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Full Length Research Paper

Role of the quorum-sensing system in biofilm formation and virulence of *Aeromonas hydrophila*

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Aeromonas hydrophila **is a pathogen that causes disease in a wide range of homeothermic and poikilothermic hosts due to its multifactorial virulence. The production of many of these virulence determinants is associated with high cell density, a phenomenon that might be regulated by quorum sensing. The quorum sensing system regulates the expression of several virulence factors in a wide variety of pathogenic bacteria. To investigate the pathogenic role of quorum sensing system in *A. hydrophila*, We constructed an *ahyI* mutant strain of a fish-clinical isolate YJ-1, named** YJ-1∆**AhyI. Compared with the wild-type strain, the *ahyI* mutant strain exhibited a significant decrease of total extracellular virulent activity, and decreased in biofilm formation, intraperitoneal LD_{50} of** YJ-1∆**AhyI were more than 10^9 CFU, about 10^4 times higher than the parent strain. These results suggest that *A. hydrophila* is able to regulate its extracellular virulent factors and biofilm formation by quorum sensing systems, and indicate that disruption of quorum sensing could be a good alternative strategy to combat infections caused by *A. hydrophila*.**

Key words: *Aeromonas hydrophila*, quorum sensing, biofilm, virulence factors.

INTRODUCTION

Aeromonas hydrophila is a ubiquitous Gram-negative bacterium of aquatic environments, which has been implicated as a causative agent of motile aeromonad septicemia in a variety of aquatic animals especially freshwater fish species (Hänninen et al., 1997). It causes gastrointestinal and extraintestinal infections in humans, including septicemia, wound infections, gastroenteritis and peritonitis (Daskalov, 2006). A number of virulence factors have been identified in *A. hydrophila*, such as, adhesins (e.g. pili), S-layers, exotoxins such as hemolysins and enterotoxin, and a repertoire of exoenzymes which digest cellular components such as proteases, amylases, and lipases (Cahill, 1990; Pemberton, 1997).

Quorum sensing (QS) (Fuqua et al., 1994) is a mechanism for controlling gene expression in response to an expanding bacterial population. In many Gram-negative bacteria, the diffusible quorum sensing signal molecule is a member of the N-acylhomoserine lactone (AHL) family (Fuqua et al., 1994; March and Bentley, 2004). Several virulence-associated phenotypes in pathogens have been shown to be controlled by their quorum sensing systems (Winzer and Williams, 2001). These phenotypes include biofilm formation (Croxatto et al., 2002), the production of virulence factors such as proteases (Swift et al., 1997, 1999; Croxatto et al., 2002), haemolysin (Kim et al., 2003), a type III secretion system (Henke and Bassler, 2004), extracellular toxin (Manefield et al., 2000) and a siderophore (Lilley and Bassler, 2000). *A. hydrophila* has been found to have homologues of the *Vibrio fischeri* quorum sensing genes *luxI* and *luxR*, designated *ahyI* and *ahyR* (Swift et al., 1997). In this study, we explored the role of the *ahyRI* dependent QS system of *A. hydrophila* by construction an analysis of the *ahyI* mutant.

We evaluated whether deletion of the *ahyI*

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Table 1. Characteristics of bacterial strains and plasmids used in this study.

Strains or plasmid	Relevant characteristic(s)	Source or reference
A. hydrophila		
YJ-1	Virulent	Chu, 2001
YJ-1ΔahyI	ahyI mutant strain of YJ-1	This study
YJ-1ΔahyIRC	Complement strain of YJ-1ΔahyI	This study
E. coli		
DH5α	recA gyrA	Laboratory stock
CC118	λpir lysogen of CC118 (Δ(ara-leu) araD ΔlacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1)	Dennis et al., 1998
S17-1	Smr Spr hdsR RP4-2 kan : : Tn7 tet : : Mu, integrated in the chromosome	Simon et al., 1983
C. violaceum		
CV026	double mini-Tn5 mutant derived from *C. violaceum* ATCC31532, Hg^r cviI::Tn5 xylE Km^r, plus spontaneous Sm^r	McClean et al., 1997
Plasmids		
pGEMT-Easy	Cloning vector, Amp^r resistant	Promega
pFS100	Km^r, Pgp704 suicide vector	Rubires et al., 1997
p*ahyI*$^-$	pFS100 harboring with an internal fragment of ahyI gene	This study
pGEMT-ahyIR	harboring a 1972bp DNA fragment containing the ahyIR gene	This study

