression of virulence factors of many important pathogens, the interference with these signaling mechanisms, known as quorum-sensing inhibition (QSI), has been proposed as a promising anti-pathogenic strategy for the control of pathogens virulence (Bjarnsholt and Givskov 2007; Dong et al. 2007; LaSarre and Federle 2013; Williams et al 2007). Moreover, the inhibition of QS systems has other important applications, like the control of membrane fouling (Lade et al. 2014). Two main strategies have been explored to interfere with AHL-mediated QS systems: the use of inhibitory substances that block signal reception and the enzymatic degradation of signals. Several molecules with inhibitory or antagonist activity of QS signal reception have been described (Hentzer and Givskov, 2003; LaSarre and Federle 2013). Among these, the furanones, the first QSI molecules described, are produced by the red seaweed *Delisea pulchra* and are capable of mimicking the AHLs produced by biofilm-forming bacteria by blocking signal reception, avoiding in this way surface colonization of the seaweed (Givskov et al. 1996). Furanones can protect rainbow trout from infection with *Vibrio anguillarum*, but result toxic for the fishes (Rasch et al. 2004). Recently, andrographolide, a QS inhibitor known to interfere with the AI-2 signal QS system has been demonstrated to reduce cell damage caused by avian pathogenic *E. coli* (Guo et al. 2014) demonstrating the potential of the QSI strategies to control the expression of virulence factors in *E. coli.*

The enzymatic inactivation of AIs, also known as quorum quenching (QQ) is another QSI alternative that has been largely explored. Among the different possibilities of AHL cleavage/modification mechanism, acylases and lactonases are the best characterized QS enzymes. Acylases separate the HSL ring from the fatty acid while lactonases hydrolyze the HSL ring, a reaction that also occurs spontaneously at high pH values (Yates et al. 2002). AiiA from *Bacillus* sp. strain 240B1 was the first identified enzyme with AHL-lactonase activity (Dong et al. 2000). Up to now, several lactonases have been described in genus *Bacillus* sp. (Chen et al. 2010; Liu et al. 2008; Momb et al. 2008). All of them share more than 90 % identity and are classified into the metallo-β-lactamase (MBL) superfamily. Metallo-β-lactamases (MBL) present common structural characteristics such as the tertiary structure (αββα), the conserved zinc-binding motif and two key residues to interact with metal ions to carry out their catalytic activity (Bebrone 2007; Murphy et al. 2003). In spite of the MBLs representing the most abundant cluster among AHL-degrading enzymes, several unrelated families of enzymes with AHL-lactonase activity belonging to the phosphotriesterase (PTE) family or α/β-hydrolase-fold family have been described (Romero et al. 2014). To date, many AHL-degrading genes have been cloned and characterized from bacteria, fungi, mammalian cells, and metagenomic libraries constructed from environmental soil samples (Romero et al. 2014). Enzymes with AHL degradation activity have been tested as quorum quenchers in different areas with special mention marine fish farming industry. Degradation of AHLs by AiiA enzyme expressed in *Pichia pastoris* increased the survival of carp when co-injected with *Aeromonas hydrophila* (Chen et al. 2010), and the inclusion of the thermostable AiiA from *Bacillus* sp. strain AI96 in fish feed also reduced *A. hydrophyla* infection in zebra fish (Cao et al. 2012). Heterologous expression of AiiD acylase reduced

significantly *P. aeruginosa* virulence in the nematode *Caenorhabditis elegans* (Lin et al. 2003). The effectivity of the QQ strategy has been also demonstrated alto in mammal models, since the inhalation of a lactonase reduced mortality in an acute lethal model of *P. aeruginosa* pneumonia (Hraiech et al. 2014).

In a previous work, strain *Tenacibaculum* sp. 20J (CECT 7426) was identified presenting with a wide-spectrum QQ activity (Romero et al. 2011a) which confers these strain important advantages in comparison with *Bacillus* strains presenting QQ capacity (Romero et al., 2014). *Tenacibaculum* sp. strain 20J, a member of the *Cytophaga-Flexibacter-Bacteroides* (CFB) group, presents a 99 % 16S rRNA gene sequence identity with *Tenacibaculum discolor* DSM 18842, which causes the fish Bgliding bacterial disease^ or tenacibaculosis/flexibacteriosis (Piñeiro-Vidal et al. 2008), but strain 20J can grow in the absence of marine salts which excludes its classification as member of this species (Piñeiro-Vidal et al. 2008). The QQ activity of strain 20J is constitutive and is cell-bound and has been demonstrated to be able to quench the production of AHLs by the fish pathogen *Edwarsiella tarda* (Romero et al. 2014), therefore presenting a high potential to control pathogens related to aquaculture, human health, animals, and plants or to avoid the biofilm formation (Romero et al. 2014).

Recently, three other QQ enzymes have been characterized belonging to the CFB cluster (Tang et al. 2015; Wang et al. 2012; Zhang et al. 2015) presenting unique characteristics. In this study, we cloned, over-expressed, and characterized the enzyme responsible for the QQ activity in strain 20J that resulted to be a wide-spectrum AHL-lactonase of the MBL family with very strong thermo resistance capacity and investigated the use of this enzyme to counteract the activation of the glutamate-dependent acid resistance mechanisms in *E. coli* K-12 triggered by exogenous AHLs.

Materials and methods

Bacterial strains and growth conditions

Selected bacterial strains and plasmids used in this study are listed in Table 1. *Tenacibaculum* sp. strains were routinely cultured at 22 °C on marine agar or broth (MA/MB, Difco). *E. coli* EPI300™, XL1blue, BL21(DE3) plysS, and K-12 strains were grown in Luria-Bertani (LB) medium supplemented with appropriate antibiotic when required. *Chromobacterium violaceum* AHL biosensor strains were routinely cultured on LB medium at 30 °C. Antibiotics were added at final concentrations of 200 μg/mL ampicillin, 12.5 μg/mL chloramphenicol, 25-50 μg/mL kanamycin, and 25 μg/mL tetracycline as required.

Cloning of AHL-lactonase Aii20J

A genomic fosmid library from *Tenacibaculum* sp. 20J was constructed with the CopyControl™ Fosmid Library Production Kit (Epicentre, Madison, WI). Briefly, purified chromosomal DNA from 20J was randomly sheared by passing it through a 200-μL small-bore pipette tip, treated enzymatically to generate end-repair blunt ends, fragments of 40 kb were then selected by pulsed field gel electrophoresis and ligated intro the cut/linearized and dephosphorylated fosmid vector pCC2FOS. After in vitro packaging into lambda phages and infection of *E. coli* EPI300™, the bacterial cells were plated on LB supplemented with 12.5 μg/mL chloramphenicol. The plates were incubated at 37 °C for 24 h and transfected *E. coli* colonies were transferred to 96-well plates. Functional screening was carried out with *C. violaceum* CV026 and VIR07 (McClean et al. 1997; Morohoshi et al. 2008) to detect *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-decanoyl-L-homoserine lactone (C10-HSL) degradation in concentration 60 μM.

Confirmation of QQ activity of positive clones was performed by HPLC-MS. For the analysis, crude cell extract (CCE) from positive clone was obtained from a 24-h 15-mL LB culture of *E. coli* EPI300™ cells. The culture was centrifuged for 5 min at 2000×*g* in order to separate the biomass from the culture media. The pellet was washed with 15 mL of phosphate buffered saline (PBS) pH 6.7, 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 was filtered through a 0.20 μm and stored at 4 °C prior to use. Aliquots of 500 μL were incubated with 10 μM C6- and C10-HSLs for 24 h at 22 °C with shaking. Then, the reaction mixtures were extracted with or without acidification to pH 2.0. The remaining AHLs were extracted three times with the same volume of ethyl acetate, evaporated under nitrogen flux and resuspended in 400 μL of acetonitrile for HPLC-MS analysis and quantification as previously described (Romero et al. 2014). PBS and *E. coli* EPI300™ with pCC2FOS without the insert plus the same amount of C6 or C10-HSL were used as controls.

The fosmid from the only clone with confirmed AHL degradation activity was sequenced using a GS-FLX Titanium Plus sequencer (Roche) and sequencer contigs assembled with Phrap software. The insert in the positive clone was sheared and fragments between 3 to 5 kb subcloned in pBluescript KS II (+) and transformed in *E. coli* EPI300™ to detect the gene responsible for QQ activity as explained before. Nine positive subclones were sequenced to confirm the responsible genetic sequence of QQ activity.

