Ann Microbiol (2016) 66:1117–1126
DOI 10.1007/s13213-016-1196-4

ORIGINAL ARTICLE

Quorum sensing regulated phenotypes in *Aeromonas hydrophila* ATCC 7966 deficient in AHL production

Adriana dos Reis Ponce-Rossi[1] · Uelinton Manoel Pinto[2] · Andrea de Oliveira Barros Ribon[3] · Denise Mara Soares Bazzolli[4] · Maria Cristina Dantas Vanetti[4]

Received: 18 July 2015 / Accepted: 20 January 2016 / Published online: 13 February 2016
© Springer-Verlag Berlin Heidelberg and the University of Milan 2016

Abstract The virulence of *Aeromonas hydrophila* is positively regulated by a quorum sensing (QS) system based on the *ahyRI* genes. The aim of this study was to evaluate the phenotypic characteristics related to virulence in the *A. hydrophila* strain ATCC 7966 in order to check if virulence traits are still functioning, despite the absence of a well characterized QS system. As expected, we were not able to detect quorum signaling AHL molecules in *A. hydrophila* ATCC 7966 by cross-streaking or thin layer chromatography with AHL biosensor strains. Although *A. hydrophila* ATCC 7966 did not produce AHLs, transcription of the *ahyI* and *ahyR* genes was observed by RT-PCR. Phenotypes generally recognized as QS-regulated in *A. hydrophila* were maintained in ATCC 7966; these phenotypes included proteolytic activity on casein and gelatin and amylolytic, lipolytic, and β-hemolytic activities, as well as the ability to adhere to surfaces and to form biofilms. Moreover, the addition of exogenous AHLs did not induce changes in the evaluated phenotypes. The results suggest that although this strain does not produce AHL as evaluated in this study, it maintains the phenotypes commonly associated with QS in this species indicating that other regulatory mechanisms independent of *ahyRI* may be in place.

Keywords *Aeromonas hydrophila* · Quorum sensing · Biofilm · Hydrolytic activity

✉ Uelinton Manoel Pinto
uelintonpinto@usp.br

Maria Cristina Dantas Vanetti
mvanetti@ufv.br

[1] Institute of Biological Science, Federal University of Pará, Belém, PA 66075-110, Brazil

[2] Department of Food and Experimental Nutrition, University of São Paulo, São Paulo 05508-000, Brazil

[3] Department of Biochemistry and Molecular Biology, Federal University of Viçosa, Viçosa, MG 36570-000, Brazil

[4] Department of Microbiology, Federal University of Viçosa, Viçosa, MG 36570-000, Brazil

Introduction

Aeromonas hydrophila is a gram-negative pathogen that is capable of infecting a wide variety of hosts, which include terrestrial and aquatic animals in addition to humans. This pathogen is widely distributed in nature and is found in water and many foods, such as fish, shellfish, and chicken, as well as raw foods, such as milk, vegetables, and meat (Gobat and Jemmi 1993). Bacterial infections caused by *A. hydrophila* are among the most common and troublesome diseases of fish (Natrah et al. 2012). Furthermore, *A. hydrophila* is a pathogen present in raw milk and is an important spoilage bacterium due to its ability to grow and to present proteolytic activity in chilled foods.

Similar to many other gram-negative bacteria, *A. hydrophila* is able to regulate its gene expression in response to cell density; this ability is referred to as quorum sensing (QS). In this species, the QS signaling molecules are *N*-acyl-homoserine lactones (AHLs), which are produced and released in the surrounding environment where they can accumulate to a critical concentration with subsequent binding to a receptor protein. The complex AHL-receptor protein can either activate or inhibit transcription of target genes. *A. hydrophila* contains the *ahyRI* genes which are homologous to the *luxRI* genes from *Vibrio fischeri* and are related to the synthesis and response to AHL molecules (Swift et al. 1997; Kirke et al. 2004; Jangid

et al. 2007; Kozlova et al. 2011). The major signaling molecule synthesized by AhyI is butanoyl-homoserine lactone (C4-AHL), and a second molecule, hexanoyl-homoserine lactone (C6-AHL), is produced at a lower concentration (Swift et al. 1997; Jangid et al. 2007; Chu et al. 2013; Nagar et al. 2015). Species from the genus *Aeromonas* are capable of inducing a wide variety of AHL biosensors (Swift et al. 1997; Medina-Martinez et al. 2006; Pinto et al. 2007). Jangid et al. (2007) showed that over 97 % of the *Aeromonas* species tested were able to induce an AHL-mediated response in, at least, one of the three biosensor strains that were used, and these species contained the *luxRI* homologues with strong sequence identities, suggesting that these genes are highly conserved in the genus. A study from Kozlova et al. (2011) has revealed the presence of two additional *ahyR* genes in the genome of *A. hydrophila* SSU, namely, *ahyR*2 and *ahyR*3. However, the contribution of these two *ahyR* homologs to the QS system of *A. hydrophila* SSU remains unknown (Kozlova et al. 2011). The presence of the *luxS* gene, which is responsible for the synthesis of AI-2, a furanosyl borate diester signaling molecule, was also observed in *A. hydrophila* (Kozlova et al. 2008, 2011). Various phenotypes regulated by QS in *A. hydrophila* have been identified, including proteolytic activity (Swift et al. 1999; Bi et al. 2007; Khajanchi et al. 2009), biofilm formation (Lynch et al. 2002; Bi et al. 2007; Kozlova et al. 2008; Khajanchi et al. 2009) and expression of virulence factors (Bi et al. 2007; Kozlova et al. 2008; Khajanchi et al. 2009).

A. hydrophila ATCC 7966, which was isolated from a tin of milk with a fishy odor, harbors the *ahyRI* genes and its genome sequence is available (Seshadri et al. 2006; Jangid et al. 2007). Although *A. hydrophila* ATCC 7966 contains the QS system genes *ahyR* and *ahyI*, this strain was unable to induce an AHL-mediated response as detected by the AHL biosensors *C. violaceum* CV026, *E. coli* JM109 pSB403 and *E. coli* JM109 pJBA82 (Jangid et al. 2007) as well as *Agrobacterium tumefaciens* A136 (Pinto et al. 2007), indicating that it is not able to synthesize AHLs, even though the *ahyI* coding region seems to be identical to other functional *ahyI* genes of *A. hydrophila* strains that produce AHLs (unpublished results). Therefore, the inability to produce AHLs in this strain is not understood. According to Jangid et al. (2007) there is a CAT insertion at position 402 in the *ahyR* gene of *A. hydrophila* ATCC 7966, and we believe that this insertion could lead to the formation of a non-functional AhyR protein, but this assertion lacks experimental confirmation. After confirming the inability of *A. hydrophila* to produce AHL, we decided to evaluate whether this inability would compromise the expression of phenotypes typically regulated by quorum sensing, as previously shown for other *A. hydrophila* strains (Swift et al. 1999; Lynch et al. 2002; Bi et al. 2007; Kozlova et al. 2008; Khajanchi et al. 2009). Therefore, our objective was to evaluate the activity of hydrolytic enzymes and the formation of biofilm by *A. hydrophila* ATCC 7966, which despite having *ahyRI* genes seems to lack a functional AHL dependent QS system.