gene affected biofilm formation, motility, extracellular virulence and the pathogenicity in a fish model of infection.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

The bacteria and plasmids used are listed in Table 1. *Escherichia coli* $DH_{5\alpha}$, plasmid-containing *E. coli* strains, *A. hydrophila* and its derivative strains were grown in LB medium, *E. coli* strains were grown at 37°C, while *A. hydrophila* strains were routinely grown at 28°C, *Chromobacterium violaceum* CV026 was kindly provided by Dr. McLean (Texas State University) and was grown in LB medium at 30°C. Media were solidified with 1.5% (wt/vol) agar as needed. Antibiotics were added as required at the following final concentrations: ampicillin, 100 μg ml^{-1}; kanamycin, 50 μg ml^{-1}.

DNA manipulation

Genomic DNA of *A. hydrophila* YJ-1 was prepared as previously described (Sambrook et al., 1989). Plasmid DNA from *E. coli* was extracted using a plasmid purification kit (Shanghai Shenggong Co. Shanghai) according to the manufacturer's instructions. Taq DNA polymerase and restriction enzymes were obtained from Takara (Takara Bio. Inc., Dalian, China); and incubation conditions were as recommended by the suppliers.

Construction of *A. hydrophila ahyI* mutant

To obtain single defined insertion mutant in gene *ahyI*, we used a method based on the suicide pFS100 (Rubires et al., 1997). Briefle, an internal fragment of the selected gene was amplified by polymerase chain reaction (PCR) using *A. hydrophila* YJ-1 chromosome, ligated into pGEM-Teasy (Promega). Oligonucleotides ahyI-F (5′ -CACGGGCAAAACGTTCATC-3') and ahyI-R (5′ -ACGAGCTTTATCGCTTCCG-3') were used to amplify the internal fragment of *ahyI* gene from *A. hydrophila* YJ-1 by PCR. The PCR product was ligated to pGEM-T vector (Promega) and transformed into *E. coli* DH5α. The internal fragment was recovered by EcoRI restriction digestion, and finally ligated into EcoRI digested suicide plasmid pFS100 plasmid vector. The ligation product was transformed into *E. coli* CC118 (λpir) and selected for kanamycin resistance. The recombinant plasmid was isolated and transformed into the *A. hydrophila* YJ-1 strain to obtain the *ahyI* insertion mutant. The insertion of plasmid on the chromosomes of the mutant was confirmed by PCR with appropriate primers.

Complementation of the *A. hydrophila* YJ-1 *ahyI* mutant

To complement the *ahyI* mutant strain of *A. hydrophila*, a 1972 bp fragment containing *ahyRI* open-reading frame (ORF), including its promoter, was amplified from *A. hydrophila* genomic DNA by using two primers *ahyIR*-F/*SalI* 5'-GGGGTCGACAGCAGCTTGTAATCCAACGC-3' and *ahyIR*-R/*EcolR*I 5'-GGGGAATTCATGAACCGTCCAGCAGAGTGA-3'. The amplified product was ligated into pGMT-Easy vector creating pGEMT-*ahyIR*. pGEMT-*ahyIR* was then introduced into the YJ-1ΔAhyI strain by electroporation. Clones exhibiting resistance to ampicillin (100 μg ml^{-1}) were chosen for further study. The presence of luxS on pGEMT-*ahyI* was confirmed by sequencing. To exclude the possible influence from the vector, the empty vector was electroporated into *A. hydrophila* YJ-1ΔAhyI as a control strain.

