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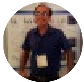

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Effect of salinity and incubation time of planktonic cells on biofilm formation, motility, exoprotease production, and quorum sensing of *Aeromonas hydrophila*

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ABSTRACT

The aim of this study was to determine the effect of salinity and age of cultures on quorum sensing, exoprotease production, and biofilm formation by *Aeromonas hydrophila* on stainless steel (SS) and crab shell as substrates. Biofilm formation was assessed at various salinities, from fresh (0%) to saline water (3.0%). For young and old cultures, planktonic cells were grown at 30 °C for 24 h and 96 h, respectively. Biofilm formation was assessed on SS, glass, and crab shell; viable counts were determined in R2A agar for SS and glass, but *Aeromonas*-selective media was used for crab shell samples to eliminate bacterial contamination. Exoprotease activity was assessed using a Fluoro™ protease assay kit. Quantification of acyl-homoserine lactone (AHL) was performed using the bioreporter strain *Chromobacterium violaceum* CV026 and the concentration was confirmed using high-performance liquid chromatography (HPLC). The concentration of autoinducer-2 (AI-2) was determined with *Vibrio harveyi* BB170. The biofilm structure at various salinities (0–3 %) was assessed using field emission electron microscopy (FESEM). Young cultures of *A. hydrophila* grown at 0–0.25% salinity showed gradual increasing of biofilm formation on SS, glass and crab shell; swarming and swimming motility; exoproteases production, AHL and AI-2 quorum sensing; while all these phenotypic characters reduced from 0.5 to 3.0% salinity. The FESEM images also showed that from 0 to 0.25% salinity stimulated formation of three-dimensional biofilm structures that also broke through the surface by utilizing the chitin surfaces of crab, while 3% salinity stimulated attachment only for young cultures. However, in marked contrast, salinity (0.1–3%) had no effect on the stimulation of biofilm formation or on phenotypic characters for old cultures. However, all concentrations reduced biofilm formation, motility, protease production and quorum sensing for old culture. Overall, 0–0.25% salinity enhanced biofilm formation and expression of quorum sensing regulatory genes in young cultures, whereas these responses were reduced when salinity was >0.25%. In old cultures, salinity at any concentrations (0.1–3%) induced stress in *A. hydrophila*. The present study provides insight into the ecology of *A. hydrophila* growing on fish and crustaceans such as shrimp and crabs in estuarine and seawater.

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1. Introduction

Aeromonas hydrophila is an emerging pathogen of animals, reptiles, and humans (Austin and Adams, 1996; Kirov, 2003; Janda and Abbott, 2010). The importance of *A. hydrophila* for food safety (Daskalov, 2006), fish diseases (Beaz-Hidalgo and Figueras, 2013), human infections (Janda and Abbott, 2010), quorum sensing, and biofilm formation (Chopra et al., 2009) has been reviewed and it has been found that by formation of biofilms, *A. hydrophila* contributes to diseases. Free-living *A. hydrophila* is ubiquitous in fresh and estuarine water (Ottaviani et al., 2011) and is associated with fish (Hossain et al., 2014), crabs (Nielsen et al., 2001), shrimps

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(Deng et al., 2013), and mollusks (Ottaviani et al., 2006) which is emerging as a potential foodborne pathogen transmitted by consumption of contaminated water or foods (Carvalho et al., 2012; Janda and Abbott, 2010; Khajanchi et al., 2010). Therefore, seafood contaminated with unpurified water are the primary vehicles for *A. hydrophila* infection in humans especially children and immunocompromised individuals (Janda and Abbott, 2010). The pathogenesis of *A. hydrophila* infection is caused by the production of multiple virulence factors such as cytotoxic enterotoxin (Act), hemolysin, protease, and lipase (Chopra et al., 2009).

Microbial biofilms are sessile microbial communities that attach to biotic and abiotic surfaces and survive as self-organizing, three-dimensional structures by producing an extracellular polymeric matrix (EPS). Like many other microorganisms, *A. hydrophila* has been shown to attach and form biofilms on stainless steel (Lynch et al., 2002) and vegetables (Jahid et al., 2014a) in laboratory settings. Initial contamination levels with osmoadaptation (Hingston et al., 2013), cultural age (Chorianopoulos et al., 2010), sublethal concentrations (Gravesen et al., 2005) modulate the biofilms formation on foods and food contact surfaces.

Quorum sensing involves secretion of specific molecules like autoinducers (AIs) by microorganisms to communicate and regulate their intraspecies and interspecies density, which contributed to food safety (Bai and Rai, 2011; Skandamis and Nychas, 2012; Smith et al., 2004). *A. hydrophila* produces *N*-3-butanoyl-DL-homoserine lactone synthase, encoded by *ahyI*, and *N*-3-hexanoyl homoserine lactone synthase, and secretes*N*-3-butanoyl-DL-homoserine lactone (C4-AHL) and *N*-3-hexanoyl homoserine lactone (C6-AHL) (Swift et al., 1999). *A. hydrophila* also produces autoinducer-2 (AI-2), which is involved in interspecies communication (Kozlova et al., 2008). The presence of *luxS* gene and the intraspecies quorum sensing molecules, C4-AHL and C6-AHL of *A. hydrophila* modulate biofilm formation, motility, protease production, and virulence gene expression (Kozlova et al., 2008; Jahid et al., 2013a; Khajanchi et al., 2009; Swift et al., 1999). Acyl-homoserine lactone (AHL) quorum sensing has been found to regulate *A. hydrophila* virulence in fish (Natrah et al., 2012). Several studies have reported a correlation between quorum sensing and protease activity (Swift et al., 1999; Vivas et al., 2004). Pretreatment with AHL has been shown to enhance the innate immune response in mice and to kill *A. hydrophila* (Khajanchi et al., 2011 The importance of quorum sensing in *A. hydrophila* has been reported in terms of protease production and virulence in natural settings such as water and fish samples (Chu et al., 2013; Natrah et al., 2012; Styp von Rekowski et al., 2008).

The effect of sodium chloride (NaCl) on biofilms has been reported in many bacteria, including foodborne pathogens such as *Listeria monocytogenes* (Jensen et al., 2007), *Staphylococcus aureus* (Lim et al., 2004), *Salmonella typhimurium* (Xu et al., 2010), *Escherichia coli* (Jubelin et al., 2005), *Vibrio cholerae* (Shikuma and Yildiz, 2009), and *Vibrio vulnificus* (McDougald et al., 2006). Most of these studies showed that NaCl enhances biofilm formation. However, few data are available on biofilm formation, motility, and quorum sensing in bacterial species that grow on shellfish and mollusks, which live at different salinities common in the estuarine environment. Several studies have independently demonstrated the relationship of salinity with AHL quorum sensing (Medina-Martínez et al., 2006), AI-2 quorum sensing (Kim and Shin, 2012), and protease production (Khan et al., 2007; Takahashi et al., 2011). However, the influence of salinity on biofilm formation and quorum sensing is poorly understood in regards to food and food contact surfaces. In this paper, we addressed the effects of salinity and age of *A. hydrophila* cultures on motility, quorum sensing, protease activity, and biofilm formation on stainless steel (SS), glass, and crab shell.