Materials and methods

Bacterial strains and culture conditions

The strains of *A. hydrophila* ATCC 7966 and *A. hydrophila* Embrapa 029 were selected for this study. The latter strain was isolated from refrigerated raw milk and was generously provided by Embrapa Dairy Cattle (Embrapa Gado de Leite, Juiz de Fora, MG, Brazil). The stock cultures were prepared in Luria broth (Himedia, Mumbai, India) supplemented with 20 % sterile glycerol and stored at -80 °C. Before each experiment, the cells were cultured two consecutive times in Luria broth and incubated at 30 °C for 18 h with stirring.

Evaluation of signaling molecule (AHLs) production by A. hydrophila

Acyl homoserine lactone production was investigated by cross-streaking *A. hydrophila* strains against the biosensor strain *C. violaceum* CV026 (McClean et al. 1997) and *Escherichia coli* DH5α pSB403 (Winson et al. 1998). Bioluminescence of *E. coli* biosensor strain was observed as previously described by Pinto et al. (2007). Detection of AHL was also investigated by extraction of autoinducers from 100 mL supernatants of *A. hydrophila* cultures, after incubation at 30 °C and 180 rpm for 24 h, using ethyl acetate acidified with 0.5 % formic acid as described by Shaw et al. (1997). The extract was concentrated on a rotary evaporator (model Q344B, Quimis, São Paulo, Brazil), resuspended in 1 mL of ethyl acetate and stored at -20 °C. The presence of AHLs in these extracts was determined by thin layer chromatography (TLC) (C_{18} reversed phase, silica gel impregnated with hydrocarbons, 20 x 20 cm, Analtech uniplate™). TLC was performed using *C. violaceum* CV026 as the reporter strain. A result was considered positive for the presence of AHL by *C. violaceum* CV026 when the production of violacein, a purple pigment, was observed. N-Hexanoyl-DL-homoserine lactone (C_6-AHL) (Sigma-Aldrich, St. Louis, MO, USA) and N-butyryl-DL-homoserine lactone (C_4-AHL) (Sigma-Aldrich, St. Louis, MO, USA), previously identified autoinducers produced by *A. hydrophila* strains (Swift et al. 1997), were used as positive controls to induce the biosensor strains.

Detection of transcripts from ahyI and ahyR by RT-PCR

To evaluate the transcription of *ahyI* and *ahyR* genes, *A. hydrophila* ATCC 7966 and Embrapa 029 were cultured

for 16 h at 30 °C, and total RNA was extracted with the Trizol Reagent® (Sigma-Aldrich, St. Louis, MO, USA). Approximately 10^8 CFU were transferred to Eppendorf tubes and centrifuged at 6000 *g* for 5 min at 4 °C. Next, 1 mL Tri Reagent® was added to the pellet. Subsequent steps were performed following the manufacturer's instructions. The RNA obtained was resuspended in 40 μL diethylpyrocarbonate (DEPC)-treated water and stored at -80 °C. Quantification of RNA was performed by reading the absorbance at 280 and 260 nm (A280/A260) in a spectrophotometer Ultrospec® 3000 (Pharmacia Biotech, Buckinghamshire, England), and its purity and integrity were evaluated on a 1 % agarose gel. The amplification of 16S rDNA was performed using the oligonucleotides rDNA16S F and rDNA16S R (Table 1) in a thermocycler (model PTC-100, MJ. Research Inc. MA, USA) as described by Bi et al. (2007), and the PCR products were evaluated on a 1 % agarose gel. The synthesis of cDNA was performed by using the *ImProm-II*™ Reverse Transcription System (Promega, Madison, WI, USA) as described by the manufacturer. *ahyI* and *ahyR* gene amplification was performed by using the cDNA as a template and the oligonucleotide primers described in Table 1. PCR reactions were prepared with GoTaq ® Green Master Mix (M7122) (Promega, Madison, WI, USA) as described by the manufacturer, and amplification was also performed following the manufacturer's instructions in a thermocycler (model PTC-100) with an initial step of denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min; followed by a last extension at 72 °C for 5 min.

Evaluation of hydrolytic activity

The activity of gelatinase from *A. hydrophila* ATCC 7966 and Embrapa 029 was evaluated in nutrient broth (BD Difco™, Franklin Lakes, NJ, USA) containing 12 % gelatin as a substrate with or without the addition of 20 μM butyryl-homoserine lactone (C_4-AHL) (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 7 days at 30 °C, the tubes were transferred to 7 °C, and the hydrolysis of gelatin was identified by a lack of solidification of the medium. The assay was performed thrice, with duplicates in each assay.

Proteolytic activity was evidenced by the presence of a clarification halo in plate count agar (PCA; Himedia, Mumbai, India) supplemented with 2 % reconstituted skim milk with or without the addition of 20 μM C_4-AHL, inoculated with 20 μL of a suspension of cells standardized to an optical density ($OD_{600\ nm}$) of 0.1. Quantification of proteolytic activity was performed as described by Christensen et al. (2003) through reaction with 2 % azocasein (w / v) using filter-sterilized culture supernatants. Proteolytic activity from strains *A. hydrophila* ATCC 7966 and Embrapa 029 cultured in TYEP (tryptone 10 g l^{-1}, yeast extract 2.5 g l^{-1}, K_2HPO_4 1 g l^{-1}, KH_2PO_4 1 g l^{-1}) + $CaCl_2$ 2.5 g l^{-1} (Pinto et al. 2010) were quantified by determining the absorbance at 450 nm with a Microplate Thermoplate Reader (Molecular Devices, CA, USA). Absorbance values were converted to units of enzymatic activity, considering that one enzyme unit corresponds to the amount of enzyme capable of promoting an absorbance increase of 0.01 per hour of incubation. Proteolytic activity was also assessed by coagulation of 12 % reconstituted skim milk (RSM) inoculated with 1 % active *A. hydrophila* ATCC 7966 and Embrapa 029 cultures standardized to an $OD_{600\ nm}$ of 0.1. During the 30 °C incubation period, visual observations of milk coagulation were made following the protocol of Christensen et al. (2003). The assays of proteolytic activity were performed twice, with triplicates in each occasion. Zymography with azocasein as a substrate is another technique that was used to determine the proteolytic activity of cell-free supernatants of *A. hydrophila* ATCC 7966 and Embrapa 029 (Christensen et al. 2003). A positive control zymogram was performed by using the cell-free supernatant of the strain *Pseudomonas fluorescens* 041 (Martins et al. 2015).