AHL degradation in genus *Tenacibaculum*

C. violaceum-based solid plate assays were carried out to detect C6- and C10-HSLs degradation activity in *Tenacibaculum* sp. strains as described before (Romero et al. 2010). Fifteen

Table 1 Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
Chromobacterium violaceum		
CV026	AHL biosensor; Km^r	McClean et al. 1997
VIR07	AHL biosensor, Km^r	Morohoshi et al. 2008
Escherichia coli		
BL21(DE3) plysS	F- *omp*T *hsd*S_B (r_B-, m_B-) *dcm gal* λ(DE3) pLysS Cm^r	Promega
EPI300™ Electrocompetent cells	*F⁻ mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ*80dlacZ*Δ*M15* Δ*lacX74 recA1 endA1 araD139* Δ(*ara*, *leu*)*7697 galU galK* λ⁻ *rpsL* (*Str*^{R}) *nupG trfA dhfr*	Epicentre
K-12	MG-1655, wild type	
XL1blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′ proAB lacIq Z*Δ*M15* Tn10 (Tetr)]	Agilent
Tenacibaculum sp.		
Tenacibaculum sp. 20J CECT 7426	Isolated from sediment of fish culture tank, Spain	Romero et al. 2011a
T. aestuarii JCM 13491^T	Isolated from tidal flat sediment, Korea	CECT[a]
T. discolor DSM 18842^T	Isolated from *Solea senegalensis*, Spain	CECT[a]
T. gallaicum CECT 7122^T	Isolated from *Psetta máxima*, Spain	CECT[a]
T. lutimaris DSM 16505^T	Isolated from tidal flat, Korea	CECT[a]
T. maritimum CECT 4276	*Isolated from Acanthopagrus schlegeli*, Japan	CECT[a]
T. maritimum NCMBI 2154^T	Isolated from *Pagrus major*, Japan	NCIMB[c]
T. soleae CECT 7292^T	Isolated from *Solea senegalensis*, Spain	CECT[a]
Plasmids		
pBlue Script KS II (+)	Cloning vector, Ap^r	Agilent
pCC2FOS	Cloning vector, Cm^r	Epicentre
pET28c(+)	Cloning vector; Km^r	Novagen
pET28c(+)-aiiA	pET28c(+) containing *aiiA* gene from *Bacillus* sp.	This study
pET28c(+)-aii20J	pET28c(+) containing *aii20J* gene from *Tenacibaculum* sp. 20 J	This study
pET28c(+)-aiiTA	pET28c(+) containing *aiiTA* gene from *T. aestuarii* JCM 13491^T	This study
pET28c(+)-aiiTD	pET28c(+) containing *aiiTD* gene from *T. discolor* DSM 18842^T	This study
pET28c(+)-aiiTG	pET28c(+) containing *aiiTG* gene from *T. gallaicum* CECT 7122^T	This study
pET28c(+)-aiiTL	pET28c(+) containing *aiiTL* gene from *T. lutimaris* DSM 16505^T	This study
pET28c(+)-aiiTM	pET28c(+) containing *aiiTM* gene from *T. maritimum* NCMBI 2154^T	This study
pET28c(+)-aiiTS	pET28c(+) containing *aiiTS* gene from *T. soleae* CECT 7292^T	This study
pGEM-T easy-aiiA	pGEM-T easy containing *aiiA* gene from uncultured *Bacillus* sp.	This study

[a] Colección Española de Cultivos Tipo (Valencia, Spain)

[b] American Type Culture Collection

[c] National Collection of Industrial, Food and Marine Bacteria (Aberdeen, UK)

milliliters of 24-h cultures of *Tenacibaculum* sp. strains in MB were centrifuged at 2000×*g* for 5 min. Supernatants were then collected, acidified to pH 6.7, and filter-sterilized (0.20 μm) prior to use. Simultaneously, pellets were washed with phosphate buffered saline (PBS) pH 6.7 and resuspended in another 5 mL of the same buffer. Part of these cell suspensions were then used for live cells AHL degradation assays, in which aliquots of 250 μL were mixed with C6 or C10-HSL (10 μM) and incubated for 3 h at 22 °C with shaking. The remaining *Tenacibaculum* cell suspensions in PBS were disrupted by sonication on ice to obtain the crude cell extracts (CCE) that were assayed either directly or after pasteurization at 60 °C for 2 h. As for live cell assays, serial dilutions of CCEs obtained in this way and culture supernatants (250 μL) were mixed with 10 μM of each AHL and incubated for 3 h at 22 °C with shaking. In order to detect AHL inactivation activity, 100 μL of the reaction mixtures was spotted in wells made in LB plates overlaid with 5 mL of a 1/100 dilution of an overnight culture of *C. violaceum* CV026 or VIR07 in soft LB (0.8 % agar). For live cell activity assays, the reaction

mixtures were centrifuged and only the surpernatants were tested in the bioassay. Plates were incubated for 24 h at 30 °C, and the production of violacein was examined. PBS buffer plus the same amount of each AHL was used as control in all plates. Protein in the CCEs was measured by the Lowry method (modified from Lowry et al. 1951), and the minimum active concentration (MAC) of CCEs was stablished as the protein concentration in the highest decimal dilution being able to completely removed the AHLs in 3 h.

Aii20J homologous identification in genus *Tenacibaculum*

The genomic DNA from *Tenacibaculum* strains was used as template for PCR detection of Aii20J homologous. Genomic DNAwas extracted with Wizard® Genomic DNA Purification Kit (Promega). Primers Lact20JF (5′-GATTAACCATGGTAAAAAAAATATTTTTATTAGC-3′) and Lact20JR (5′-GCTATGAATTCAACTTTTTTAATAGATTTTGTAAT-3′) were used for all strains except for AiiTG for which primers Lact20JF and LactTG2R (5′-GCTATGTCGACCTTTTTTAATAGATTTTGTAAT-3′) were used to avoid the presence of an *Eco*RI restriction site. PCR conditions included denaturation at 94 °C, 5 min; 30 cycles of 94 °C, 45 s; 55 °C, 45 s; and 72 °C, 45 s, with a final extension for 10 min. PCR products (about 850 bp) were then sequenced, and a phylogenetic tree of Aii20J homologous was built by the neighbor-joining method using the MEGA 6 phylogenetic tool software package (Tamura et al. 2013) using the default parameters. SignalP 4.1 software was used to predict the signal peptide in the Aii20J sequence and glycosylation prediction was performed by NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

Expression and purification of AHL-lactonases

The PCR products from *Tenacibaculum* sp. strain 20J (Aii20J) and homologous from *Tenacibaculum maritimum* NCMBI 2154^{T} (AiiTM) were purified, digested with *Eco*RI and *Nco*I (Thermo Scientific), and cloned into the *Eco*RI and *Nco*I sites of vector pET28c(+) using T4DNA ligase (Thermo Scientific), to introduce six histidine residues in the C terminus of the protein, and transformed by electroporation into *E. coli* BL21(DE3) plysS. AiiA (accession number CAD44268.1) from *Bacillus* sp. was also cloned to compare the activity of the AHL-lactonase from *Tenacibaculum* with AiiA from *Bacillus* sp. using primer aiiAFWD (5′-GATTAACCATGGTAACAGTAAAGAAGCTTTATTTCG-3′) and aiiARev (5′-GCTATGAATTCTTTATATATTCAGGGAACACTTTAC-3′). The *E. coli* BL21(DE3) plysS strains expressing recombinant proteins were inoculated into fresh LB medium with kanamycin (25 μg/mL) at 37 °C with shaking. After the OD_{600} of the culture reached 0.6, the protein expression was induced by the addition of isopropyl-D-thiogalatopyranoside (IPTG) to a final concentration of 0.1 mM followed by further incubation for 5 h. After incubation, cells were harvested by centrifugation, resuspended with 20 mL of PBS buffer, lysed by sonication on ice, and centrifuged at 4 °C (2000×*g* for 5 min). Due to the formation of inclusion bodies, pellets were washed two times in PBS buffer supplemented with 1 % Triton X-100 and six his-lactonases were purified using the His GraviTrap™ affinity column (GE Healthcare) protein purification kit under denaturing conditions by adding 6 M urea. Recombinant proteins were then refolded by dialysis in PBS with progressively decreasing concentrations of urea. For comparison, the enzyme was also dialyzed in autoclaved diluted marine water (10 ‰ salinity) in order to check the effect of the presence of metallic ions on the refolding of the enzyme. Purified proteins were measured by a UV-Vis Spectrophotometer Q5000 (Quawell) and then stored with an equivalent amount of pure glycerol at −80 °C and analyzed with 12 % SDS-PAGE.

Aii20J characterization

Thermal resistance of recombinant Aii20J in CCEs from *E. coli* BL21(DE3) plysS was evaluated with *C. violaceum*-based assays. CCE at a total protein concentration of 46.8 μg/mL (10 times higher than the minimal amount of CCE necessary to completely hydrolyze 10 μM of AHL in 3 h) was heat-treated at 22, 60, 80, and 100 °C for 10 min in thermoblock. After heat treatment, CCEs were mixed with C6-HSL 10 μM at 22 °C for 3 h and the remaining signal was detected in solid plate assay with *C. violaceum* CV026 as explained before.

Activity of purified recombinant Aii20J after heat treatment was also quantified by HPLC-MS. Aii20J (20 μg/mL, 10xMAC) was mixed with C6-HSL 50 μM in PBS pH 6.7. Two hundred-microliter aliquots were then taken at different times (0, 30, and 90 min) in triplicate, extracted three times with an equal volume of ethyl acetate; the solvent was evaporated under nitrogen flux and resuspended in 400 μL of acetonitrile for HPLC-MS analysis and quantification of remaining C6-HSL. Controls of PBS with the same amount of AHL were processed and extracted in the same way.

AHL degradation specificity of purified Aii20J (20 μg/mL) was evaluated with synthetic signals: *N*-butanoyl-L-homoserine lactone (C4-HSL), C6-HSL, *N*-octanoyl-L-homoserine lactone (C8-HSL), C10-HSL, *N*-dodecanoyl-L-homoserine lactone (C12-HSL), *N*-tetradecanoyl-L-homoserine lactone (C14-HSL), *N*-oxohexanoyl-L-homoserine lactone (OC6-HSL), and *N*-oxododecanoyl-L-homoserine lactone (OC12-HSL) at 10 μM in PBS. After 1 h of exposure, AHLs were extracted and processed as explained before for HPLC-MS analysis and quantification.