2. Materials and methods

2.1. Bacterial strain, culture media and conditions

In the present study, we used the following strains: *A. hydrophila* KCTC 11533 (isolated from surface water), KCCM 32586 (a clinical isolate), *Chromobacterium violaceum* CV026, and *Vibrio harveyi* BB120 and BB170. The bioreporter strain CV026 was provided by the Animal, Plant, and Fisheries Quarantine and Inspection Agency, Korea. The bacteria were grown in liquid nutrient broth (NB) (Difco Laboratories; Detroit, MI, USA). Modified Luria–Bertani (LB) medium (Difco) without sodium chloride (0%) was used for the violacein production assay. Prior to each experiment, the cultures were activated by transferring them from the −80 °C freezer on nutrient agar plates to 30 °C for overnight incubation. A single colony from each plate was inoculated in 5 mL NB and incubated overnight at 30 °C with shaking at 220 rpm; "young cultures" were incubated for 24 h and "old cultures" were incubated for 96 h. Young and old cultures of *A. hydrophila* were centrifuged at 11,000 × g for 10 min, washed, and resuspended in fresh LB broth to produce a final optical density at 600 nm (OD600) of 1.0. These "young" and "old" cultures were diluted as required and used in subsequent experiments for planktonic growth, biofilms formation, exoprotease assay, motility assay, and quorum sensing assay. From hereafter, these cultures will be called as "standardized culture". A 20% (w/v) sodium chloride (Merck; Darmstadt, Germany) solution was prepared by filter sterilization using 0.22-μm filters (Millipore Corporation; Billerica, MA, USA). Sodium chloride (NaCl) solutions of varying concentrations (0, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0% (w/v)) were prepared by addition of sterile modified LB. Bacteria were grown at 30 °C unless otherwise indicated. For growth and biofilm formation on crab shell, *cyanobacteria* BG-11 fresh water solution (Sigma Aldrich, Inc., St. Louis, MO, USA) was diluted with sterile deionized water representative of fresh water. Artificial sea salts (Sigma, USA) was diluted and used to prepare water of varying salinity representative of estuarine and sea water.

2.2. Biofilm formation on stainless steel

SS coupons (2 × 2 × 0.1 cm, type: 302) were processed as described previously (Shen et al., 2012). The standardized culture was diluted 1:50 and inoculated into LB containing various concentrations (0, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0% (w/v)) of NaCl in 50-mL Falcon tubes; each tube contained an SS coupon that was completely submerged in 7 mL LB to enable biofilm formation. The tubes were incubated without shaking at 30 °C for 72 h to allow formation of biofilms on the SS coupons. Following incubation, each SS coupon was transferred to a small petri dish (55 × 12 mm) that contained 2 mL of 0.1% peptone water (PW), scrubbed, and then transferred to a test tube and ultrasonicated for 1 min in a sonicator (power, 380 W, 37 kHz 37; Elma; Elmasonic P; Germany) to disperse the biofilm (Jahid et al., 2014b). The cells were vortexed and diluted in PW for counting. The samples were then spread onto R2A agar (Difco; USA) and cultures were counted after incubation at 30 °C for 24 h. The cells grown in modified LB broth with various concentrations of NaCl was considered as planktonic counts (3days at 30 °C) and determined to find out the effect of salinity on planktonic cells.

2.3. Biofilm formation on glass

Glass tubes (1-cm diameter) were autoclaved, and an *A. hydrophila* standardized cultures were diluted to 1:50 and 3 mL LB containing various concentrations of NaCl. The tubes were then incubated at 30 °C for 72 h without shaking. Following incubation,

tubes were washed with sterile distilled water and a swab technique was used to remove the biofilms. The swab technique was performed as described previously (Jahid and Ha, 2014), with some modifications. Briefly, a sterile wooden cotton swab (Mogami, China) was pre-moistened in a sterile bottle containing 20 mL of PW. Swabbing was performed by wiping the biofilms in two directions: top to bottom and left to right. Swabbing was always performed by the same person so that the same pressure was applied (Jahid and Ha, 2014).

2.4. Preparation of inoculum for food samples

standardized cultures were centrifuged (11,000 × *g* for 10 min at 4 °C) and the pellets were washed twice with Dulbecco's phosphate-buffered saline (DPBS). The pellets were re-suspended in the appropriate amount of DPBS to produce the same final concentration of bacterial cells. The cell density was then determined by performing serial dilutions and plating on *Aeromonas*-selective media (Oxoid; Basingstoke, Hants, England). These inocula were used to induce biofilm formation on crab shell.

2.5. 5Biofilm formation on crab shell

The crab (*Corystes cassivelaunus*) used in the present study was purchased from a local grocery store in Anseong-Si, Korea. The crab was aseptically cut to produce coupons of 2 × 2 cm^2 using a sterile scalpel, washed with sterile distilled water, and then the flesh was removed. The coupons were used immediately after preparation. Prior to inoculation, the coupons were placed in an open sterile petri dish and subjected to ultraviolet (UV)–C treatment for 30 min on each side to minimize background flora, before being inoculated with *A. hydrophila*. Each coupon was submerged in 10 mL fresh water or in various concentrations of seawater; the prepared inocula (Section 2.4) were inoculated at a dilution of 1:2500 and the coupons were incubated at 30 °C for 24 h without shaking. Detachment of microbial populations from the crab shell coupons was performed using procedures described by Jahid et al. (2014b), with minor modifications. After incubation, the coupons were placed in 10 mL of PW (Oxoid; UK) in a sterile stomacher bag (Nasco Whirl-pak, Fort Atkinson, WI, USA) and processed using a Stomacher® (Bagmixer; Interscience; Saint Nom, France) at the highest speed for one min to release the biofilm-forming bacteria from the crab. Thereafter, the stomacher bag containing samples was ultrasonicated to disperse the biofilm from crab shell and from each other of bacterial population. Counting of *A. hydrophila* was performed by serial dilution and spread plating on *Aeromonas*-selective medium (Oxoid; UK) containing ampicillin. The plates were incubated at 30 °C for 48 h and colonies were counted and expressed as colony forming units (CFU)/cm^2. For each of the three independent experiments, two plates per dilution were used to calculate the results.