Table 1 Primers used in this study

Name	Sequence*	Amplicon size (bp)	Source or reference
rDNA16S F	5′ AGGTTGATGCCTAATACGTA3′	650	Bi et al. 2007
rDNA16S R	5′CGTGCTGGCAACAAAGGACAG3′	650	Bi et al. 2007
*ahyI-Xba*I D[1]	5′ATTTCTAGAGTTCGCGCGCCTCGT3′	577	This study
*ahyI-Sal*I R[1]	5′ATTGTCGACCCCAGATGGGAGGTA3′	577	This study
ahyR-C/*Pst*I	5′ GGGCTGCAGTTATTGCATCAGCTTGGGGA3′	783	Khajanchi et al. 2009
ahyR-N/*Sca*I	5′GGGAGTACTATGAAACAAGACCAACTGCTT3′	783	Khajanchi et al. 2009

*The underlined sequences correspond to the restriction enzyme recognition sites

[1] Constructed using the Primer 3 tool and the sequence of *ahyI* deposited in the NCBI database

Aliquots of 20 μL from strains of *A. hydrophila* ATCC 7966 and Embrapa 029 standardized to an $OD_{600\ nm}$ of 0.1 were inoculated on solid media with substrates for the enzymes of interest. The presence of amylase was evaluated on nutrient agar (Difco, Franklin Lakes, NJ, USA) supplemented with 0.2 % starch (Stamford et al. 1998), hemolytic activity was observed in Columbia agar (Oxoid, Cambridge, UK) plus 5 % defibrinated sheep blood (Murano and Hudnall 2001) and lipase activity was measured in Tween 80 agar (Haba et al. 2000). The plates were incubated at 30 °C for up to 48 h. The assays were performed twice, with duplicates.

Biofilm assay on stainless steel

Biofilm formation by *A. hydrophila* ATCC 7966 and Embrapa 029 was evaluated on coupons of stainless steel (AISI 304, finish # 4) with dimensions of 2.0 × 6.0 × 0.1 cm that were maintained in TYEP for up to 72 h at 30 °C as described by Joseph et al. (2001). During the incubation period, the coupons were removed for observation of biofilm formation by staining with acridine orange (Parizzi et al. 2004) followed by observation under an epifluorescence microscope (model PX60 Olympus, Japan) with the WG filter (excitation at 510–550 nm and emission at 590–700 nm). Coupons were also selected for quantification of adhered cells using sonication (Branson Model 1510 Ultrasonic Cleaner) for 30 min to remove the cells. The number of viable cells was subsequently determined in PCA by micro-drops method (Morton 2001). The biofilm assay was performed twice and in duplicate.

Results and discussion

Evaluation of AHL production by A. hydrophila ATCC 7966

Cross-streak tests showed that *A. hydrophila* ATCC 7966 did not induce the AHL biosensor strains *C. violaceum* CV026 and *E. coli* pSB403, in contrast to *A. hydrophila* Embrapa 029, which was determined to be an AHL-producing strain by the same test. These results were further confirmed by TLC analysis, where a lack of violacein production by *C. violaceum* CV026 was observed in the presence of extracts of *A. hydrophila* ATCC 7966 (Fig. 1). Again, violacein production was induced by extracts from *A. hydrophila* Embrapa 029, which confirmed the presence of a functional AhyI synthase in this strain (Fig. 1). The *A. hydrophila* strain ATCC 7966 did not produce detectable levels of AHL as observed by using *C. violaceum* CV026 or *E. coli* pSB403 as AHL biosensors.

The absence of AHLs in extracts of *A. hydrophila* ATCC 7966 confirms the previous findings from Jangid et al. (2007) and Pinto et al. (2007), who found that this strain was unable to induce responses that could be measured by AHL biosensor strains including *C. violaceum* CV026, *E. coli* JM109 pSB403, *E. coli* JM109 pJBA89, and *A. tumefaciens* A136. The absence of AHL production in strain ATCC 7966 is not understood and no evidence is found in the literature that explains the non-functionality of the AhyI enzyme in this strain. Although the current investigation indicates that *A. hydrophila* ATCC 7966 is impaired in QS signal production, it is not possible to rule out that it produces these signal molecules at low concentrations or AHL-like molecules that cannot be detected by using *C. violaceum* CV026, *E. coli* (pSB or pJBA), or *Agrobacterium*-based biosensors. According to Steidler and Venturi (2007), the inability of a bacterium to elicit a response in biosensor strains does not exclude AHL production as some of these signals may be produced by the tested organism at concentrations below the threshold sensitivity of the biosensor strains or quorum sensing could be regulated under conditions that are different from those tested. On this rationale, several works have shown that the bacterial physiological state and the medium composition can influence quorum sensing response. For instance, Jahid et al. (2013) found that glucose present or added to foods modulates AHL production in *A. hydrophila* strains in a concentration dependent manner. Actually, concentrations lower than 0.25 % stimulated AHL production, while concentrations higher than 0.25 % inhibited signal production as well as QS regulated phenotypes. In a following work by the same group, they have shown that salinity also influenced AHL production

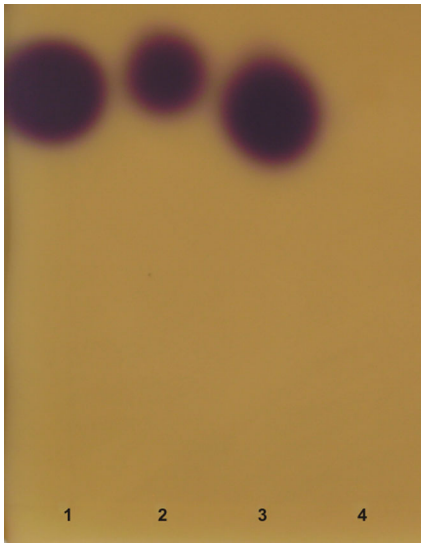

Fig. 1 Thin-layer chromatography (TLC) of AHL extracts from *A. hydrophila*. 1, Hexanoyl homoserine lactone (6 μL; 0.1 mg mL^{-1}); 2, butyryl-homoserine lactone (6 μL; 1 mg mL^{-1}); 3, Positive control (AHL) extract from *A. hydrophila* 029 (60 μL); 4, AHL extract from *A. hydrophila* ATCC 7966 (60 μL)