In order to determinate the kinetics of AHL degradation by Aii20J, C6- and C10-HSLs (50 μM) were added to a PBS solution buffer with purified Aii20J (20 μg/mL). At different

times (0, 10, 20, 30, 60, and 90 min), 200 μL of each solution was extracted three times with an equal volume of ethyl acetate, evaporated under flux of nitrogen and resuspended in acetonitrile for HPLC-MS analysis and quantification. C6- and C10-HSL degradation kinetics of purified AiiA (40 μg/mL) from *Bacillus* sp. were evaluated at 0, 30, and 90 min. All experiments were performed in triplicate and PBS plus AHLs extracted at the same times were used as controls.

Effect of pH on Aii20J AHL-lactonase activity

To study the stability of Aii20J at various pH values, 20 μg/mL of the purified enzyme were incubated in different buffers with pH values that ranged from 3.0 to 9.0 for 30 min at 22 °C. Then pH was adjusted to 6.7, and C6- and C10-HSL signals were added at a final concentration of 10 μM and incubated for 3 h at 22 °C. Aliquots of 100 μL of each reaction were used to detect the residual AHL activity with the *C. violaceum* biosensors. The buffers used were McIlvaine buffer (pH 3.0 to 5.0), PBS buffer (pH 6.0 and 7.0), and 0.05 M Tris-HCl buffer (pH 8.0 and 9.0). PBS plus each AHL was used as negative control.

Resistance of Aii20J to proteases

To determine the resistance to proteolysis, the purified Aii210J was incubated at a concentration of 20 μg/mL with α-chymotrypsin (1:60 *wt/wt*) and proteinase K (1:10 *wt/wt*) (Cao et al. 2012) for 30 and 60 min at 30 °C. Then, C6- and C10-HSLs were added at 10 μM, and AHL detection solid bioassays were carried out after 24 h of exposure. PBS and proteases supplemented with the same amount of AHLs were set as negative controls.

Interference of Aii20J with antibiotics

To check the effect of purified Aii20J on different β-lactam antibiotics (penicillin G, methicillin, amoxicillin, ampicillin, cephalothin, cefaclor, cefoxitin, ceftriaxone, cefoperazone, imipenem, and meropenem) and its susceptibility to β-lactam inhibitors (sulbactam and postassium clavulanate), a solution of enzyme (20 μg/mL) was incubated in duplicate with each antibiotic or β-lactam inhibitor in PBS buffer at 22 °C for 24 h, with constant agitation. Methicillin, amoxicillin, ampicillin, imipenem and meropenem were used at a concentration of 100 μg/mL, penicillin G at 12 μg/mL, and the rest of antibiotics and β-lactam inhibitors were used at 150 μg/mL. The remaining antibiotic activity after exposure to the enzyme was evaluated in comparison with controls maintained in PBS by antibiogram assay in Mullier-Hinton Agar plates with *Staphylococcus aureus* ATCC 25923 and *E. coli* XL1blue. Wells were made in each plate in which each reaction solution was added to compare with the antibiotics and inhibitors without the enzyme. Plates were incubated 24 h at 37 °C to observe the results. As control, Aii20J at the same concentration in PBS pH 6.7 was added to the antibiograms. Moreover, an antibiogram with *Tenacibaculum* strain 20J was performed to detect possible resistances to antibiotics and β-lactam inhibitors tested.

Quenching acid resistance in *E. coli* K-12

Cultures of *E. coli* K-12 were grown in LB broth at 30 °C for 48 h with shaking. Then, cells were inoculated in LB broth with glucose (0.4 %) to repress the AR-1 acid resistance system (Dyszel et al. 2010), and incubated at 30 °C with or without 5 μM OC6-HSL, and 20 μg/mL Aii20J. After 15 h of incubation cultures were diluted (1:1000) in MEM medium supplemented with glucose (0.4 %) and glutamate (1.6 mM) at pH 2.0 (Vogel and Bonner 1956) and incubated in the same conditions. Colony forming colonies units were estimated by plating dilutions in LB at 0, 1.5 and 3 h.

Nucleotide sequence accession number

The nucleotide sequences of the genes from *Tenacibaculum* strains have been deposited in GenBank database under the following accession numbers: KR232934 (*aii20J* gene from *Tenacibaculum* sp. 20J CECT 7426), KR232935 (*aiiTD* from *T. discolor* DSM 18842^T), KR232936 (*aiiTG* from *Tenacibaculum gallaicum* CECT 7122^T), KR232937 (*aiiTS* from *Tenacibaculum soleae* CECT 7292^T), KR232938 (*aiiTM* from *T. maritimum* NCMBI 2154^T), KR232939 (*aiiTL* from *Tenacibaculum lutimaris* DSM 16505^T), and KR232940 (*aiiTA* from *Tenacibaculum aestuarii* JCM 13491^T).

Results

Identification and cloning of the gene responsible for QQ activity in *Tenacibaculum* sp. 20J

The analysis of the QQ activity present in a purified cell extract of strain 20J separated by non-denaturing gel electrophoresis and covered with the biosensor strain CV026 and C6-HSL (20 μM) revealed a single degradation spot (data not shown), consistent with the presence of a single QQ enzyme in this bacterium (Tang et al. 2015). Different attempts were carried out for the identification of the gene responsible for AHL degradation in strain 20J. PCR amplification trials using degenerated primers designed on the basis of conserved sequences of QQ enzymes were unsuccessful. Also, the QSIS pMH655 plasmid (Rasmussen et al. 2005) was used to construct a genomic library of strain 20J that was expressed in *P. aeruginosa* PAOI in order to select the clones being able to survive in the presence of sucrose, which should carry the

gene responsible for AHL degradation, but no positive clone could be obtained. Finally, a fosmid genomic library of strain 20J was constructed in pCC2FOS (Epicentre) and used to transform EPI300™ *E. coli* cells. A functional screening on 96-well microtitter plates was carried out with *C. violaceum* biosensor CV026 (modified from Romero et al. 2010) on 6912 clones, and a single positive clone, named 13-E2, was obtained capable of inhibiting violacein production triggered by exogenous addition of C6-HSL (Fig. 1a). The enzymatic activity of the cell extract of clone 13-E2 against both C6- and C10-HSLs was confirmed by HPLC-MS, resembling the activity described for strain 20J live cells (Romero et al. 2011a). The acidification of the reaction mixture to pH 2 that drives spontaneous reformation of the lactone ring opened by lactonase activity allowed the partial recovery of the AHL (Fig. 1b). The partial recovery of restoration of AHLs after acidification has been reported previously for other lactonases

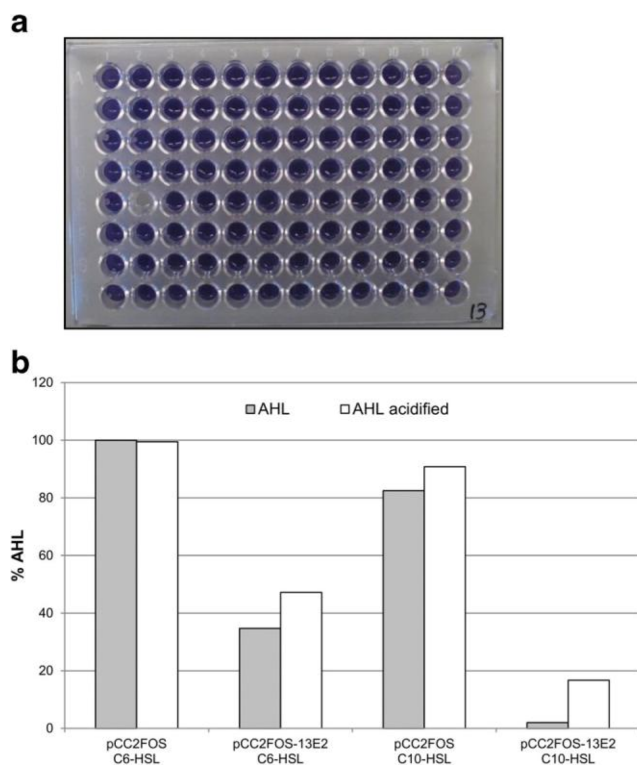

Fig. 1 Identification of the gene responsible for the QQ activity in *Tenacibaculum* sp. strain 20J. a Screening of a fosmid-based expression genomic library of *Tenacibaculum* sp. strain 20J constructed in pCC2FOS transformed in EPI300™ *E. coli*, in 96-well microtitter plates showing the only positive clone (13-E2) identified which is *boxed*. The screening was performed with *C. violaceum* CV026 biosensor. The presence of QQ activity is revealed by the absence of violacein in the well as a result of the degradation of exogenous C6-HSL (60 μM). b HPLC-MS analysis of degradation of C6-HSL and C10-HSL (10 μM) after 24 h by the crude cellular extract (*CCE*) of the positive clone 13-E2, identified in the pCC2FOS fosmid genomic library of strain 20J. Portions of the reaction mixtures were acidified to pH 2 to allow the recovery of the lactone ring after lactonolysis. EPI300™ *E. coli* extract with pCC2FOS fosmid without the insert and PBS were used as negative controls. Data are shown as percentage of the concentration retrieved in PBS treated in the same way

and has been attributed to additional degradation or modification of the ring-opened AHL (Rashid et al. 2011).