2.6. Motility assay (swimming and swarming)

Swimming is defined as flagella-directed movement in an aqueous environment, and swarming is defined as multiple, lateral, flagella-directed rapid movements on a solid surface. Both forms of motility were examined using previously described methods (Jahid et al., 2013a) with slight modifications. For swimming motility, 1.5 μL standardized cultures were spotted at the center of a plate containing 25 mL modified LB with various salinity (0, 0.1, 0.25, 0.5, 1.0, 2.0, and 3.0%) and 0.3% Bacto-agar (Difco) and incubated face-up for 15 h at 25 °C. The assay was performed after the plates were allowed to dry for 12 h. To measure swarming, 1.5-μL culture volume was inoculated on an agar plate containing 25 mL modified LB with various salinity and 0.5% Bacto-agar (Difco) and incubated at 25°C for 72 h. After incubation, the diameter of motility of the strains was measured by examining the migration of bacteria through the agar from the center toward the periphery of the plate and the plate was photographed.

2.7. Exoprotease activity assay

Exoprotease activity was assessed using a protease assay kit (G-Biosciences; St. Louis, MO, USA).*A. hydrophila* standardized cultures were diluted (1:50) with in modified LB with different concentrations of NaCl was added and the cultures were incubated for 24 h without shaking. After incubation, supernatants were collected by centrifugation at 15,000 × *g* for 10 min the supernatant (45 μL) from each NaCl condition was processed using a protease assay kit, according to the manufacturer's instructions (G-bioscience, St. Louis, MO, USA). After incubation, the absorbance was determined at 570 nm using a microplate reader (Spectra Max 190; Molecular Devices; Sunnyvale, CA, USA). The data were interpreted using the trypsin standard supplied with the kit and medium with substrate as a negative control.

2.8. Quantification of acyl-homoserine lactone production

To quantify violacein production, *A. hydrophila* cultures were grown in various concentrations of NaCl in modified LB broth at 30 °C for 24 h in 50 mL Falcon tube, after which the supernatant was collected by centrifugation at 15,000 × *g* for 15 min. The supernatant was then filter-sterilized using 0.22-μm filters (Millipore Corporation; Billerica, MA, USA). Luria–Bertani agar (LA) without NaCl was prepared and poured using the open side of a 1 mL pipette tip. Thereafter, 10 μLof overnight culture of CV026 was spread on the wall of a well in the LA platesand 100-μL sterile supernatant from each condition was added in the wells. These were then incubated at 28 °C for 24 h in an inverted position. Next, whole CV026 grown on plate were collected and were solubilized with 250 μL dimethyl sulfoxide (DMSO; Sigma Aldrich). The mixtures were then vortexed to ensure release the violacein pigment. After centrifugation at 15,000 × *g* for 15 min, 200 μL of colored DMSO from each condition was measured using a microplate reader (Spectra Max 190; Molecular Devices) at 585 nm.

2.9. Detection of AHLs by high-performance liquid chromatography (HPLC)

Detection, identification, and quantification of AHLs were performed as previously described (Truchado et al., 2009) with some modifications. Filtered sterile supernatants from *A. hydrophila* 11533 (Section 2.8) were used to detect AHL by HPLC. The AHLs were analyzed in an HPLC system using an Atlantis dc-18 column (4.6 × 200 mm, water) and a Varian ProStar HPLC (Varian; Walnut Creek, CA, USA) set at 280 nm in a diode array detector. Columns were used with water (A) and acetonitrile (B) HPLC-grade solvents (J.T.Baker, Center Valley, PA, USA). The mobile phase consisted of water (solvent A) and acetonitrile (solvent B), both of which contained 0.1% (v/v) formic acid. The isocratic profile used was10%B in A to 95% B in A for 15 min and then 100% B for 16–18 min, then 90% A and 10% B for 19 min, and finally, 90% A to 10% B for 4 min. A flow rate of 1.0 mL/min was applied using a microsplitter valve.

2.10. Field emission scanning electron microscopy (FESEM)

Biofilm formation of *A. hydrophila* was observed on crab shell and SS using FESEM. The inoculation and incubation procedures were the same as those described. Processing of FESEM samples

was performed according to procedures described previously (Jahid et al., 2014b).

2.11. AI-2 determination

AI-2 levels in *A. hydrophila* grown on crab shell at various concentrations of salinity were determined according to procedures described previously (Soni et al., 2008), with minor modifications. Briefly, the fresh and seawater samples grown on crab shell were incubated as previously described and centrifuged at 15,000 × *g* for 10 min. The cell-free culture supernatants were then passed through syringe filters (0.2 μm) (Sartorius Stedim Biotech GmbH, Goettingen, Germany) and stored at −20 °C. *V. harveyi* strain, BB120 (which produces AI-1 and AI-2) was used as a positive control. Control strains of *V. harveyi* were grown overnight at 30 °C with aeration in LB (Difco) broth, and 1 mL of cell-free supernatant of each culture was prepared as described above. The cell-free supernatants from *A. hydrophila* and *V. harveyi* BB170 were tested for the presence of autoinducers that could induce luminescence due to the presence of AI-2 in the supernatant. This reporter strain possesses sensor two but not sensor one, and is therefore capable of sensing AI-2 but not AI-1. For this bioassay, *V. harveyi* strain BB170 was grown overnight at 30 °C with aeration in autoinducer bioassay (AB) broth and diluted 1:1000 into AB medium (Bassler et al., 1993). Four-and-a-half milliliters of diluted bacteria and 500 μL of the cell-free supernatant from each sample were added to 50-mL Falcon tubes and shaken for 16 h at 220 rpm. One-hundred microliter samples were transferred to white microtiter plates and the luminescence was measured after incubation for 16 h using a computer-controlled microplate luminometer (GloMax® 96 Microplate Luminometer for Luminescence; Promega, Madison, WI, USA).

2.12. Statistical analysis

Biofilm formation, protease activity, and quorum sensing were analyzed by ANOVA for a randomized design using SAS software, version 9.2 (SAS Institute Inc.; Cary, NC, USA). When the effect was significant ($p < 0.05$), separation of the mean was accomplished with Duncan's multiple-range test.

3. Results

3.1. Planktonic inhibition and biofilms on stainless steel

The results of biofilm modulation on SS with different concentrations of NaCl using the plate count method are presented in Fig. 1. The biofilm formation seems to be stimulated at the salt-concentrations of 0.1–0.25% and repressed at concentrations of 3%. The CFU/cm^2 at 0% was not significantly different from the CFU/cm^2 obtained at 0.5–2%, for the young 11533 culture (Fig. 1).