and quorum sensing mediated phenotypes, with low salinity (0.25 %) enhancing these traits and higher salinities (0.5 to 3 %) inhibiting them (Jahid et al. 2015). Interestingly, they point out that there was correlation between AHL concentration (detected by CV026 biosensor) with motility, biofilm formation, and protease production, which are all quorum sensing, regulated phenotypes in *A. hydrophila.* However, biosensor strains have been widely used for AHL detection in bacterial isolates with many studies using multiple biosensors in order to improve AHL detection, as different biosensors respond to different AHLs, which enhance the chances of AHL recognition. In fact, studies have used similar biosensor strains in order to detect AHL production in *A. hydrophila* strains from different sources, including foods. For instance, a recent work by Nagar et al. 2015 have used *C. violaceum* CV026, *A. tumefaciens* NTL1(pZLR4) with the TraI/R QS system and *E. coli* pJBA130 containing the LuxI/R system from *V. fisheri.* Those strains are identical or use the same detection systems as the ones used in the present study and from previous ones that dealt with strain ATCC 7966 (Jangid et al. 2007 and Pinto et al. 2007). Nagar et al. (2015) have shown that the major AHLs produced by *A. hydrophila* strains are C4-HSL and C6-HSL, which further confirms many previous studies (Swift et al. 1997; Jangid et al. 2007; Pinto et al. 2007; Chu et al. 2013; Jahid et al. 2013, 2015).

The lack of response to AHLs by AhyR may be explained by a CAT insertion at position 402 of the *ahyR* gene (Jangid et al. 2007), which could lead to the production of a nonfunctional AhyR protein or a protein with altered affinities to the common AHLs produced by *A. hydrophila*. The CAT insertion does not cause frameshift or nonsense mutations, but it adds an isoleucine residue in the protein at position 134 (I134). Interestingly, the inserted I134 is located in the N-terminal domain of the AhyR protein, a region known to bind to AHLs and to mediate dimerization and proteolytic stability of LuxR type proteins (Pinto and Winans 2009). An alignment between TraR from *A. tumefaciens* C58 and AhyR from ATCC 7966 (Fig. 2) indicates that the inserted I134 residue in AhyR is equivalently located to a region of TraR that contains the residue T129, which mediates AHL binding and specificity (Zhang et al. 2002; Chai and Winans 2004). By using Phyre web server (Kelley and Sternberg 2009), we confirmed that the inserted I134 residue is fully buried in the AHL binding pocket of the modeled structure of AhyR protein from ATCC 7966 which could have changed the binding pocket of AhyR and altered its function (Fig. 3). In fact, the non-functionality of this regulator can explain the unchanged phenotype of this strain in experiments in which exogenous AHL was added to the culture (Table 2). Future analysis aimed at evaluating whether functional AhyR protein (without the I134 insertion), expressed ectopically, and in the presence of exogenous AHLs, or AHLs produced from a constitutively expressed AhyI synthase, would have any effect on the physiology of *A. hydrophila* ATCC 7966.

Detection of transcripts from ahyI and ahyR by RT-PCR

A. hydrophila ATCC 7966 was able to transcribe the *ahyI* and *ahyR* genes, as demonstrated in Fig. 4. However, two bands resulted from the amplification of *ahyI* from *A. hydrophila* ATCC 7966 (Fig. 4a), which included a fragment of 577 bp corresponding to the coding region of *ahyI* and a band of approximately 400 bp. This additional band could be the result of non-specific amplification. We identified only a single 783 bp fragment corresponding to the *ahyR* gene in this strain (Fig. 4b). We detected one transcript each for *ahyI* and *ahyR*

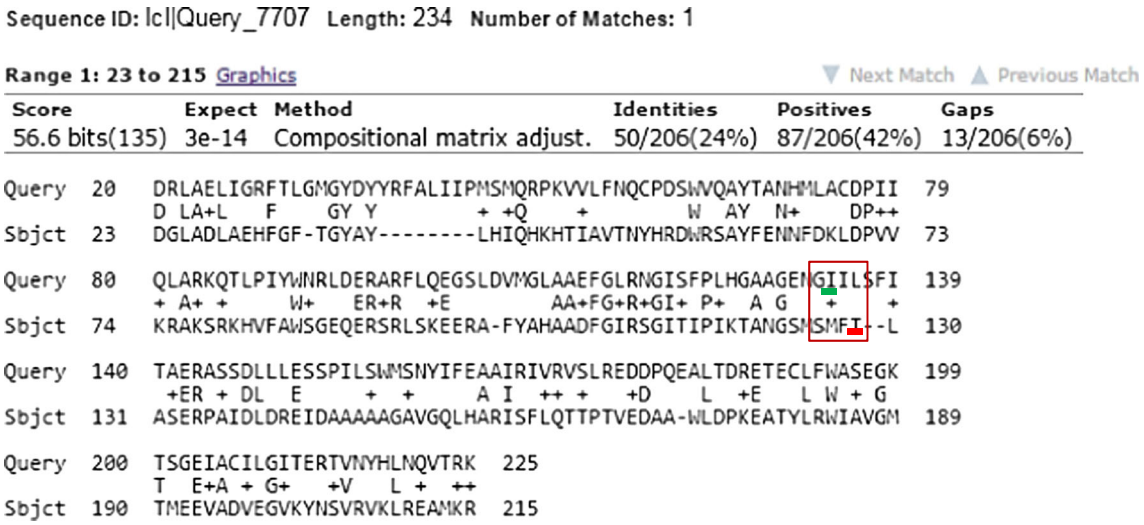

Fig. 2 Protein alignment of AhyR (query) from *A. hydrophila* ATCC 7966 and TraR (sbjct) from *A. tumefaciens* C58. The boxed region shows where the inserted I134 (highlighted in green) is located in relation to the TraR protein. Highlighted in red is T129 from TraR which mediates AHL binding and is located in the hydrophobic pocked of TraR (Chai and Winans 2004). The alignment was performed by using the Protein Blast tool for two sequences on NCBI (blastp suite-2sequences)

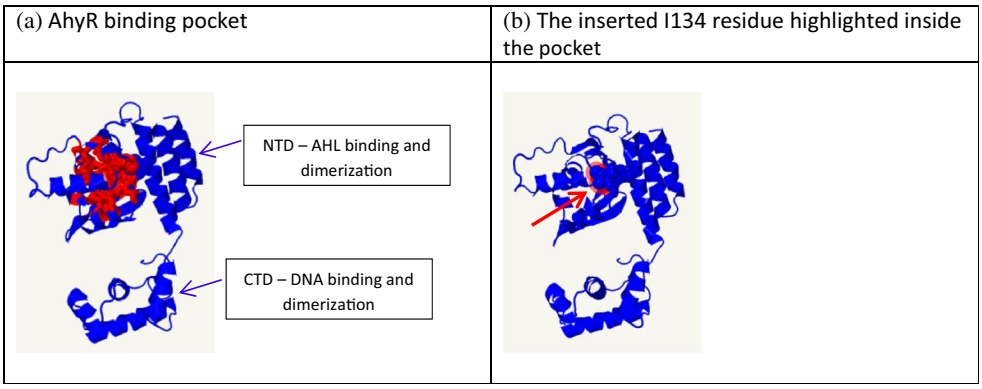

Fig. 3 Predicted structure of AhyR based on TraR as determined by using the Phyre protein fold recognition server (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). **a** Monomeric AhyR showing the AHL binding pocket in red as well as the N-Terminal Domain (NTD) and C-Terminal Domain (CTD). **b** The inserted residue I134, which differs from other AhyR proteins from *A. hydrophila,* is indicated with an arrow to show its location inside the predicted AHL binding pocket