The insert in the positive clone 13-E2 (40 kb) was pyrosequenced using GS-FLX Titanium Plus sequencer technology (Roche) and simultaneously fragmented to perform a pBlue Script KS II (+)-based sub-library with smaller insert sizes (3-5 kb). A second functional screening was carried out with biosensors CV026 and VIR07 on the clones obtained in the sub-library in order to unequivocally identify the gene responsible for the enzymatic activity present in clone 13-E2. As a result, an ORF of 861 bp was identified in all sequenced fragments that encoded a putative metallo-β-lactamase of 286 amino acidic residues that was named autoinducer inactivator gene from *Tenacibaculum* sp. 20J (*aii20J*) confirming the results obtained from the HPLC analysis of the QQ activity in the positive clone. The complete sequence of the 40-kb fragment present in clone 13-E2 confirmed that the active sequence was located on the 3′ end of the insert. A zinc-binding domain (HXHXDH) that is commonly conserved in the metallo-β-lactamase superfamily was detected in the amino acidic sequence of *aii*20J (Fig. 2), as well as other histidine and aspartic acid residues (Bebrone 2007; Dong et al. 2000; Dong et al. 2002), which confirms the data derived from biochemical analysis regarding the presence of a lactonase in strain 20J, and was classified as a new member of metallo-β-lactamase superfamily (Figs. 2 and 8). Although a putative Gram-positive signal peptide was predicted at the N terminus in the amino acid sequence of Aii20J by SignalP 4.1 analysis (Fig. 2), Aii20J is not excreted, since no QQ activity could be detected in the culture medium of strain 20J (Romero et al. 2014) or in any of the *Tenacibaculum* strains studied (see below).

QQ activity and presence of Aii20J homologous in the genus *Tenacibaculum*

The presence of QQ activity was investigated in cultures of another seven strains of the genus *Tenacibaculum* by C6- and C10-HSL degradation bioassay with *C. violaceum* biosensors. Most strains were able to degrade C6- and C10-HSL signals after 3 h of exposure (Fig. 3), showing a degradation activity in live cells similar to that of strain 20J (Romero et al. 2014). On the contrary, *Tenacibaculum aestuarii* JCM 13491^T and *T. lutimaris* DSM 16505^T could only degrade the long-chain signal C10-HSL under the same conditions. The activity was also present in cell extracts in all cases. On the contrary, the two strains of *T. maritimum* tested, *T. maritimum* CECT 4276 and NCIMB 2154^T, failed to degrade both C6- and C10-HSL signals, a result that contrasts with previous reports that found C10-HSL degradation activity in *T. maritimum* NCIMB 2154^T (Romero et al. 2010). This discrepancy may be derived from the strict control of pH in the present experiments in which the pH was maintained at pH 6.7 to discard a

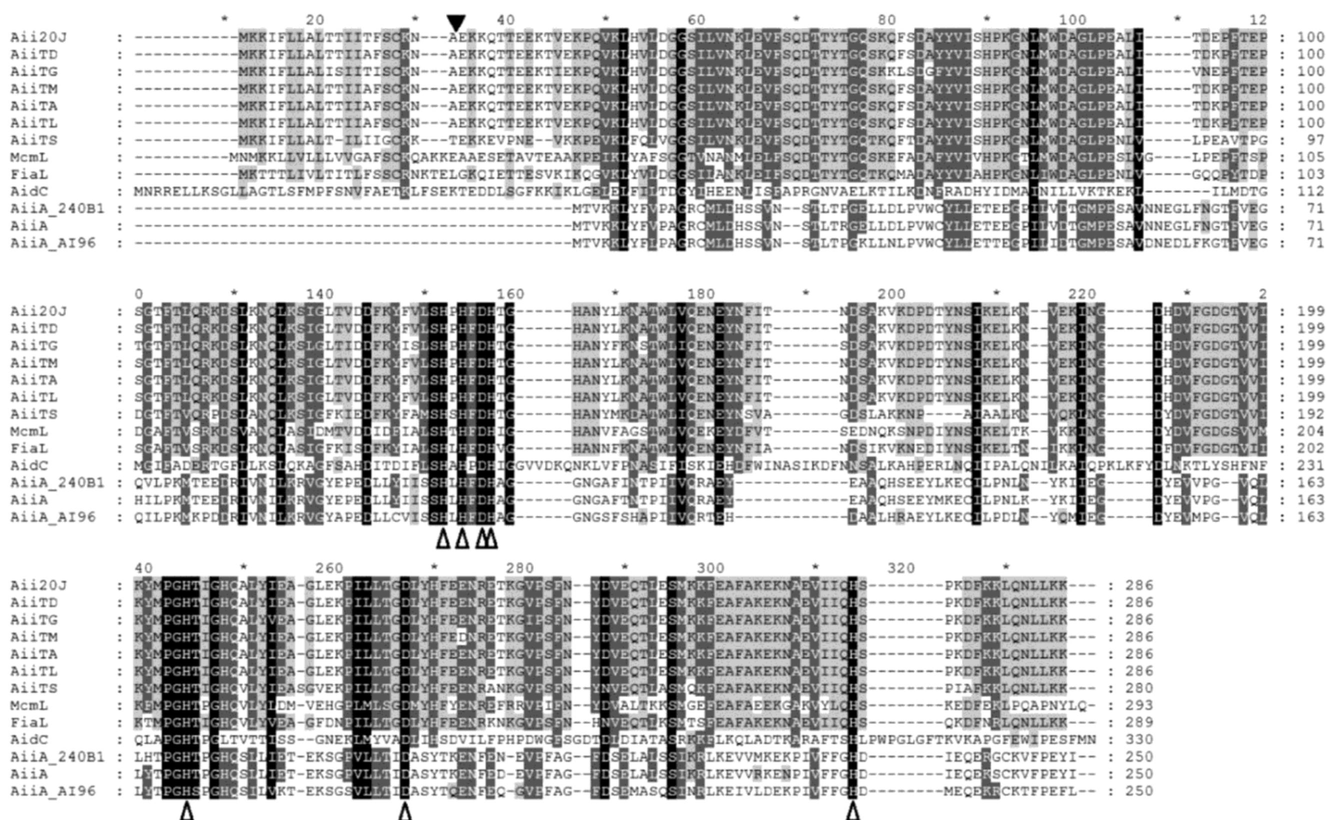

Fig. 2 Amino acid sequence alignment of AHL-lactonases from *Tenacibaculum* and AiiA from *Bacillus*. Aii20J: Aii20J sequence from strain 20J; AiiTD: sequence from *T. discolor* DSM 18842^{T}; AiiTL: sequence from *T. lutimaris* DSM 16505^{T}; AiiTA: sequence from *T. aestuarii* JCM 13491^{T}; AiiTM: sequence from *T. maritimum* NCIMB 2154^{T}; AiiTG: sequence from *T. gallaicum* CECT 7122^{T}; AiiTS: sequence from *T. soleae* CECT 7292^{T}; AiiA(240B1): AiiA sequence from *Bacillus* sp. strain 240B1 (accession number AAF62398.1); AiiA: AiiA sequence from an *uncultured Bacillus sp.* (CAD44268.1), AI96: AiiA from *Bacillus* sp. strain AI96 (ADK91097.1), AidC from *Chryseobacterium* sp. strain StRB126 (BAM28988.1), MomL from *Muricauda olearia* (AIY30473), and FiaL from *Flaviramulus ichthyoenteri* Th78^{T} (WP_034041734.1). Sequences were aligned using ClustalW program (Thompson et al., 1994) and shaded with the Genedoc program (http://www.nrbsc.org/gfx/genedoc/). The identical and similar residues are shaded in *black* and *grey*, respectively. The conserved metal binding residues are indicated by *white triangles*. Predicted cutting site for putative signal peptide is shown by a *black triangle*

spontaneously degradation of AHLs caused by alkaline pHs (Yates et al. 2002). No QQ activity was retrieved in the cell-free culture media of any of the *Tenacibaculum* strains (data not shown), revealing that as described for strain 20J, their QQ activity against C6- and C10-HSLs is cell-bound.

To investigate the presence of sequences homologous to Aii20J in other strains of the genus *Tenacibaculum*, PCR amplification was performed using primers Lact20JF and Lact20JR or LactTG2R and the genome of *Tenacibaculum aestuarii* JCM 13491^{T}, *T. discolor* DSM 18842^{T}, *T. gallaicum* CECT 7122^{T}, *T. soleae* CECT 7292^{T}, *T. lutimaris* DSM 16505^{T}, and *T. maritimum* NCIMB 2154^{T} as template. Results showed that all the *Tenacibaculum* species studied have an AHL-lactonase that shares an identity of 99 % with Aii20J sequence at amino acidic level, except AiiTG from *T. gallaicum*, that shares an identity of 93 % and AiiTS from *T. soleae*, that shares an identity of 76 % with Aii20J and clusters together with the homologous sequence found in the genome of *T. ovolyticum* (Fig. 2), in which the AHL-degrading activity remains to be confirmed. All sequences shared the same signal sequence with the same predicted cleavage site, although no QQ activity was found in the culture media of any of the strains. Despite showing no AHL degradation activity in bioassays (Fig. 3), *T. maritimum* NCIMB 2154^{T} harbors a lactonase, AiiTM, that is 99 % identical to Aii20J at the amino acidic sequence level (Fig. 2). Interestingly, the lactonase from *T. maritimum* was able to hydrolyze AHLs when transformed into *E. coli*, while live cells or culture medium from *T. maritimum* showed no AHL degradation activity (data not shown).