In contrast, old cultures did not induce biofilms at any concentration of NaCl compared to 0% salinity. In both cases, 3% NaCl inhibited biofilm formation, resulting in bacterial cell counts of approximately 4.0 log CFU/cm^2. At 0% NaCl, the average colony count was approximately 6.6 log CFU/cm^2, which increased to 8.6 log CFU/cm^{2}at 0.25% for young cultures, and was repressed to 4.0–5.0 log CFU/cm^2 at 3.0% NaCl. In general, salinity of 0.25% favored biofilm formation in young cultures but not in old cultures. For both culture types, increasing the salt concentration from 0.5% to 3.0% NaCl significantly ($p < 0.05$) decreased biofilm formation (Fig. 1). There was no significant ($p > 0.05$) difference for the planktonic populations obtained after 3 days in the LB with salt concentrations ranging from 0 to 3% NaCl. However, it is interesting that 3% NaCl inhibits the biofilm formation but not the planktonic growth. The mean viable counts were 8.57, 8.54, 8.47, and 8.43 CFU/mL at control (0% salinity) which were 8.15, 8.29, 8.21, and 8.29 CFU/mL for 3% salinity for 11533 young, 32586 young, 11533 old, and 32586 old respectively. The viable counts were impaired to 4.86, 4.48, 5.75, and 5.03 CFU/mL for 11533 young, 32586 young, 11533 old, and 32586 old respectively (Supplement Table 1).

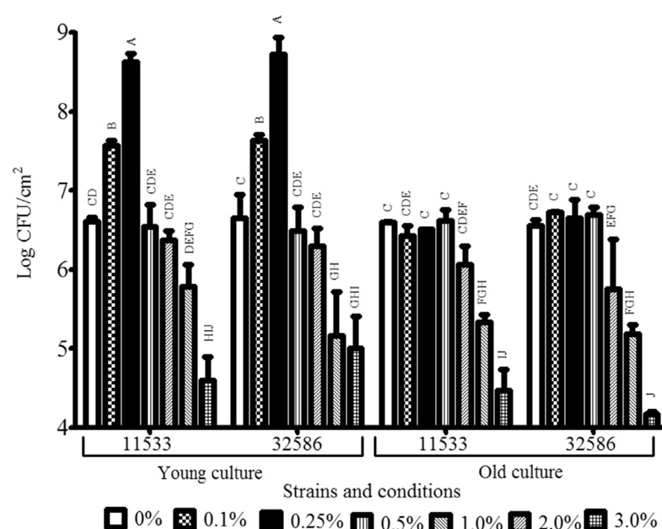

Fig. 1. NaCl concentration affects biofilm formation on stainless steel by young and old bacterial cultures. The values shown represent the means of log-values of bacterial populations ± SEM for three independent replicates. Within each treatment, values indicated with different capital letters are significantly different, according to Duncan's multiple-range test (p <0.05).

3.2. Biofilms on glass

To investigate whether NaCl modulates biofilm formation on glass, we tested *A. hydrophila* strains in the presence of various concentrations of NaCl. Biofilm production increased at concentrations from 0 to 0.25% in young cultures, as seen in Fig. 2. Biofilm formation was maximized at 0.25% NaCl, with a mean value of 7.5 log CFU/cm^2 and 8.3 log CFU/cm^2 for strains 11533 and 32586,

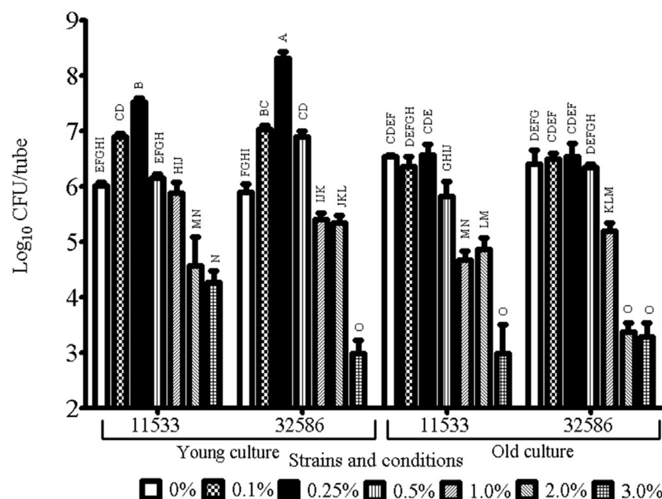

Fig. 2. NaCl concentration affects biofilm formation on glass surfaces by young and old bacterial cultures. (A) The values shown represent the means of log-values of bacterial populations ± SEM for three independent replicates. Within each treatment, values indicated with different capital letters are significantly different, according to Duncan's multiple-range test (p <0.05).

respectively. Biofilm formation remained constant until 1.0% salinity and then declined as salinity increased to 3.0% NaCl, with mean culture counts of 4.2 log CFU/cm^2 and 2.9 log CFU/cm^{2}for strains 11533 and 32586, respectively (Fig. 2). Biofilm production in old cultures remained constant from 0 to 0.5% salinity for both strains, but decreased from 1 to 3.0% NaCl. These results indicate that low concentrations of NaCl induced biofilm formation in young but not in old cultures (Fig. 2).

3.3. Biofilms on crab shell

To determine the effect of NaCl in an estuarine environment, *A. hydrophila* strains were grown in fresh water, and in artificial seawater at different salt concentrations on crab shell. The results presented in Fig. 3 show that in young cultures, biofilm formation on crab shell significantly ($p < 0.05$) increased from freshwater to 0.25% sea salts and gradually decreased from 0.5 to 3.0% sea salts. In old cultures, viable bacteria showed significantly ($p < 0.05$) higher biofilm formation in freshwater with crab shell, but no variation was observed until a 2.0% sea salt level, in which biofilm formation was reduced to 5.1 log CFU/cm^2 and 3.7 log CFU/cm^2 for strains 11533 and 32586, respectively.

3.4. Motility

Motility is known to be a key factor in biofilm formation. In young cultures, a small increase in NaCl, from 0 to 0.25% enhanced both swarming and swimming motility by 2–2.5-fold (Fig. 4). As shown in Fig. 4A, significant highest swarming motility was observed at 0.25% salinity and then decreased from 0.5% to 2% for young cultures while no significant increasing has been found from 0 to 0.25% salinity rather remained same from 0 to 0.25% salinity and then decreased from 0.5% to 2% salinity. Both strains showed no motility at 3.0% NaCl. Significant ($p < 0.05$) differences in swimming motility were observed in both strains at different NaCl concentrations (Fig. 4B). The swimming motility has stimulated from 0.1% to 0.25% and then decreased from 0.5% to 3% salinity for young cultures.