from *A. hydrophila* Embrapa 029. Despite the amplification of *ahyI* and *ahyR* in strain ATCC 7966, it is likely by our results (Fig. 1) and from others (Jangid et al. 2007; Pinto et al. 2007) that the AhyI enzyme is nonfunctional or expressed to low levels in strain ATCC 7966 (Fig. 4a). The reduced levels of *ahyI* RNA in ATCC7966 with respect to Embrapa 029 strain (despite the need for real time PCR analysis that would confirm this preliminary observation) might be due to the CAT insertion in the *ahyR* gene (Figs. 2 and 3), which would negatively impact the quorum sensing regulated genes, including *ahyI*, in this strain. Additional experiments like the expression of *ahyI* gene from *A. hydrophila* in recombinant *E. coli* strain or the expression of *ahyI* from a constitutive promoter in ATCC 7966 would further clarify these issues and may be carried out in the future.

Hydrolytic enzyme activity

Gelatinase activity, which was determined as the ability to liquefy medium containing 12 % gelatin as a gelling agent, was observed in *A. hydrophila* ATCC 7966 and Embrapa 029 grown in gelatin with or without 20 μM C_4-AHL (results not shown). It should be noted that gelatin hydrolysis by ATCC 7966 was detected after 72 h of incubation, while Embrapa 029, which has a functional QS system, exhibited detectable gelatin hydrolysis after 48 h of incubation. The addition of 20 μM C_4-AHL to the gelatin medium did not reduce the detection time in hydrolysis assays with *A. hydrophila* ATCC 7966 (results not shown).

Proteolytic activity was confirmed in *A. hydrophila* ATCC 7966 by the presence of a hydrolysis halo in PCA supplemented with 2 % skim milk (Fig. 5a). We also quantified the proteolytic activity by using azocasein as substrate (Table 2). Although *A. hydrophila* ATCC 7966 exhibits proteolytic activity, this activity was lower than that which was observed in Embrapa 029 (Table 2) and may be associated with the inability of ATCC 7966 to produce and/or respond to AHL, and therefore, to upregulate protease production. However, the addition of 20 μM C_4-AHL did not affect the proteolytic activity of *A. hydrophila* ATCC 7966, regardless of the method used. Our results further illustrate that even though *A. hydrophila* ATCC 7966 does not seem to have a functional *ahyRI* QS system, this strain does exhibit proteolytic activity which is not affected by the exogenous addition of the most common AHL molecule produced by *A. hydrophila* strains (Table 2). This result may suggest that the proteolytic activity in this strain may be regulated by mechanisms other than the AHL-mediated QS system usually found in *A. hydrophila* strains.

Aeromonas hydrophila ATCC 7966 and Embrapa 029 were able to coagulate reconstituted skim milk after 24 h of incubation at 30 °C (Fig. 5b), which confirmed the previous results showing proteolytic activity. Milk coagulation was less intense in *A. hydrophila* ATCC 7966 (Fig. 5b), indicating lower proteolytic activity. The lower proteolytic activity of ATCC 7966 was most likely responsible for the failure to detect a

Table 2 Number of cells and quantification of proteolytic activity of *A. hydrophila* cultured in TYEP with 0.25 % $CaCl_2$ (1 M) and incubated at 30 °C for 42 h with or without exogenous C4-AHL

Strain	Cultivation without exogenous AHL			Cultivation with exogenous AHL		
	Growth (CFU mL^{-1})	Proteolytic activity (U h^{-1})	Specific proteolytic activity	Growth (CFU mL^{-1})	Proteolytic activity (U h^{-1})	Specific proteolytic activity
ATCC7966	8.0×10^8	0.66	1.6	6.4×10^8	0.8	1.7
029	1.9×10^9	2.9	2.9	1.4×10^9	3.1	3.1

hydrolysis band in the zymogram (Fig. 5c). The lack of a band produced by hydrolysis of azocasein in the zymograms may be due to the type of protease produced by this strain or, alternatively, to the lack of renaturation or incorrect folding that may indicate that this strain has a more sensitive protease than that of *A. hydrophila* Embrapa 029.

The different techniques used for qualitative and quantitative detection of proteolytic activity suggest that this activity in *A. hydrophila* ATCC 7966 is still present; even though it is lower than in strain 029, possibly due to the impaired AHL quorum sensing system. This result differs from those obtained by other authors, who reported a loss of proteolytic activity in mutants of *A. hydrophila* with compromised QS systems (Swift et al. 1997; Bi et al. 2007; Khajanchi et al. 2009), although we should point out that strain ATCC 7966 is not a lab created mutant, but a naturally deficient strain for the QS system under analysis. Experiments with *Pseudomonas aeruginosa,* performed by Van Delden et al. (1998), have shown that starvation can restore protease, elastase and hamnolipd production in a *las* quorum sensing deficient mutant.