AHL bioassays

Chromobacterium violaceum CV026 was used as a biosensor to detect AHL. The AHL detection was applied by cross-streaking test

strains against *C. violaceum* CV026 on nutrient agar plate, in which the purple pigment violacein can be restored in response to the presence of AHL molecules. Briefly, strain CV026 was streaked at the center of the nutrient agar plate, the target bacteria were streaked on the same plate against CV026 line, if the target bacteria have AHL-producing ability, diffusible AHL produced by the target bacteria induces strain CV026 to produce a purple pigment (McClean et al., 1997). *C. violaceum* CV026 (a mini-Tn5 mutant) was used as an indicator strain for the detection of C4 and C6-HSLs.

Motility assay

LB medium containing 0.3% (wt/vol) agar was used to characterize the motility phenotype of wild type (wt) *A. hydrophila* YJ-1 and its *ahyI* mutant strain. The plates were then wrapped with Saran Wrap to prevent dehydration and incubated at 30°C for 12 to 14 h, and the motility was assessed by examining migration of bacteria through the agar from the center towards the periphery of the plate.

Detection of extracellular virulence factors

Some extracellular virulence factors activities were detected by patching bacteria on LB agar plates supplemented with different substrates (Swift et al., 1999). All strains were tested in duplicate, and when results were different, a third experiment was carried out to resolve the discrepancies.

Hemolytic activity was tested on agar base (Oxoid) supplemented with 5% sheep erythrocytes. The culture was streaked onto the plates and incubated at 27℃ for 24 to 36 h, The presence of a clear colourless zone surrounding the colonies indicated β-hemolytic activity. Protease production and proteolytic activity was detected on 1.2% agar plates supplemented with 10% (v/v) sterile skimmed milk (105℃ for 30 min). The cultures were streaked on the skim milk agar plates and incubated at 27°C for 24 to 36 h. Proteolytic strains caused a clearing zone around the colonies. Lipase activity was assayed on 0.5% tributyrin (Panreac,Barcelona, Spain) agar emulsified with 0.2% Triton X-100 and incubated at 27°C for 24 to 36 h. The presence of a transparent zone around the colonies indicated lipase activity. Extracellular nucleases (DNases) were determined on Dnase agar plates (Difco) with 0.005% methyl green. The culture was streaked onto the plates and incubated at 27°C for 24 to 36 h, a pink halo around the colonies indicated nuclease activity.

SDS-PAGE analysis of extracellular proteins

To prepare extracellular proteins, *A. hydrophila* YJ-1 and YJ-1ΔluxS were grown for 15 h and inoculated into 8 ml of fresh LB (1% inoculum). After incubation for 24 h, the cells were removed by centrifugation at 12,000 x g for 5 min and 4 ml of the separated culture supernatant was combined with 800 µl of 10% trichloroacetic acid. After 10 min at room temperature, the mixture was centrifuged and residues were solubilized in sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS. The protein samples were analyzed by SDS-PAGE using 8% gel and stained with Coomassie Brilliant Blue G-250.

Morphological changes in epithelioma papillosum cyprini (EPC) cells induced by *A. hydrophila*

Cytotoxicity of *A. hydrophila* strains was assayed with EPC cells. The EPC cells were grown as a monolayer at 25°C in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% fetal calf serum in a 5% CO_2 atmosphere incubator, and harvested with trypsin ethylenediaminetetraacetic acid. A 900 µl aliquot of the cell suspension was inoculated to each well in a 24 well culture plate. After incubation for 24 h, EPC monolayers were infected with *A. hydrophila* cells (wt and QS mutant) suspended in phosphate-buffered saline (PBS) at a multiplicity of infection (MOI) (number of bacteria per cultured cell) of 1 and incubated for 30 min, after infection, the EPC cells were washed three times with PBS. The cell morphology were examined using an Axiover 25CFL phase-contrast inverted microscope (Carl-Zeiss) at 200 magnifications.