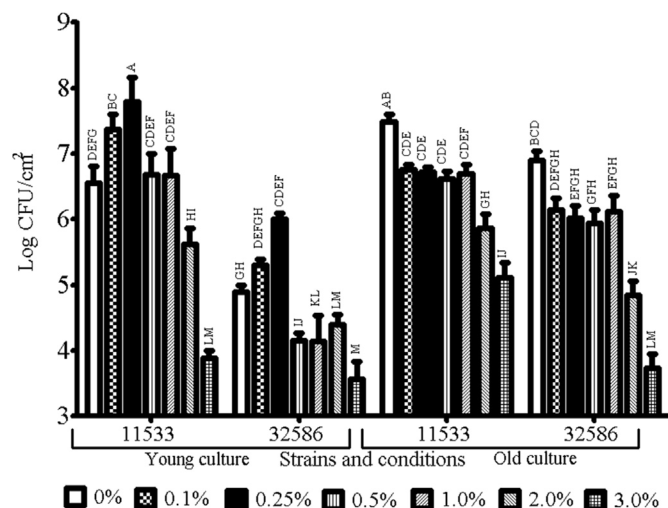

Fig. 3. Biofilm formation on crab shell by young and old bacteria cultured in various salinities. The values shown represent the means of log values of bacterial populations ± SEM for three independent replicates. Within each treatment, values indicated with different capital letters are significantly different, according to Duncan's multiple-range test (p <0.05).

3.5. Protease assay

We next assessed whether osmolarity had an effect on the ability of *A. hydrophila* to secrete proteases by using NaCl as an osmotic agent. It was not surprising that young cultures had significantly ($p < 0.05$) increased protease production from 0 to 0.25% NaCl, which decreased at higher concentrations of NaCl, from 0.5 to 3.0% (Table 1). In old cultures, compared to the control (0%), NaCl did not enhance protease production at any concentration rather decreased at any concentrations of salinity (Table 1).

3.6. AHL quorum sensing

Young cultures had significantly ($p < 0.05$) enhanced AHL production from 0.25 to 0.5% NaCl, which subsequently decreased from 1.0 to 3.0% NaCl. In old cultures, none of the samples containing NaCl stimulated the production of AHL compared to the control, but rather decreased the production of AHL (Fig. 5). It was also noteworthy that old cultures secreted significantly ($p < 0.05$) less AHL than young cultures. Production of AHL was observed to be strain-dependent; *A. hydrophila* 11533 produced more AHL than strain 32586 (Fig. 5). The current findings suggest that biofilm formation, motility, and protease production are all controlled by quorum sensing. The violacein production increased for young culture from 0% salinity to 0.5% salinity and at the same ways, the biofilms formation, motility, protease production enhanced from 0% to 0.25% which indicates the correlation of AHL quorum sensing with motility, biofilms protease productions.

3.7. AHL determination by HPLC

Violacein production at various concentrations of NaCl in LB broth by strain 11533 was confirmed by HPLC (Supplement Fig. 1). As shown in Supplement Fig. 1, the HPLC profile, retention time, and UV/visible spectra of standard C4-HSL matched the corresponding peaks in the supernatants of *A. hydrophila*, and the retention time was 6.91 min (standard data not shown). As shown in Supplement Fig. 1a, the highest peak was found at 0.25% NaCl; reduced peaks were detected at 0.10% NaCl and in the control (0%), and the peak was effectively absent from 3.0% NaCl. In marked contrast, in old cultures, the highest peak was observed for controls (0%), and reduced peaks were observed at increasing concentrations of NaCl (Supplement Fig. 1b). Although *A. hydrophila* produces C6-HSL, we did not observe any C6-HSL at the retention time of 12 min (data not shown). Therefore, it could be hypothesized that salinity control C4 and C6-HSL as well as biofilms, motility and exoproteases.

3.8. AI-2 quorum sensing assay

To improve our understanding of biofilm regulation by AI-2, we examined AI-2 expression using *V. harveyi* BB170 and monitored AI-2 production in supernatants of *A. hydrophila* grown on crab shell at various salinities. AI-2 expression by young and old cultures grown at different salinities on crab shell is shown in Table 2. In general, the results suggest that AI-2 expression varied widely at the different salinities and culture ages tested. In young cultures, higher expression was observed from 0 to 0.25% NaCl, and expression decreased from 0.5 to 3.0% salinity. In old cultures, the secretion of AI-2 at any salinity (0.1–3.0%) was always significantly ($p < 0.05$) less than the control (0% salinity) (Table 2). So, salinity modulates biofilms formation, motility and exoproteases by controlling the AI-2 quorum sensing.

3.9. FESEM

Because *A. hydrophila* biofilm formation was affected by salinity, we speculated that salinity might also affect the architecture of

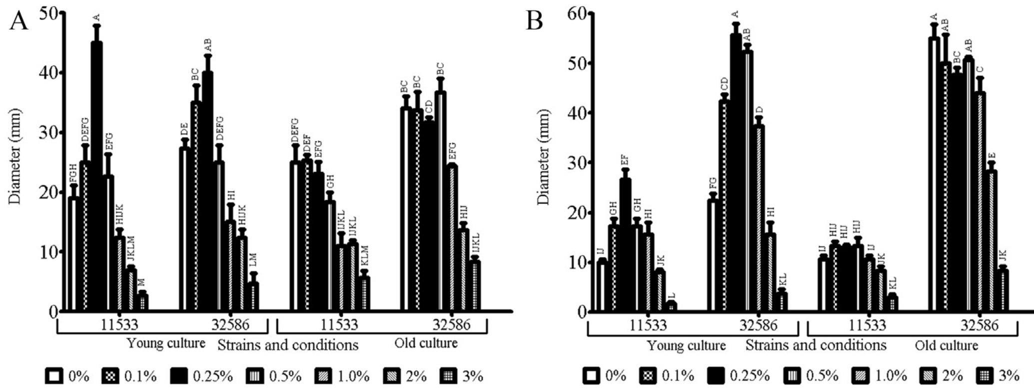

Fig. 4. Motility assay of *A. hydrophila* at various concentrations of NaCl. (A) Swarming motility. (B) Swimming motility. Values shown are the mean ± SEM of three independent experiments. Within each variable, values with the same letter are not significantly different according to Duncan's multiple-range test ($p < 0.05$).

biofilms on crab shell. FESEM images of biofilms formed at various salinities are shown in Fig. 6. Biofilm formation of young *A. hydrophila* was vigorous in fresh water containing crab shell. Mushroom-like three-dimensional structures were observed (Fig. 6A). At 0.25% salinity, the bacteria also formed three-dimensional structures and subsequently degraded the chitin surface of crab shell (Fig. 6B). At higher salinity (3%), the bacteria did not form strong biofilms, but rather formed individual bacterial attachments (Fig. 6C). Old cultures showed almost identical trends in biofilm formation on crab shell as young cultures. In freshwater, the bacteria formed three-dimensional structures, degraded the crab shell, and formed a strong EPS matrix (Fig. 6D). At 0.25% salinity, the bacteria degraded the crab shell but did not form three-dimensional biofilms; instead, they formed multiple layers of aggregated cells (Fig. 6E). The structures did not appear as strong as those of young cultures at 0.25% (Fig. 6E). Similarly, at 3% salinity, the bacteria did not form any significant biofilm structures or degrade the crab shell surface (Fig. 6F). Biofilm formation by *A. hydrophila* on SS is shown in Fig. 7. Without NaCl in LB (0%), the bacteria formed strong biofilms on SS (Fig. 7A) and young cultures showed increased biofilm formation at 0.25% NaCl (Fig. 7B), whereas at 3% NaCl, the bacteria attached but did not form any three-dimensional structures (Fig. 7C). In old cultures, FESEM showed little difference between biofilms on SS at 0% (Fig. 7D) and 0.25% NaCl (Fig. 7E). At 3% NaCl, the bacteria did not form biofilms, but did form small aggregations on the surface (Fig. 7F). Closer inspection revealed the presence of an EPS matrix (Fig. 7 A, B, D, and E) when cells were grown at 0 and 0.25% NaCl concentrations. No EPS was observed at 3% salinity on crab shell (Fig. 6C, F and I) or SS (Fig. 7C and F). Decreased salinity induced the formation of cracks on crab shell and the formation of biofilms (Fig. 6G and H). The shell was utilized by the bacteria at salinities of 0 and 0.25% (Fig. 6G and H, respectively).