Overexpression and purification of Aii20J

aii20J was amplified by PCR with primers Lact20JF and Lact20JR using genomic DNA of 20J as template. The amplification product was digested with *Nco*I and *Eco*RI and ligated into the overexpression plasmid pET28c(+) linearized with the same restriction enzymes. Since pET28c(+) adds an hexa-

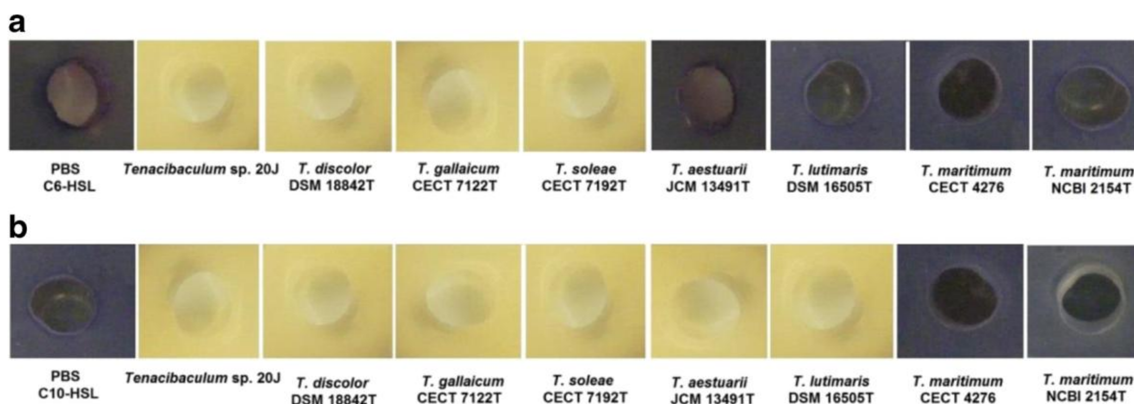

Fig. 3 Solid plate assay to detect AHL-QQ activity in live cells of different *Tenacibaculum* strains with the AHL biosensors *C. violaceum* CV026 (a) and *C. violaceum* VIR07 (b) in cultures of *Tenacibaculum aestuarii* JCM 13491^{T}, *T. discolor* DSM 18842^{T}, *T. gallaicum* CECT 7122^{T}, *T. soleae* CECT 7292^{T}, *T. lutimaris* DSM 16505^{T}, *T. maritimum* CECT 4276, and *T. maritimum* NCIMB 2154^{T}. Remaining AHL activity was evaluated after 3 h of exposure of C6- or C10-HSLs 10 μM in PBS pH 6.7 to live cells. A positive control was also set in the plates with strain *Tenacibaculum* sp. 20J

histidine tail to the recombinant protein, Aii20J could be purified after overexpression in *E. coli* BL21(DE3) plysS. For comparison, AiiA from *Bacillus* sp. (Carlier et al. 2003; accession number CAD44268.1) was also cloned and overexpressed in the same way. When overexpressed in *E. coli* BL21(DE3) plysS, Aii20J and AiiA formed inclusion bodies under the tested conditions, and therefore, urea denaturation followed by refolding by dialysis in PBS buffer 6.5 pH was needed in order to purify these lactonases. Interestingly, when the signal peptide sequence was removed from the sequence, no inclusion bodies were formed and the resulting truncated Aii20J was retrieved in the soluble fraction of cell extracts (Fig. 4a). A similar effect of the signal peptide has been already described for MomL (Tang et al. 2015). However, in order to facilitate the comparison with the *Bacillus* enzyme, the un-truncated form of Aii20J was used through the study. The SDS-PAGE analysis showed that purified Aii20J migrates as a single band with molecular mass slightly lower than 35 kDa, which is consistent with its predicted molecular mass of 34.9 kDa, including the histidine tag (Fig. 4b).

Characterization of Aii20J

Substrate specificity

The minimum active concentration (MAC) of purified Aii20J (complete sequence) was calculated as the minimal concentration of purified enzyme necessary to completely hydrolyze 10 μM of C6-HSL in 3 h as detected by the *C. violaceum* solid plate assay (data not shown) and was stablished as 2 μg/mL (the same amount for C10-HSL). Ten times this concentration (20 μg/mL) was used in all the assays for the physical-chemical characterization of the enzyme. Under the same conditions, 100 μg/mL of AiiA was necessary to completely hydrolyze the same amount of C6-HSL and 40 μg/mL of C10-HSL, indicating a higher specific activity of Aii20J.

The capacity of Aii20J to hydrolyze different unsubstituted (C4-HSL, C6-HSL, C8-HSL, C10-HSL, C12-HSL, C14-HSL) and substituted (OC6-HSL and OC12-HSL) AHLs after 1 h of exposition at 22 °C was measured by HPLC-MS analysis in triplicate. Results revealed a broad and unspecific substrate spectrum for purified Aii20J, since a concentration of enzyme of 20 μg/mL was sufficient to completely degrade 10 μM of all the tested signals in 1 h except for C4-HSL that was reduced by 60 % and OC6-HSL that was reduced by 80 % in the same period (data not shown). Aii20J was also able to eliminate the activity of OC10-HSL, OHC10-HSL, OC12-HSL, OHC12-HSL, OC13-HSL, and OC14-HSL as detected by *C. vilaceum* bioassays (data not shown).

AHL degradation kinetics

The degradation kinetics of purified Aii20J (20 μg/mL) and AiiA (40 μg/mL) against C6-HSL and C10-HSL were investigated by sampling the mixture reactions at different times, extracting and quantifying the AHLs by HPLC-MS. C10-HSL was completely eliminated by purified Aii20J after 20 min (Fig. 5), while less than 40 % of the initial amount of C10-HSL had been degraded by AiiA after 30 min. It should be noted that the amount of AHL used for the degradation kinetics is at least one order of magnitude higher than the concentration usually found in bacterial cultures (Romero et al. 2014). Results also showed a preference of Aii20J for long AHL signals as the short signal C6-HSL was completely degraded only after 60 min. In comparison, AiiA was able to reduce C6-HSL and C10-HSL by 50 and 60 %, respectively, after 90 min of exposure (Fig. 5). On the basis of these results, the specific activity of purified Aii20J, defined as the hydrolyzed micromoles of AHL per minute per milligram of AHL-

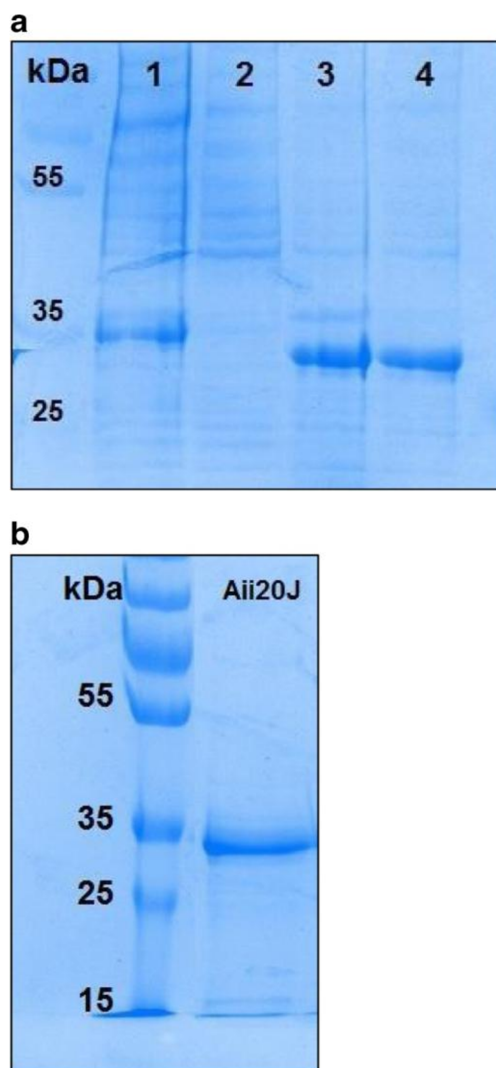

Fig. 4 a SDS-PAGE gel stained with Coomasie blue of cell extracts of *E. coli* BL21(DE3) plysS transformed with pET28c(+) harboring the full sequence of *aii20J* (*lanes 1 and 2*) and with the sequence without the signal peptide (*lanes 3 and 4*). *Lanes 1 and 3* show non-centrifuged extracts. *Lanes 2 and 4* show centrifuged extracts. b SDS-PAGE gel of the purified lactonase Aii20J (full sequence, *right lane*). *Left lane*, protein marker. The protein, including the poly-his tag and the signal peptide, has an expected molecular weight of 34.9 kDA

lactonase was 142 U/mg towards C6-HSL and 230 U/mg towards C10-HSL, while the specific activity of AiiA was 16 U/mg towards C6-HSL and 21 U/mg towards C10-HSL, which are similar to the specific activity values reported in the literature for AiiA from *Bacillus* sp. 240 (Wang et al. 2004).