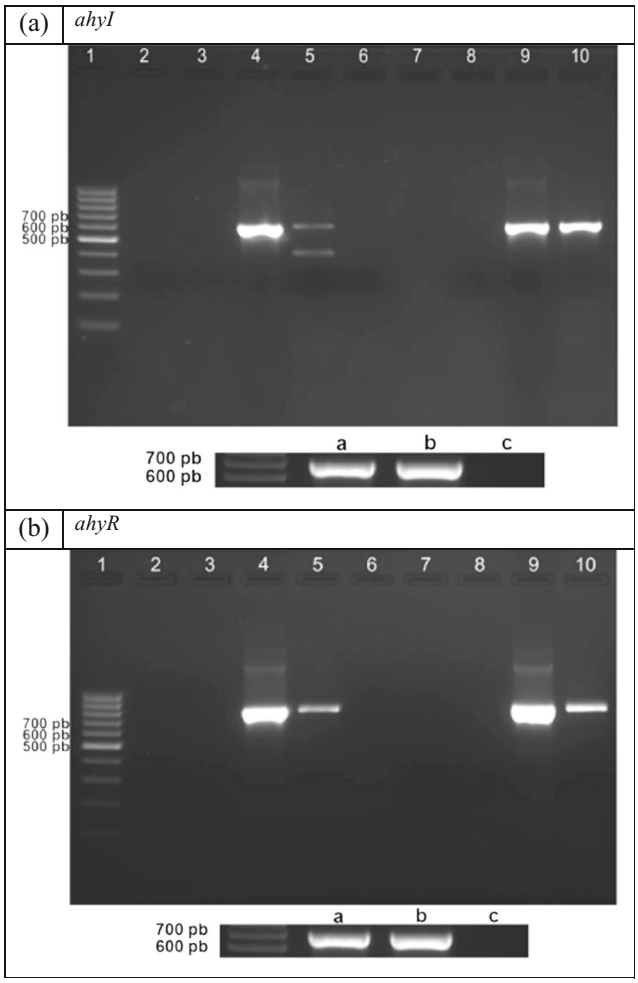

Fig. 4 Confirmation of gene amplification of *ahyI* (**a**) or *ahyR* (**b**) using cDNA templates from *A. hydrophila* ATCC 7966 and Embrapa 029. Reaction products were subjected to 1.5 % agarose gel electrophoresis. Lane 1, DNA marker (100 bp Gene Ruler^{TM}; Fermentas); Lane 2, negative PCR control (without genomic DNA); Lane 3, negative control for *A. hydrophila* ATCC 7966 cDNA (without reverse transcriptase); Lane 4, positive control for *ahyI* or *ahyR* using genomic DNA from *A. hydrophila* ATCC 7966 as a template; Lane 5, *ahyI* (**a**) or *ahyR* (**b**) using cDNA of *A. hydrophila* ATCC 7966 as a template; Lane 6, empty; Lane 7, negative PCR control (without genomic DNA); Lane 8, negative control for *A. hydrophila* Embrapa 029 cDNA (without reverse transcriptase); Lane 9, positive control for *ahyI* or *ahyR* using genomic DNA from *A. hydrophila* Embrapa 029 as a template, Lane 10, *ahyI* (**a**) or *ahyR* (**b**) using cDNA from *A. hydrophila* 029 as a template. Lane a, 16S rDNA using cDNA from *A. hydrophila* ATCC 7966 as a template; Lane b, 16S rDNA using cDNA from *A. hydrophila* Embrapa 029 as a template; Lane c, negative PCR control

Other hydrolytic activities were also present in both the ATCC 7966 strain and the Embrapa 029 strain. Specifically, we observed the activities of amylase (Fig. 6a) and lipases (Fig. 6b) along with β-hemolysis, all of which were verified by the presence of a clear halo surrounding the colonies. *A. hydrophila* ATCC 7966 showed amylolytic, lipolytic, and β-hemolytic activity, suggesting that these activities are not regulated by ahyI/R QS in this particular strain. Different results were obtained by Bi et al. (2007), who found that an *A. hydrophila* J-1 *ahyR* mutant did not produce detectable quantities of amylase or hemolysins. To the best of our knowledge, there is no evidence that lipolytic activity is regulated by QS in *A. hydrophila*. Additionally, there are no detailed studies on the regulation of genes encoding for lipases in *A. hydrophila.* Taken together, these results suggest that the AHL mediated QS is not the only regulator of the expression of genes related to the evaluated hydrolytic activities in *A. hydrophila* ATCC 7966.

Biofilm assay on stainless steel

A. hydrophila ATCC 7966 adhered to the stainless steel coupons from the initial time of inoculation, and a biofilm was formed during the 72 h incubation period (Fig. 7a, c, e, and g). Similar results were observed with *A. hydrophila* Embrapa 029, which was used as a positive control for the QS system (Fig. 7b, d, and f). Despite its compromised QS system, *A. hydrophila* ATCC 7966 presented a similar or even greater number of adhered cells compared to *A. hydrophila* Embrapa 029. The initial adhesion of both strains was similar and ranged from 2.5 to 3 log CFU cm^{-2}. However, in the samples taken at 24 and 48 h of incubation, *A. hydrophila* ATCC 7966 exhibited an adhered cell count that was approximately 1 log cycle greater than that which was observed in *A. hydrophila* Embrapa 029, which was used as a positive control for QS mediated phenotypes (data not shown). These results suggest that the *ahyRI* QS system of *A. hydrophila* ATCC 7966 does not interfere in this phenotype, under the evaluated conditions. Contrasting results were found by Lynch et al. (2002), who observed that the *ahyI*⁻ mutant was unable to form a mature

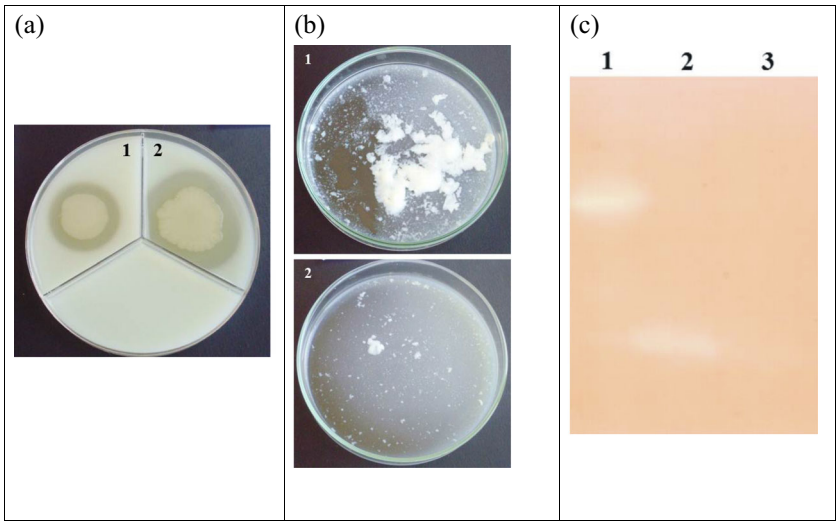

Fig. 5 Proteolytic activity of *A. hydrophila* strains. (**a**) PCA agar plates supplemented with 2 % skim milk powder and incubated at 30 °C for 72 h. (a_1) *A. hydrophila* ATCC 7966; (a_2) *A. hydrophila* Embrapa 029. (**b**) Coagulation of reconstituted skim milk powder (12 %) after 24 h at 30 °C. (b_1) *A. hydrophila* ATCC 7966; (b_2) *A. hydrophila* Embrapa 029. (**c**) Zymogram on 12 % SDS-PAGE using 2 % azocasein as a substrate. Cell-free supernatant of (c_1) *P. fluorescens* 041 (c_2) *A. hydrophila* 029 (10 μg of protein); (c_3) *A. hydrophila* ATCC 7966 (10 μg of protein)

biofilm. *A. hydrophila* SSU, which is unable to synthesize AI-2, also exhibited an altered architecture and altered dynamics of biofilm formation (Kozlova et al. 2008). In a double knock-out mutant of *A. hydrophila* SSU (Δ*ahyRI*), biofilm formation on a solid surface was significantly decreased compared to the wild-type strain (Khajanchi et al. 2009). Because *A. hydrophila* ATCC 7966 does not accumulate detectable AHLs as observed in our study and others, these results illustrate that the formation of biofilms, which depends on multiple factors for development (not only quorum sensing), may be independent of the common AHL-mediated signaling in this strain.