Table 1
Exoprotease assay (30 °C) in *A. hydrophila* strains for 48 h at various concentrations of NaCl (0–3%) in modified Luria–Bertani (LB) medium.

Salinity (%)	11533 young culture (ng/mL ± SEM[a])	32586 young culture (ng/mL ± SEM)	11533 old culture (ng/mL ± SEM)	32586 old culture (ng/mL ± SEM)
0	5.81 ± 0.59CDE	6.80 ± 0.04BCD	7.04 ± 1.20BCD	4.67 ± 0.38EFG
0.1	8.15 ± 0.72AB	7.82 ± 0.23AB	6.57 ± 1.24CDEF	4.03 ± 0.75FG
0.25	9.32 ± 0.06A	8.91 ± 0.12A	5.83 ± 1.19DEFG	3.92 ± 0.42FGH
0.50	3.55 ± 0.08HI	3.62 ± 0.44IJK	4.86 ± 0.76HI	1.37 ± 0.004IJ
1.0	1.80 ± 0.72JK	0.39 ± 0.20JK	1.38 ± 0.11IJK	ND
2.0	0.16 ± 0.02JK	ND[b]	0.32 ± 0.08IJK	ND
3.0	ND	ND	ND	ND

[a] The values are mean ± SEM of 3 independent experiments. The values with same letters within a column were not significant ($p < 0.05$) according to Duncan's multiple-range test.
[b] ND is not detected.

4. Discussion

A. hydrophila is an emerging foodborne pathogen that has been isolated from fresh and estuarine water and can cause disease in humans and animals, particularly in animals with chitin shells such as crabs. *A. hydrophila* can cause bacteremia in both humans and aquatic animals such as crabs (Wang, 2011). *A. hydrophila* is a chitinolytic bacterium, and is involved in shell diseases in aquatic animals (Wang, 2011). Although it has been demonstrated that *A. hydrophila* cannot survive longer than two days in seawater (Brandi et al., 1999), estuarine seafood has often been reported to be contaminated by this bacterium. This raises the question of how the bacteria are surviving in this hostile environment. We hypothesized that *A. hydrophila* could form biofilms in fish and on seafood and survive to cause disease (Fig. 3). In freshwater, *A. hydrophila* can survive for >100 days, and we observed strong biofilms on crab (Fig. 3). Our observation that biofilm formation is higher at lower salinities (<0.1%) is concordant with observations that the isolation

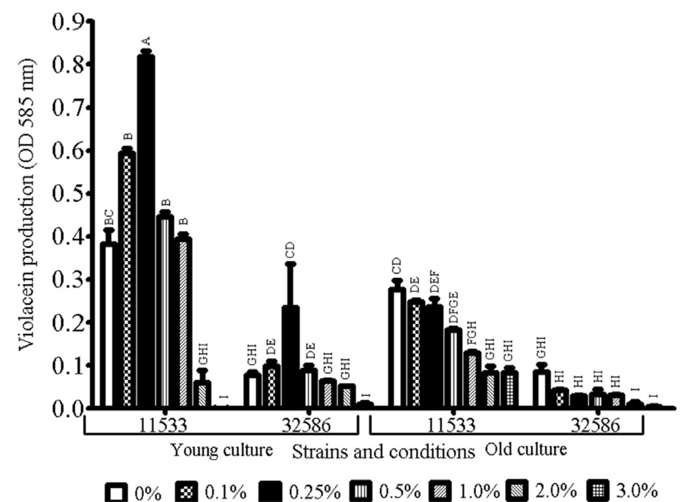

Fig. 5. Violacein production in *A. hydrophila* at different concentrations of NaCl. Values shown are the mean ± SEM of three independent experiments. Within each treatment, values marked with the same letter are not significantly different according to Duncan's multiple-range test ($p < 0.05$).

Table 2
AI-2 production by *A. hydrophila* formed biofilms on crab in fresh and saline water (0.1–3%) and incubated at 30 °C with bioreporter strain *Vibrio harvei* BB170.

Salinity (%)	11533 young culture (RLU±SEM[b])	32586 young culture (RLU ± SEM)	11533 old culture (RLU ± SEM)	32586 old culture (RLU ± SEM)
0	$4.22 \times 10^5 \pm 1.45 \times 10^4$GHI[a]	$7.53 \times 10^5 \pm 1.81 \times 10^5$DEFG	$1.15 \times 10^6 \pm 1.17 \times 10^5$BCD	$1.51 \times 10^6 \pm 2.9 \times 10^5$ABC
0.1	$4.22 \times 10^5 \pm 2.53 \times 10^4$GHI	$1.17 \times 10^6 \pm 2.23 \times 10^5$ABCD	$3.51 \times 10^5 \pm 1.24 \times 10^5$I	$5.33 \times 10^5 \pm 4.02 \times 10^5$EFGH
0.25	$1.74 \times 10^6 \pm 3.11 \times 10^5$AB	$2.03 \times 10^6 \pm 1.96 \times 10^4$A	$3.52 \times 10^5 \pm 2.58 \times 10^5$HI	$5.60 \times 10^5 \pm 1.41 \times 10^5$EFGH
0.50	$8.96 \times 10^5 \pm 1.87 \times 10^5$DEFG	$1.45 \times 10^6 \pm 2.21 \times 10^5$BCDE	$2.83 \times 10^5 \pm 1.96 \times 10^5$HI	$4.99 \times 10^5 \pm 3.99 \times 10^5$FGHI
1.0	$1.08 \times 10^6 \pm 2.75 \times 10^5$DEFG	$1.19 \times 10^6 \pm 2.88 \times 10^5$BCDE	$4.12 \times 10^5 \pm 2.73 \times 10^4$HI	$3.59 \times 10^5 \pm 2.79 \times 10^5$HI
2.0	$6.34 \times 10^5 \pm 9.97 \times 10^3$FGH	$9.90 \times 10^5 \pm 2.32 \times 10^5$DEFG	$2.92 \times 10^5 \pm 2.30 \times 10^5$HI	$4.50 \times 10^5 \pm 3.63 \times 10^4$HI
3.0	$6.34 \times 10^5 \pm 6.14 \times$ 104EFGH	$7.01 \times 10^5 \pm 1.12 \times 10^5$DEFGH	$1.69 \times 10^5 \pm 6.79 \times 10^4$HI	$2.62 \times 10^5 \pm 3.67 \times 10^4$HI