Animal experiments

50±3 g (mean ±SD) *Carassius auratus gibelio* were obtained from a aquaculture farm in Nanjing, Jiangsu Province, P. R. China. The *C. auratus gibelio* were kept in 100 L tanks supplied with aerated fresh water and fed with commercial pelleted diet twice a day. The water temperature was kept at (25±1)°C. Before manipulation, the fish were anesthetized with 1:15,000 tricaine methane sulfonate MS-222 (Sigma) in water. For 50% lethal dose (LD50) determinations, six groups of 10 fish were intraperitoneally (i.p.) injected with 0.1 ml of washed culture of *A. hydrophila* YJ-1 and of *A. hydrophila ahyI* mutant, emulsified in sterile phosphate-buffered saline containing 10^3 to 10^9 CFU. The fish were observed for 7 days, and any dead specimen was removed for routine bacteriological examination. The experiment was carried out three times in duplicate, and the LD_{50} was calculated by the statistical approach of Reed and Muench (1938).

Biofilm assay

A quantitative biofilm formation experiment was performed in a microtiter plate as described previously (O'Toole and Kolter, 1998), with minor modification. Briefly, bacteria were grown on LB agar, and several colonies were gently re-suspended in LB (with or without the appropriate antibiotic); 100 µl aliquots were placed in a microtiter plate (polystyrene) and incubated 48 h at 28°C without shaking. After the bacterial cultures were poured out, the plate was washed extensively with water, fixed with 2.5% glutaraldehyde, washed once with water, and stained with a 0.4% crystal violet solution. After solubilization of the crystal violet with ethanol-acetone (80:20, vol/vol) the absorbance at 570 nm was determined using a microplate reader (Bio-Rad, Hercules, Calif.).

Statistical analysis

For animal studies, statistical analyses were performed using Fisher's exact test. For all other studies, Student's t test was used.

RESULTS

Characterization of *ahyI* mutant strain of *A. hydrophila* YJ-1

An *ahyI* mutant strain YJ-1ΔAhyI was constructed with a deletion of 147 bp of *ahyI* (GenBank accession no.X89469). The successful mutant of the *ahyI* gene was confirmed by PCR and DNA sequencing (data not shown). The CV026 bioassay revealed that the YJ-

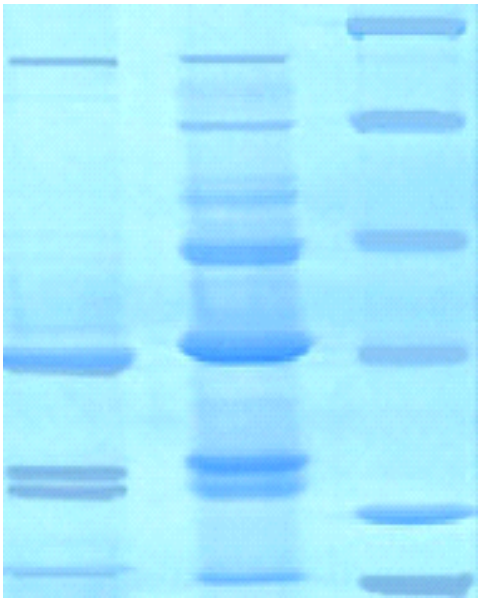

Figure 1. SDS–PAGE analysis of extracellular proteins of *A. hydrophila* strains. Lane 1, YJ-1ΔAhyI; lane 2, YJ-1, lane 3 Molecular weight markers.