Conclusions

Quorum signaling AHL molecules were not detected in *A. hydrophila* ATCC 7966 by cross-streaking or thin layer chromatography with the use of AHL biosensor strains, as previously reported. Although *A. hydrophila* ATCC 7966 did not produce detectable AHLs, transcription of the *ahyI* in low quantity and *ahyR* genes was observed by RT-PCR.

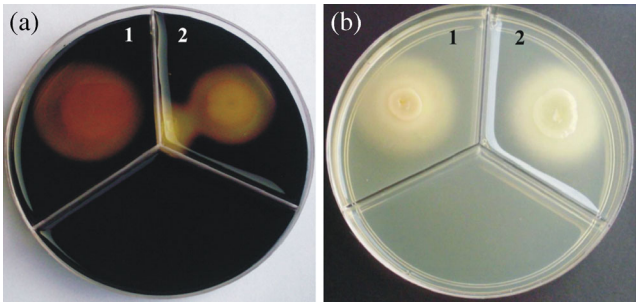

Fig. 6 Hydrolytic activities of *A. hydrophila* strains incubated at 30 °C for 72 h. (**a**) Amylolytic activity on nutrient agar supplemented with 0.2 % starch and (**b**) Lipolytic activity on Tween 80 agar; (1) *A. hydrophila* ATCC 7966; (2) *A. hydrophila* 029

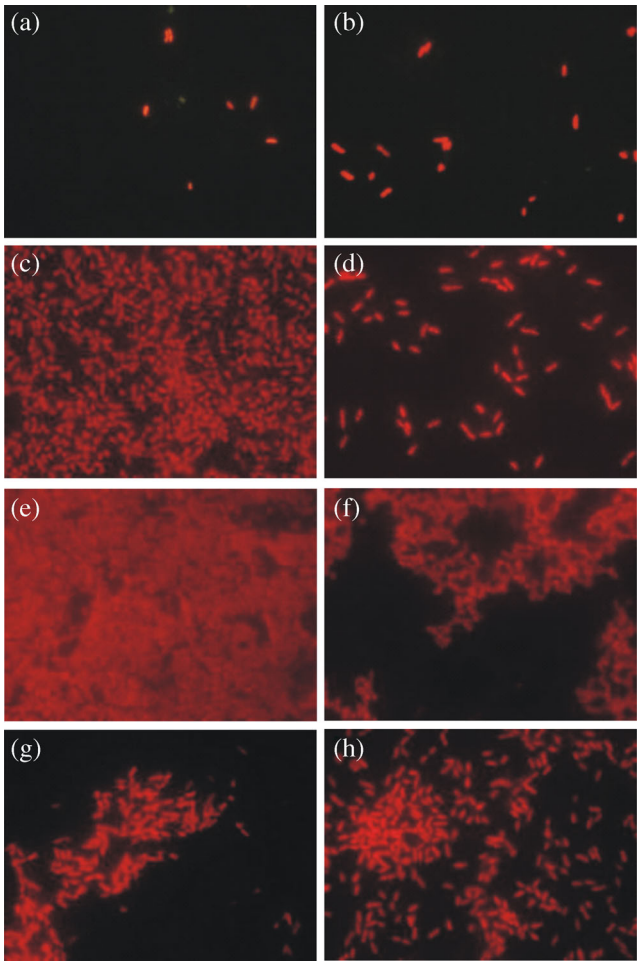

Fig. 7 Epifluorescence micrographs of biofilms formed by *A. hydrophila* incubated on stainless steel coupons immersed in TYEP at 30 °C for 0 to 72 h and subsequently stained with acridine orange. Magnification = 1000X. *A. hydrophila* ATCC 7966 at 16 h (**a**); 24 h (**c**); 48 h (**e**); 72 h (**g**) and *A. hydrophila* 029 at 16 h (**b**); 24 h (**d**); 48 h (**f**); 72 h (**h**)

Phenotypes generally recognized as QS-regulated in *A. hydrophila* were maintained in ATCC 7966; these phenotypes included proteolytic, amylolytic, lipolytic and β-hemolytic activities, as well as the ability to adhere to surfaces and to form biofilms.

The comparison of the results found in *A. hydrophila* ATCC 7966 with those described in the literature for different QS mutants of *A. hydrophila* suggests that there might be different regulatory strategies being used by this bacterium that still need to be clarified. It is likely that *A. hydrophila* ATCC 7966 may have adapted to the absence of AHL signaling response by developing other mechanisms of gene regulation on phenotypes commonly associated with *ahyRI* quorum sensing. This adaptation may apply to other strains that have naturally compromised QS systems in the genus *Aeromonas*.

Acknowledgments A.R. Ponce-Rossi was supported by a CNPq-Brazil fellowship, and the FAPEMIG and CAPES provided research funds. We thank the Embrapa Gado de Leite for providing the strain *Aeromonas hydrophila* Embrapa 029. We acknowledge Jose C. Huguet-Tapia for his insights in the genomic comparisons among *A. hydrophila* strains. We also thank two unidentified reviewers from the journal who have greatly contributed to the improvement of our text.

References

Bi ZX, Liu YJ, Lu CP (2007) Contribution of AhyR to virulence of *Aeromonas hydrophila* J-1. Res Vet Sci 83:150–156

Chai Y, Winans S (2004) Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: alteration of autoinducer specificity. Mol Microbiol 51(3):765–776

Christensen AB, Riedel K, Eberl L, Flodgaard LR, Molin S, Gram L, Givskov M (2003) Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a. Microbiology 149:471–483

Chu W, Liu Y, Jiang Y, Zhu W, Zhuang X (2013) Production of N-acyl homoserine lactones and virulence factors of waterborne *Aeromonas hydrophila*. Indian J Microbiol 53(3):264–268

Gobat P, Jemmi T (1993) Distribution of mesophilic *Aeromonas* species in raw and ready-to-eat fish and meat products. Int J Food Microbiol 20:117–120

Haba E, Bresco O, Ferrer C, Marqué A, Busquets M, Manresa A (2000) Isolation of lipase-secreting bacteria by deploying used frying oil as selective substrate. Enzyme Microbiol Tech 26:40–44

Jahid IK, Lee N-Y, Kim A, Ha S-D (2013) Influence of glucose concentrations on biofilm formation, motility, exoprotease production, and quorum sensing in *Aeromonas hydrophila*. J Food Prot 70(2):239–247