[a] The values are mean ± SEM of 3 independent experiments. The values with same letters within a column were not significant ($p < 0.05$) according to Duncan's multiple-range test.
[b] RLU means relative light intensity of luminescence.

rate of *Aeromonas* spp. is higher at low salinity (<1.0%) and lower during dry seasons when the salinity is higher than 1.0% (Marcel et al., 2002). Most foodborne pathogens such as *L. monocytogenes*, *S. aureus*, *Shigella boydii*, and *S. typhimurium* have been found to form biofilms at NaCl concentrations as high as 10% (Xu et al., 2010). However, we observed biofilm inhibition in young cultures of *A. hydrophila* at salinities >0.25% (Figs. 1–3), and any concentration of salinity inhibited biofilm formation in old cultures (Figs. 1–3). NaCl could modulate the curli expression of *E. coli* and a high NaCl content resulted in low curli production (Jubelin et al., 2005). We also hypothesized that *A. hydrophila* would show a complete reduction in swarming and swimming motilities at high NaCl concentrations, thus inhibiting biofilm formation at high concentrations of NaCl (3%) (Fig. 4). In general, more motile *A. hydrophila*

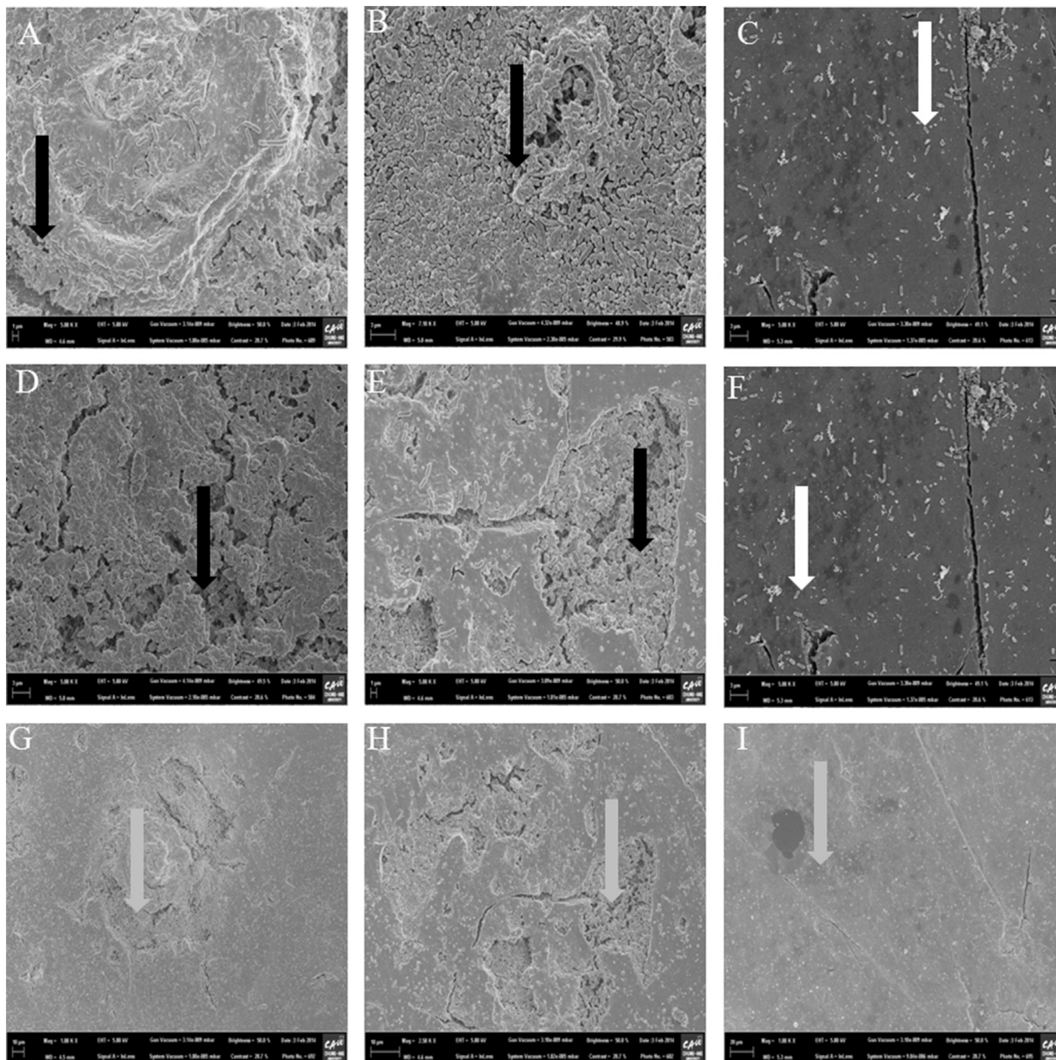

Fig. 6. FESEM images of biofilm formation on crab shell in *A. hydrophila* at different salinities. The figure shown is a representative result for strain KCCM KCTC 11533. (A) 0% salinity, young culture; (B) 0.25% salinity, young culture; (C) 3.0% salinity, young culture; (D) 0% salinity, old culture; (E) 0.25% salinity, old culture; (F) 3.0% salinity, old culture. (G) Surface topography of *A. hydrophila* grown on crab shell in freshwater. (H) Surface topography of *A. hydrophila* grown on crab shell in 0.25% salinity. (I) Surface topography of *A. hydrophila* grown on crab shellin 3.0% salinity.