1ΔAhyI does not produce AHL signal molecules (data not shown). The motility of YJ-1ΔAhyI was significantly less compared to that of the WT or the complemented strain. These results suggested that the quorum sensing system played an important role in *A. hydrophila* motility. The deletion of the *ahyI* gene did not alter bacterial growth kinetics over a tested period of 24 h. The analysis of extracellular enzyme activities revealed that the *ahyI* mutant could not produce the detectable extracellular proteases, haemolysin, amylase and Dnase, while *A. hydrohila* YJ-1 had a high level of extracellular enzyme activities. The extracellular protein profiles of YJ-1 and YJ-1ΔAhyI were surveyed by SDS–PAGE analysis (Figure 1). Compared with the case of YJ-1, many protein bands were decreased clearly in YJ-1ΔAhyI.

Morphological changes of EPC cells induced by *A. hydrophila* ECP

The cytotoxicity of *A. hydrophila* strains against *A. hydrophila* were EPC cells was further assessed, upon incubated with YJ-1, the ECP cells underwent a series of morphological changes. An monolayer EPC cells incubated with YJ-1ΔAhyI appeared as a smooth sheet with the cells adhere tightly to the neighbors, while incubated with YJ-1, the cells first became slightly detached from one another, the smooth appearance was lost and then large holes separated cells, and last the cells became rounded and the spindle connections were lost (Figure 2).

Role of quroum sensing in biofilm formation of *A. hydrophila*

Biofilm formation of *A. hydrophila* wild-type and *ahyI* mutant strain, YJ-1ΔAhyI was monitored in microtiter plates. As shown in Figure 3, biofilm formation of YJ-1ΔAhyI was significantly decreased, compared with that of wild-type strain YJ-1, while the complemented strain of YJ-1ΔAhyI, YJ-1ΔAhyIC can form biofilm. Thus, quorum sensing has a distinct influence on biofilm formation in *A. hydrophila.*

Fish infection

To ascertain the role of quorum sensing system in the pathogenesis of *A. hydrophila*, the LD50 was determined for *A. hydrophila* YJ-1 and YJ-1ΔAhyI by intraperitoneal challenge of *C. auratus gibelio*. As showed in Table 2, the LD50 values were more than 1.0 x 10^9 CFU bacteria for YJ-1ΔAhyI and 6 x 10^5 CFU bacteria for wild-type respectively. Fish injected with the parental strain died more rapidly than those injected with YJ-1ΔAhyI. All recorded deaths occurred within 4 days when the fish were injected with the wild type; however, deaths were recorded up to 6 days following injection when the fish were injected with YJ-1ΔAhyI. The *ahyI* mutation led to a significant decrease in strain virulence, indicating that quorum sensing system has a role in the pathogenic mechanism of *A. hydrophila*.

Examination of mortality showed typical clinical signs of hemorrhagic septicemia, mainly external lesions (abdominal distension at the injection site) and internal hemorrhages. To confirm stability of the insertional inactivated *ahyI* mutant gene, bacteria were isolated from dead fish inoculated with YJ-1ΔAhyI, all conferring a Kan phenotype.

DISCUSSION

In animal and plant pathogens, such as *Agrobacterium tumefaciens*, *Erwinia chrysanthemi*, *Pseudomonas aeruginosa*, and *Vibrio anguillarum*, AHL systems control the expression of a number of exported products that are proven or putative virulence factors. For example, quorum sensing through AHLs has been shown to be involved in biofilm formation (Kjelleberg and Molinm, 2002), competitive or cooperative bacterial interactions (Keller and Surette 2006) and virulence factors secretion. As described earlier, *A. hydrophila* produces a wide range of virulence factors. These virulence factors are expressed differently, depending on environmental and metabolic aspects of its current habitat. The regulation of many of these virulence factors is based on cell density-dependent cell-to-cell signaling, termed quorum sensing (Lynch et al., 2002; Bi et al., 2007; Khajanchi et al.,