Response to physical-chemical conditions: thermostability, resistance to pH changes and proteases

Resembling the thermostability of the QQ activity present in the CCEs of strain 20J, the enzymatic activity present in crude cell extracts (CCE) of *E. coli* BL21(DE3) plysS transformed with pET28c(+)-aii20J was extremely thermostable, being able to resist a treatment of 100 °C during 10 min even if the extract had been previously pasteurized for 2 h at 60 °C (Fig. 6a).

On the contrary, the purified enzyme Aii20J showed significant lower thermo resistance, being able to resist a treatment of 80 °C for 10 min (data not shown). Activity was still present in the purified enzyme after a treatment at 60 °C during 10 min but was significantly reduced in comparison with the untreated enzyme (Fig. 6b). When the denaturized, purified enzyme was refolded in sterilized seawater instead of PBS; the enzyme thermostability increased from 60 to 80 °C (data not shown).

To establish the feasibility of Aii20J as an animal feed additive that has to be ingested orally, we also assessed how the exposure during 30 min to different pHs in the range 3-9 affected the catalytic activity of Aii20J against C6- and C10-HSLs. Aii20J was fully active against both AHLs after exposure to all the pH range tested (data not shown).

The resistance of purified Aii20J (20 μg/mL) to α-chymotrypsin and proteinase K was also tested and evaluated with the biosensors *C. violaceum* CV026 and VIR07 to detect AHL degradation of C6- and C10-HSLs. Aii20J was strongly resistant to both proteases, being able to degrade the signals after 30- and 60-min incubation with proteases (data not shown).

Interference with β-lactam antibiotics

Due to the sequence similarity of Aii20J and other AHL-lactonases with β-lactamases, which are responsible for the antibiotic resistance of numerous bacteria (Bebrone 2007), the activity of Aii20J against different β-lactam antibiotics including penicillin G, methicillin, amoxicillin, ampicillin, cephalothin, cefaclor, cefoxitin, ceftriaxone, cefoperazone, imipenem, and meropenem was evaluated. Moreover, Aii20J susceptibility to β-lactamase inhibitors like sulbactam and postassium clavulanate was studied. Each antibiotic was incubated with a solution of Aii20J (20 μg/mL) for 24 h and residual activity was evaluated against *Staphylococcus aureus* ATCC 25923 and *E. coli*. No changes in the activity of antibiotics treated with Aii20J lactonase were observed (data not shown). Moreover, AHL degradation activity of Aii20J was not affected by the β-lactam inhibitors tested (data not shown). In addition, strain 20J showed no resistance to the same antibiotics.

Quenching acid resistance in E. coli K-12

In order to check whether the lactonase Aii20J could revert the protective effect of AHLs on *E. coli* K-12 when exposed to low pH, an acid resistance assay was performed. The addition of 5 μM OC6-HSL or C6-HSL resulted in a 2- to 50-fold increase in acid resistance in

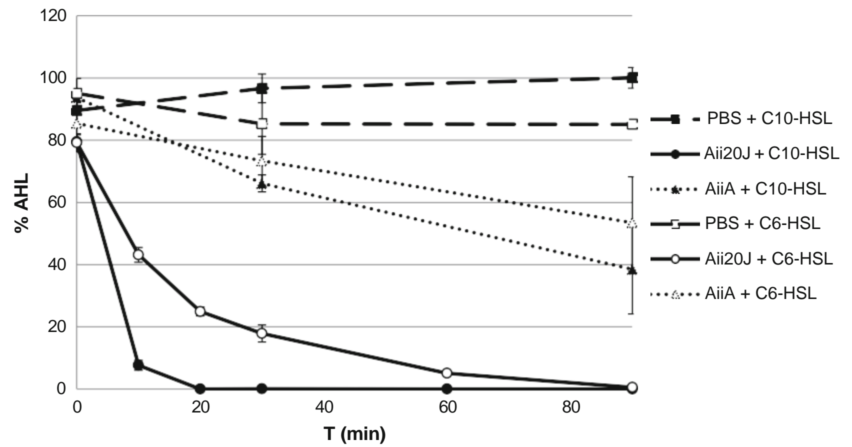

Fig. 5 Kinetics of degradation of C6-HSL and C10-HSL (50 μM) by purified Aii20J (20 μg/mL) from *Tenacibaculum* sp. 20J and purified AiiA (40 μg/mL) from *Bacillus* sp. (Carlier et al. 2003) by HPLC-MS. Controls were set with the signals in PBS. Data are means ± SD of three independent degradation reactions. AHL concentrations were measured by HPLC-MS using external calibration curves

E. coli in different experiments (Fig. 7, data shown for OC6-HSL activation). The addition of Aii20J (20 μg/mL) to cultures without signal had no significant effect on *E. coli* resistance to acidic environment while the addition of Aii20J to cultures to which AHL was added resulted in a significant reduction (around 50 %) in cell viability in comparison with cultures that were acid-resistant due to the presence of AHLs (Fig. 7).

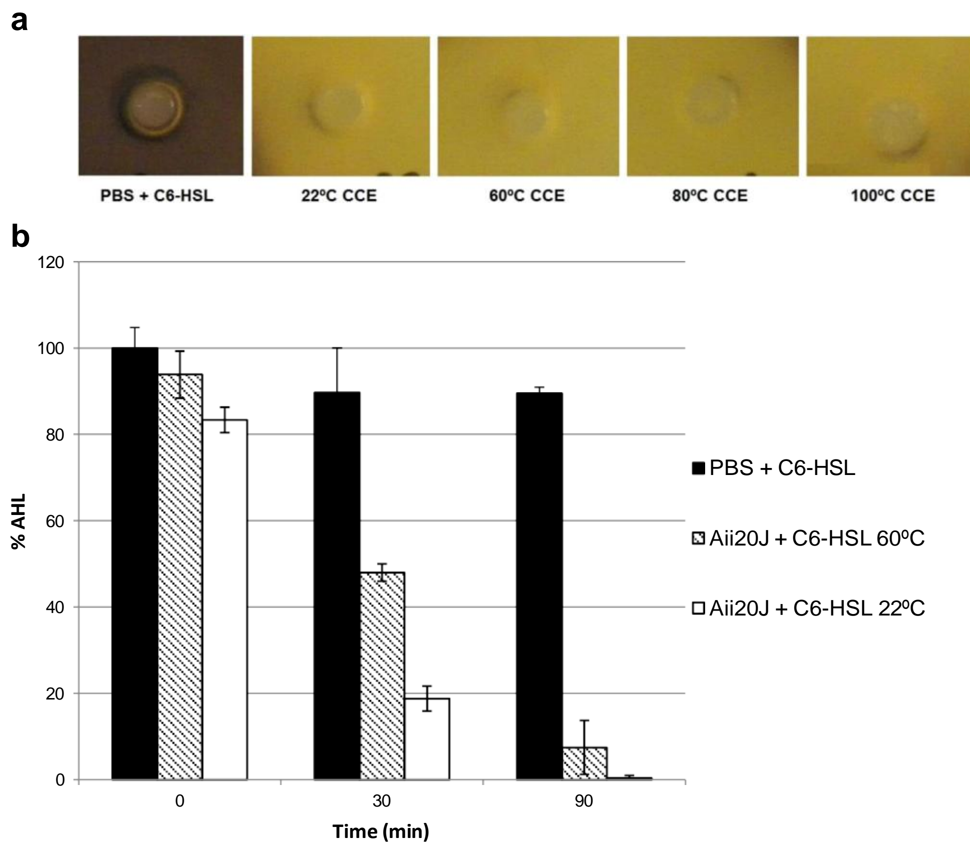

Fig. 6 a Thermostability of pasteurized (60 °C, 2 h) CCE from *E. coli* BL21(DE3) plysS transformed with pET28c(+)-aii20J, heat treated at 22, 60, 80, and 100 °C for 10 min. Solid plate assay was performed with 10 μM C6-HSL and detected by biosensor *C. violaceum* CV026 after 3 h of exposure. PBS plus the same amount of C6-HSL was used as negative control. b Thermostability of purified Aii20J (20 μg/mL) from *Tenacibaculum* sp. 20J treated previously at 60 °C for 10 min. Remaining C6-HSL after 90 min concentration was measured by HPLC-MS. PBS with the same amount of C6-HSL (50 μM) was set as control. Data are means ± SD of three independent degradation reactions. Differences in the thermo resistance of cell extracts and purified enzyme are derived from the denaturation process applied to solubilize the inclusion bodies, since the soluble enzyme showed the same thermostability profile than CCEs (data not shown)

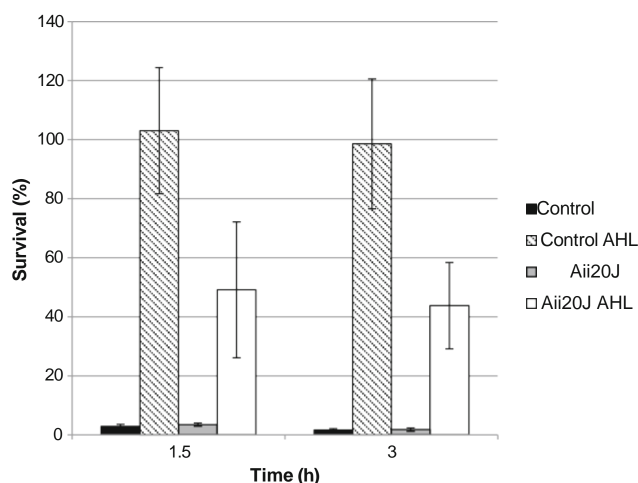

Fig. 7 Effect of the quorum-quenching lactonase Aii20J on AHL-induced acid resistance of *E. coli* K-12. Cells were grown in LB glucose with 5 μM OC6-HSL (Control AHL), with Aii20J (20 μg/mL) (Aii20J), with Aii20J and OC6-HSL (Aii20J AHL) or *E. coli* K-12 alone (Control) at 30 °C and then subcultured into pre-warmed MEM with glucose and glutamate at pH 2.0 at the same temperature. *E. coli* survival was determined by plating for CFU/mL at 0, 1.5, and 3 h and was expressed as percent in regard to viable cell counts at 0 h. Each condition was assayed in triplicate and *error bars* represent standard deviation