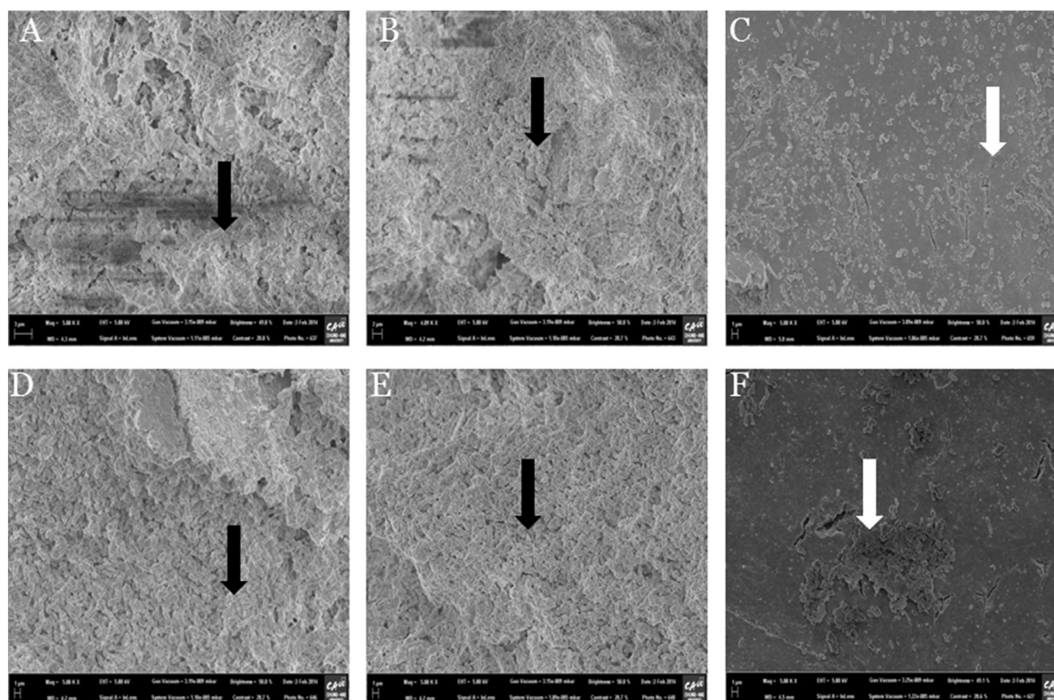

Fig. 7. FESEM images of biofilm formation of *A. hydrophila* on SS at different salinities. The figure shown is a representative result for strain KCCM KCTC 11533. (A) 0% salinity, young culture; (B) 0.25% salinity, young culture; (C) 3.0% salinity, young culture; (D) 0% salinity, old culture; (E) 0.25% salinity, old culture; (F) 3.0% salinity, old culture.

have been found in natural fresh water (Pablos et al., 2009) and in animals, including fish and crabs, living in water (Nielsen et al., 2001).

It could be hypothesized that salinity control C4 and C6-HSL as well as biofilms, motility and exoproteases. Previously, Medina-Martínez et al. (2006) reported that 3% NaCl completely inhibited AHL production by *A. hydrophila* isolated from food samples, which is concordant with our results (Fig. 5 and Supplement Fig. 1). C4-HSL showed a peak at a retention time of 6.91 min, which also correlates with other findings for *A. hydrophila* (Truchado et al., 2009). Because the supernatant was not concentrated, we did not observe any C6-HSL; its concentration is 70 times less than that of C4-HSL (Swift et al., 1999). Although several authors (Bi et al., 2007; Jahid et al., 2013a; Swift et al., 1999) have noted a correlation of quorum sensing with protease activity, other studies have reported contradictory results (Vivas et al., 2004). We observed a correlation between protease production and quorum sensing (Fig. 5 and Table 1), consistent with previous studies that reported that quorum sensing modulated protease production (Bi et al., 2007; Jahid et al., 2013a; Swift et al., 1999). Bi et al. (2007) also reported that AHL quorum sensing controls not only protease induction, but other virulence and pathogenic factors of *A. hydrophila* as well, including amylase, DNase, hemolysin, and S layer. However, it has been reported that AHL quorum sensing is not essential for growth and survival of *A. hydrophila* in lake water (Styp von Rekowski et al., 2008) rather we found that salinity control the quorum sensing including biofilm formation, motility and protease production. Recent results from our laboratory have shown that biofilm formation, AHL quorum sensing, motility, and protease production in *A. hydrophila* are controlled by glucose (Jahid et al., 2013a). Several authors have reported that 1.5% NaCl inhibits the protease activity of *Aeromonas sobria* (Khan et al., 2007; Takahashi et al., 2011). Khan et al. (2008) reported decreased protease production in seawater compared with river water. We have found that in both conditions tested; more than 1% NaCl completely inhibited the protease activity of *A. hydrophila* (Table 1). Extracellular protease is very important for bacterial survival in the wild, as proteases degrade the proteins of biotic surfaces, such as fish and crabs, and produce amino acids and oligo-peptides, which bacteria can use as nutrients. Salinity modulates biofilms formation, motility and exoproteases by controlling the AI-2 quorum sensing. It is worth noting that AI-2 production may also be correlated with biofilm formation, protease production, and other virulence factors, as it is controlled by salinity (Table 2). This observation and the correlation between salinity and AI-2 regulation in *V. vulnificus* (Kim and Shin, 2012) has been attributed to the significant impact of AI-2 and other virulence factors, including biofilm formation of *A. hydrophila* (Table 2). Although, AI-2 and biofilms would be related to each other, we did not get strong correlation at various salinity with AI-2 and biofilms as the procedures of AI-2 was too sensitive and highly variable for different independent experiments (Table 2). It has been noted that early stage of *Salmonella enterica* Serovar Enteritidis biofilm formation inhibited by quorum sensing compounds containing cell free supernatant of *Hafnia alvei* but not stationary phase (Chorianopoulos et al., 2010). In our study, salinity modulate the quorum sensing with bacterial cultural stage and modulate the quorum sensing regulated functions such as biofilm, protease production, and motility. Still this study raises the question of how the enhanced biofilm formation and virulence of young cultures might be explained. Enhancement of biofilm formation, motility, protease production, and quorum sensing have been proposed as a possible explanation of higher AMP production correlated with protease production (Takahashi et al., 2011), as young cultures may contain more deposited polyphosphate compared to old cultures (Jahid, 2013b; Jahid et al., 2006). Therefore, we hypothesize that salinity controls motility, biofilm formation, quorum sensing, and virulence factors of *A. hydrophila* in natural environmental water in the presence of phosphate. The present study had some limitations: our findings were limited to two strains, and the mechanism by which 0.25% salinity enhances biofilm formation and quorum sensing in young cultures remains unknown.

Overall, these data suggest that: (i) salinity, at any concentration, may decrease biofilm formation of *A. hydrophila* on food and food contact surfaces, if we consider old cultures as representative of environmental bacterial physiology; (ii) low salinity (0.25%) enhances biofilm formation, virulence, and quorum sensing in young cultures as representative of environmental bacterial physiology; (iii) biofilms control, motility, exoprotease production and quorum sensing phenotypes differ significantly between strains, (iv) bacterial physiology modulate quorum sensing as well other stationary phase genes such as biofilms, protease activity and motility. This study highlights novel aspects of *A. hydrophila* food ecology and clarifies that *A. hydrophila* can forms more biofilms in estuarine water but less in salt water, and thus would be expected to cause less disease in crustaceans or cross-contamination of food with salt water. Further studies using molecular biology and proteomics methods may help to answer these questions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2015.01.016.

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