Jahid IK, Mizan MFR, Ha AJ, Ha S-D (2015) Effect of salinity and incubation time of planktonic cells in biofilm formation, motility, exoprotease production, and quorum sensing of *Aeromonas hydrophila*. Food Microbiol 49:142–151

Jangid K, Kong R, Patole MS, Shouche YS (2007) *luxRI* homologs are universally present in the genus *Aeromonas*. BMC Microbiol 7:93–101

Joseph B, Otta SK, Karunasagar I (2001) Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. Int J Food Microbiol 64:367–372

Kelley LA, Sternberg MJE (2009) Protein structure prediction on the web: a case study using the phyre server. Nat Protoc 4:363–371

Khajanchi BK, Sha J, Kozlova EV, Erova TE, Suarez G, Sierra JC, Popov VL, Horneman AJ, Chopra AK (2009) N-Acyl homoserine lactones involved in quorum sensing control the type VI secretion system, biofilm formation, protease production, and in vivo virulence in a clinical isolate of *Aeromonas hydrophila*. Microbiology 155:3518–3531

Kirke D, Swift S, Lynch MJ, Williams P (2004) The *Aeromonas hydrophila* LuxR homologue AhyR regulates the N-acyl homoserine lactone synthase AhyI positively and negatively in a growth phase-dependent manner. FEMS Microbiol Lett 241:109–117

Kozlova EV, Popov VL, Dha J, Foltz SM, Erova TE, Agar SL, Hornema AJ, Chopra AK (2008) Mutation in the S-ribosylhomocysteinase (*luxS*) gene involved in quorum sensing affects biofilm formation and virulence in a clinical isolate of *Aeromonas hydrophila*. Microb Pathog 45:343–354

Kozlova EV, Khajanchi BK, Sha J, Chopra AK (2011) Quorum sensing and c-di-GMP-dependent alterations in gene transcripts and virulence-associated phenotypes in a clinical isolate of *Aeromonas hydrophila*. Microb Pathog 50(5):213–223

Lynch MJ, Swift S, Kirke DF, Keevit W, Dodd CER, Williams P (2002) The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ Microbiol 4(1):18–28

Martins ML, Pinto UM, Riedel K, Vanetti MCD (2015) Milk-deteriorating exoenzymes from *Pseudomonas fluorescens* 041 isolated from refrigerated raw milk. Braz J Microbiol 46(1):207–217

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Camara M, Daykin M, Lamb JH, Swift S, Bycroft BW, Stewart GS, Williams P (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of N-acylhomoserine lactones. Microbiology 143:3703–3711

Medina-Martinez MS, Uyttendaele M, Demolder V, Debevere J (2006) Influence of food system conditions on N-acyl-L-homoserine lactones production by *Aeromonas* spp. Int J Food Microbiol 112:244–252

Morton RD (2001) Aerobic plate count. In: Downes FP, Ito K (eds) Compendium of methods for the microbiological examination of foods, 4th edn. APHA, Washington, pp 183–193

Murano EA, Hudnall JA (2001) Media, reagents, and stains. In: Downes FP, Ito K (eds) Compendium of methods for the microbiological examination of foods, 4th edn. APHA, Washington, pp 601–648

Nagar V, Sinha V, Bandekar JR (2015) Diverse profiles of N-acyl homoserine L-lactones, biofilm, virulence genes and integrons in food-borne *Aeromonas* isolates. J Food Sci 80(8):M1861–M1870

Natrah FMI, Alam MI, Pawar S, Harzevili AS, Nevejan N, Boon N, Sorgeloos P, Bossier P, Defoirdt T (2012) The impact of quorum sensing on the virulence of *Aeromonas hydrophila* and *Aeromonas salmonicida* towards burbot (Lotalota L.) larvae. Vet Microbiol 159:77–82

Parizzi SQF, Andrade NJ, Silva CAS, Soares NFF, Silva EAM (2004) Bacterial adherence to different inert surfaces evaluated by epifluorescence microscopy and plate count method. Braz Arch Biol Technol 47:77–83

Pinto UM, Winans SC (2009) Dimerization of the quorum-sensing transcription factor TraR enhances resistance to cytoplasmic proteolysis. Mol Microbiol 73(1):32–42

Pinto UM, Viana ES, Martins ML, Vanetti MCD (2007) Detection of acylated homoserine lactones in gram-negative proteolytic psychrotrophic bacteria isolated from cooled raw milk. Food Control 18:1322–1327

Pinto UM, Costa ED, Mantovani HC, Vanetti MCD (2010) The proteolytic activity of *Pseudomonas fluorescens* 07A isolated from milk is not regulated by quorum sensing signals. Braz J Microbiol 41:91–96

Seshadri R, Joseph SW, Chopra AK, Sha J, Shaw J, Graf J, Haft D, Wu M, Ren Q, Rosovitz MJ, Madupu R, Tallon L, Kim M, Jin S, Voung H, Stine C, Ali A, Horneman AJ, Heidelberg JH (2006) Genome sequence of *Aeromonas hydrophila* ATCC 7966T: Jack of all trades. J Bacteriol 188(23):8272–8282

Shaw PD, Ping G, Daly SL, Chung C, Cronan JE, Rinehart KL, Farrand SK (1997) Detection and characterizing N-acil-homoserine lactone signal molecules by thin-layer chromatography. Proc Natl Acad Sci U S A 94:6036–6041

Stamford TLM, Araújo JM, Stamford NP (1998) Enzymatic activity of microorganisms isolated from *Pachyrhizuserosus* L. Urban (Atividade enzimática de microrganismos isolados do jacatupé (*Pachyrhizuserosus* L. Urban). Cienc Tecnol Aliment 18(4):382–385

Steidler L, Venturi V (2007) Detection of quorum-sensing N-acyl homoserine lactone signal molecules by bacterial biosensors. FEMS Micrbiol Lett 266:1–9

Swift S, Karlyshev AV, Fish L, Durant EL, Windon 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-acyl homoserine lactone signal molecules. J Bacteriol 179(17):5271–5281

Swift S, Lynch MJ, Fish L, Kirke DF, Tomés JM, Stewart GSAB, Williams P (1999) Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*. Infect Immun 67(10):5192–5199

Van Delden C, Pesci EC, Pearson JP, Iglewski BH (1998) Starvation selection restores elastase and rhamnolipid production in a Pseudomonas aeruginosa quorum sensing mutant. Infect Immun 66(9):4499–4502

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-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Lett 163:85–192

Zhang R-G, Pappas KM, Brace JL, Miller PC, Oulmassov T, Molyneaus JM, Anderson JC, Bashkin JK, Winans SC, Joachimiak A (2002) Structure of a bacterial quorum-sening transcription factor complexed with pheromone and DNA. Nature 417:971–974