Discussion

The enzyme responsible for the quorum-quenching activity in *Tenacibaculum* sp. 20J, Aii20J, shares only 26-31 % identity at the amino acid level across the entire length of AiiA from different species of *Bacillus* (Cao et al. 2012; Carlier et al. 2003; Dong et al. 2000), by BLAST analysis (Fig. 2). The identity shared with other AHL-lactonases belonging to the metallo-β-lactamases superfamily is in some cases even lower, including 31 % with AttM from *Agrobacterium tumefaciens* (Zhang et al. 2002), 26 % with AhlK from *Klebsiella pneumoniae* (Park et al. 2003), 24 % with AhlS from *Solibacillus silvestris* (Morohoshi et al. 2012), 23 % with AhlD from *Arthrobacter* sp. (Park et al. 2003), and the thermostable AiiT from *Thermaerobacter marianiensis* (Morohoshi et al. 2014). Recently, three different lactonases have been identified in bacteria belonging to the *Bacteroidetes* phylum that includes the genus *Tenacibaculum*; Aii20J shares 28 % identity at amino acid level with AidC from *Chryseobacterium* sp. strain StRB126 (Wang et al. 2012), 59 % with MomL from *Muricauda olearia* (Tang et al. 2015), and 71 % with FiaL (gene GL001211) from *Flaviramulus ichthyoenteri* Th78^{T} (Zhang et al. 2015).

A sequence highly homologous to *aii*20J was found in all the strains of *Tenacibaculum* studied. All of them share an identity of 99 % with Aii20J sequence at amino acidic level, except for AiiTG from *T. gallaicum* that shares an identity of 93 % and AiiTS from *T. soleae* that shares an identity of 76 % with Aii20J (Fig. 2). Despite the presence of the sequence in all the *Tenacibaculum* strains, differences were found in the QQ activity of live cells and cell extracts were observed, since two of them, *T. aestuarii* JCM 13491^{T} and *T. lutimaris* DSM 16505^{T}, could only degrade the long-chain signal C10-HSL. No significant difference was found in the sequence and/or conserved domains of these strains to justify the differences in degradation activity with the other strains, and therefore differences could be derived from differences in the level of expression of the lactonase among species. Despite the presence of a sequence 99 % identical to Aii20J, the two *T. maritimum* strains tested, *T. maritimum* CECT 4276 and NCIMB 2154^{T}, failed to degrade both C6- and C10-HSL signals. On the contrary, the enzyme was active when expressed in *E. coli*, indicating that the AiiTM lactonase-encoding gene could be regulated by an upstream region or promoter, which avoids gene expression, at least under the culture conditions tested, or produces a non-functional protein in the *T. maritimum* strains tested. This result has important implications for the evaluation of QQ activity in natural samples solely on the basis of the presence of QQ sequences (Romero et al. 2012).

All the *Tenacibaculum* AHL-lactonase sequences cluster together as a separate branch within the metallo-β-lactamases, being closely related to the other MBLs identified from species of the CFB cluster (Fig. 8), sharing the unique characteristic of the presence of a Gram-positive signal peptide. The presence of a signal peptide seems to be a unique characteristic of AHL-lactonases from the *Bacteroidetes* group, since a signal peptide with the same cutting site can also be predicted in the sequences of MomL, AidC, and FiaL (Fig. 2). Despite the existence of a predicted signal peptide and unlike MomL that is an extracellular enzyme (Tang et al. 2015), Aii20J is not excreted, since no QQ activity could be detected in the culture medium of strain 20J (Romero et al. 2014) or in any of the *Tenacibaculum* strains studied. Therefore, the presence of a signal peptide in Aii20J sequence and its homologous could represent a reminiscence indicating horizontal transfer of such activity.

The characterization of the QQ activity of Aii20J revealed a much higher specific activity than AiiA from *Bacillus* (Fig. 5). Only 2 μg/mL of enzyme was necessary to hydrolyze 10 μM of C6-HSL in 3 h as detected by the *C. violaceum* solid plate assay (data not shown) in comparison with the 100 μg/mL of AiiA necessary to carry out the same reaction. Aii20J is characterized by a wide range of substrates, being able to degrade all the unsubstituted and substituted AHLs tested, with a slight preference for long-chain AHLs (Fig. 5). These results differ from profiles registered for AidC from *Chryseobacterium* sp., classified as a member of the CFB group, which shows slightly higher degradation activity against oxo-substituted AHLs than against unsubstituted AHLs and was more effective degrading C6- and C8-HSLs than C10- and C12-HSLs (Wang et al. 2012). Several studies

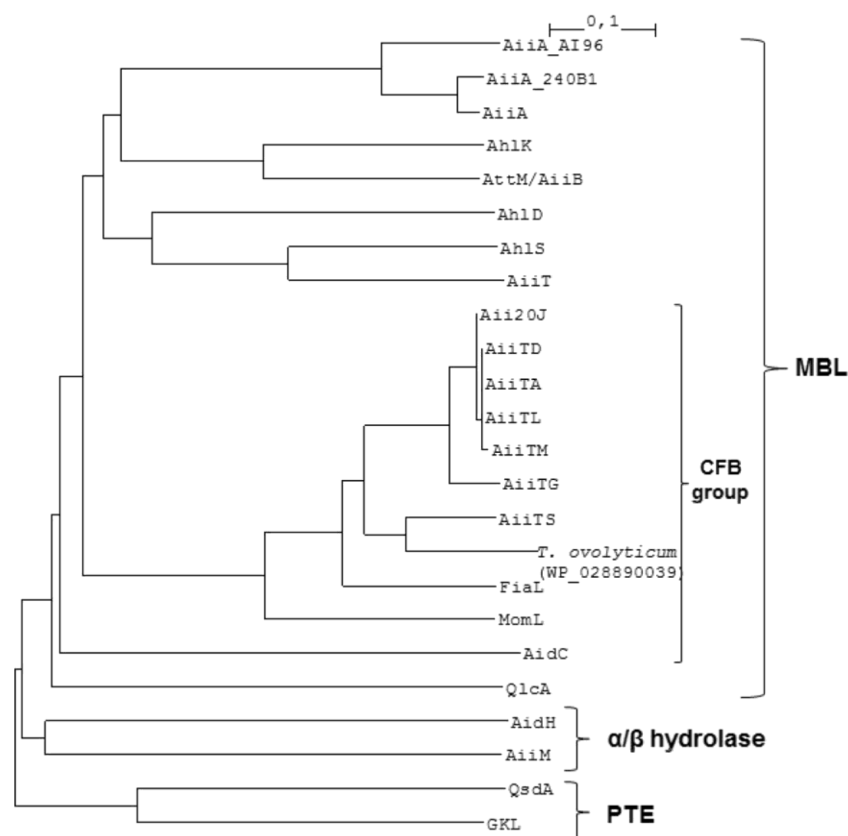

Fig. 8 Phylogenetic tree based on amino acid sequences of the AHL-lactonases Aii20J, AiiTD, AiiTG, AiiTS, AiiTA, AiiTL, and AiiTM from *Tenacibaculum* spp. and the homologous sequence found in the genome of *T. ovolytic*um (accession number WP_028890039, Zhang et al. 2015), AhlD from *Arthrobacter* sp. (AAP57766.1, Park et al. 2003), AhlK from *K. pneumoniae* (AAO47340.1, Park et al. 2003), AhlS from *Solibacillus silvestris* (BAK54003.1, Morohoshi et al. 2012), AidC from *Chryseobacterium* sp. strain StRB126 (BAM28988.1, Wang et al. 2012), AidH from *Ochrobactrum* sp. T63 (ACZ73823.1, Mei et al. 2010), AiiA from *Bacillus* sp. strain 240B1 (AAF62398.1, Dong et al. 2000), AiiA from uncultured *Bacillus* sp. (CAD44268.1, Carlier et al. 2003), AiiA from *Bacillus* sp. strain AI96 (HM750248.1, Cao et al. 2012), AiiM from *Microbacterium testaceum* strain StlB037 (YP_004225655.1, Morohoshi et al. 2011), AttM/AiiB from *A. tumefaciens* (AAL13075.1, Zhang et al. 2002), QlcA from *Acidobacteria bacterium cosmid* p2H8 (ABV58973.1, Riaz et al. 2008), QsdA from *Rhodococcus erythropolis* (AAT06802.1 , Uroz et al. 2008), Aii T from *Thermaerobacter marianensis* (AB935246.1, Morohoshi et al. 2014), FiaL from *Flaviramulus ichthyoenteri* Th78^T (WP_034041734.1, Zhang et al. 2015), MomL from *Muricauda olearia* (AIY30473, Tang et al. 2015), and GKL from *Geobacillus kaustophilus* (WP_011231002.1, Chow et al. 2010). Sequences are divided as members of the metallo-β-lactamase family (MBL), α/β-hydrolase family and phosphotriesterase family (PTE). The dendrogram was constructed by the neighbor-joining method using ClustalW. The *scale bar* represents 0.1 substitutions per amino acid position

have demonstrated that AiiA from *Bacillus* also shows strong preference for long-chain AHLs (Liu et al. 2013; Momb et al. 2008). The AiiA enzyme used in this study for comparison (Carlier et al. 2003) also shows preference for C10-HSL. The strong ability of Aii20J to degrade all range of AHLs makes it one of the most active QQ enzymes described. It should be noted that in the present study, the pH conditions were always controlled to discard a spontaneously degradation of HSL ring of signals due to alkaline pH (Yates et al. 2002).

The thermo resistance of the QQ enzymes is a valuable characteristic that enables a wider range of biotechnological applications. When the complete sequence of Aii20J was overexpressed in *E. coli*, the protein formed inclusion bodies and urea denaturation was required for purification. The denaturation and refolding process strongly affected the thermo resistance of the enzyme. Residual enzymatic activity was found in the purified, full sequence Aii20J after a treatment of 80 °C for 10 min, but only when reconstituted in the presence of metallic ions, which indicates that the presence of metal ions is beneficial for the correct folding and catalytic activity of the protein, as described previously for other metallohydrolases (Cao et al. 2012; Chen et al. 2010; Momb et al. 2008; Tang et al. 2015; Wang et al. 2012) and confirms that correct folding is a key factor in the thermostability of the denaturized enzyme. Activity was still present in the purified enzyme after a treatment at 60 °C during 10 min but was significantly reduced in comparison with the untreated enzyme (Fig. 6b). No potential *N*-glycosylation site was identified by NetCGlyc 1.0 Server, which is a characteristic related to thermo resistance of enzymes (Chen et al. 2010), and therefore the loss of thermo resistance of the purified enzyme cannot be associated with improper glycosylation in the

overexpression strain. When the signal peptide is eliminated from the sequence, the enzyme is produced as a soluble protein in *E. coli* and no denaturalization is necessary for purification. The native, truncated Aii20J was able to resist a treatment of 100 °C for 10 min without significant reduction in the activity, presenting a much higher thermostable profile than the closely related lactonase MomL that retained 30 % activity after being heated to 60 °C for 30 min (Tang et al. 2015) and indicating that the presence of the signal peptide is not involved in the extremely high thermostability of the enzyme. Nevertheless, even after denaturation and refolding, the thermostability of reconstituted Aii20J was significantly higher than that of AiiA from *Bacillus* sp. 240 which is inactive at >45 °C (Wang et al. 2004) or than Aid C from *Chryseobacterium* sp. which reduces its relative activity after incubation at temperatures over 50 °C and its enzymatic activity is greatly reduced over 60 °C (Wang et al. 2012). In the case of the thermostable $AiiA_{AI96}$ from *Bacillus* sp. strain AI96, it has been described that it retains 20 % of its activity at 60 °C (Cao et al. 2012) similarly to denatured Aii20J (Fig. 6b); nevertheless, the thermostability profile of $AiiA_{AI96}$ was examined at pH 8.0 and therefore the AHLs could be degraded spontaneously (Yates et al. 2002). An increased number of proline or reduced number of glycine residues has been proposed as one of the factors sustaining a higher thermostability in phosphotriesterase-like lactonases (Hawwa et al. 2009). In the thermostable lactonase AiiT from *Thermaerobacter marianensis* that presents a maximal AHL-degrading activity at approximately 70-75 °C, corresponding to the optimal growth condition of the parent strain, the number of arginine and aspartate residues in the non-conserved domains increased in comparison with the closely related thermo-sensitive AhlS (Morohoshi et al. 2014), providing stabilization to the structure of the protein through the existence of ion pairs among oppositely charged residues. None of these two characteristics is present in Aii20J in comparison with the amino acidic sequence of the close thermo-sensitive AidC (Wang et al. 2012) or the thermostable MomL (Tang et al. 2015), and therefore, the thermostability found in Aii20J should be derived from different features.

The highly thermostable profile of Aii20J and the wide spectrum of substrates are important characteristics for the industrial application of this enzyme, guarantying its use under aggressive physical-chemical conditions such as feed fabrication. Another important characteristics of Aii20J are the resistance to proteinases and extreme pH values (3-9) in comparison with AiiA from *Bacillus* sp., which completely lost enzymatic activity at pHs lower than 5 (Wang et al. 2004). AidC from *Chryseobacterium* sp. (Wang et al. 2012) and the thermostable $AiiA_{AI96}$ lactonase were unstable at pHs lower than 6 (Cao et al. 2012). Also, the activity of MomL, from *Muricauda olearia*, was completely lost when incubated at pH lower than 7 (Tang et al. 2015), which confirms the unique characteristics of Aii20J. These results suggest that this enzyme could be able to retain its activity after passing the digestive tract of animals, and therefore it would be possible to use it as a functional ingredient in feeds for the control of bacterial pathogens with AHL-based QS.

The family of metallo-β-lactamases includes broad-spectrum enzymes able to hydrolyze most clinically useful β-lactam antibiotics, including carbapenems (Bebrone 2007). Due to the sequence similarity of Aii20J and other AHL-lactonases with β-lactamases, which are responsible for the antibiotic resistance of numerous bacteria (Bebrone 2007), the activity of Aii20J against different β-lactam antibiotics was evaluated, confirming the absence of interference. AHLs have been proposed to have other different properties, functioning as iron quelants or antibiotics (Kaufman et al. 2005; Qazi et al. 2006; Romero et al. 2011b). Reversely, in a natural environment, antibiotics are often present in subinhibitory concentrations and therefore have been proposed to act like molecules involved in QS process and biofilm formation (Sengupta et al. 2013). The absence of activity of Aii20J towards a wide range of β-lactam antibiotics indicates that in this case, Aii20J lactonase is an enzyme with a well-defined action spectrum excluding antibiotics.

Acid resistance is an important virulence feature of gastrointestinal pathogens as *E. coli* (Lin et al. 1996), and *sdiA* has a key role in the regulation of the glutamate-dependent resistance acid system as well as controlling the production of other virulence factors in a QS-dependent manner in *E. coli* K-12 and EHEC (Dyszel et al. 2010; Kanamaru et al. 2000; Sharma and Bearson 2013). In our experiments, the addition of 5 μM OC6-HSL or C6-HSL resulted in a 2- to 50-fold increase in acid resistance in *E. coli* (Fig. 7). These data are in deep contrast with those obtained by Dyszel et al. (2010) that reported no effect of 1 μM OC6-HSL on acid resistance of either wild-type or SdiA mutants of *E. coli*, suggesting that a different signal molecule could be responsible for the activation of the glutamate-dependent acid-resistant system. This discrepancy can be derived from the higher amount of AHL used in the present experiments. The addition of Aii20J (20 μg/mL) to cultures to which AHL was added resulted in a 50 % reduction in cell viability in comparison with cultures that were acid-resistant due to the presence of AHLs (Fig. 7). Therefore and even though Aii20J presents relatively low affinity towards OC6-HSL in comparison with non-substituted AHLs, the enzyme is able to counteract the effect of the signal on SdiA, even when such high concentration of AHL is used.

Although most of the experiments regarding the role of SdiA on the expression of virulence factors in *E. coli* have been carried out through the overexpression of the gene (Kanamaru et al. 2000; Rahmati et al. 2002; Van Houdt et al. 2006; Yamamoto et al. 2001) and therefore cannot be extrapolated to its role under natural conditions and signal specificity, several studies indicate that AHLs or AHL-like

molecules can interact with the SdiA system in *E. coli*, resulting in the modification of gene expression. When Shiga toxin- and verotoxin-producing *E. coli* were transformed with the *yen*I gene of *Yersinia enterocolitica* to produce AHLs, the transgenic strain showed impaired expression of flagella, decreased motility, reduced biofilm formation etc., as well as attenuated adherence and invasion of IPEC-12 cells (Yang et al. 2013). Also, organic extracts obtained from aerobic granules from membrane-partitioned bioreactors used for wastewater treatments promote adhesion and growth of *E. coli*, and such activity has been attributed to the presence of AHLs in the liquid phase of granular sludge (Ren et al. 2013). Present results clearly demonstrate that the presence of OC6-HSL strongly promotes the acid resistance mechanisms in *E. coli* K-12, and this effect can be partially quenched by the addition of the lactonase Aii20J. Aii20J was not able to completely restore the acid sensitivity of the cultures, which may indicate that a very little amount of AHL is necessary for the activation of SdiA or that another molecule produced by *E. coli* should be able to activate SdiA or another downstream regulator, promoting acid resistance. Recently, a new type of signal molecules, the dialkylresorcinols, has been identified being able to activate LuxR-type orphan receptors (Brameyer et al. 2015). This or another autoinductor could be responsible for the activation of SdiA receptor in *E. coli* in the absence of AHLs.

Our results confirm the potential of the use of Aii20J in the control of the expression of virulence and adaptation genes in important human pathogens, as demonstrated for acid resistance in *E. coli*. Unique features like extreme temperature and pH resistance, resistance to proteases, wide substratum spectrum, and high specific activity enable the application of this QQ enzyme in a wide range of biotechnological applications.

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