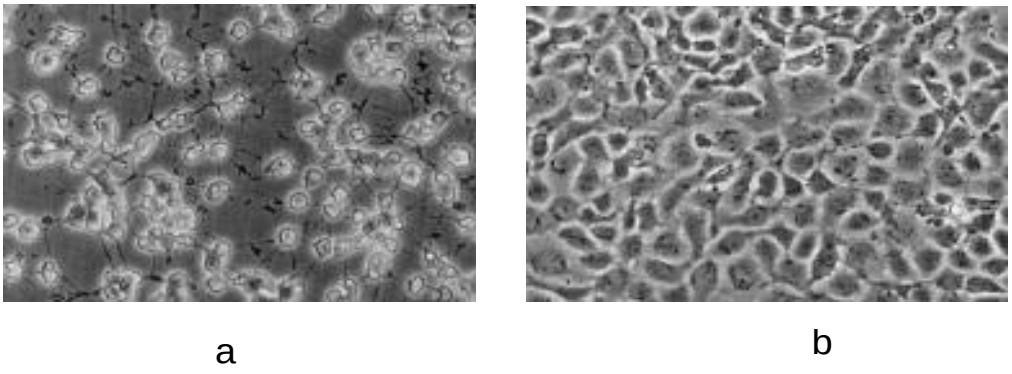

Figure 2. Micrographs of EPC cells infected with *A. hydrophila* YJ-1 (a) and YJ-1ΔAhyI (b) at 5 h post infection.

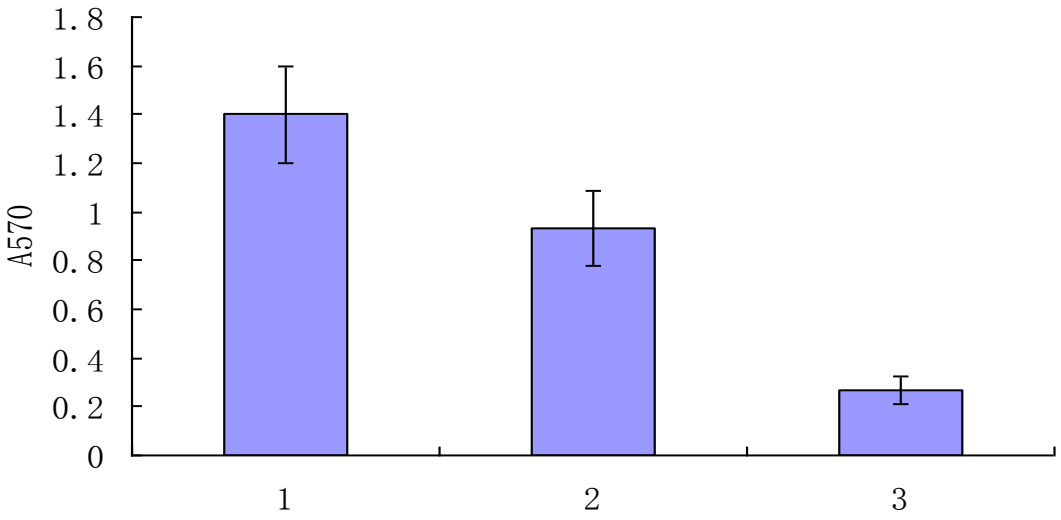

Figure 3. The amount of biofilm formation for each strain was quantified by solubilizing the stained biofilm with ethanol : acetone and measuring the OD_{570}. Each strain was tested in quadruplicate at each time point. Error bars indicate standard deviation. Lane 1, wide type Ah YJ-1, lane 2, YJ-1ΔAhyIRC, lane 3, YJ-1ΔAhyI. The results are representative of three experiments.

Table 2. Calculations of LD_{50} strain YJ-1 and the *ahyI* mutant.

Bacteria CFU/0.1 ml	Mortality (no. dead/no. total)		Day of death (no. dead/no. total)	
	YJ-1	*AhyI* mutant	YJ-1	*AhyI* mutant
10^9	10/10	1/10	1(5/10) 2(8/10) 3(10/10)	6(1/10)
10^8	10/10	1/10	1(5/10) 2(7/10) 4(10/10)	6(1/10)
10^7	8/10	0/10	1(4/10) 2(7/10) 4(8/10)	NA*
10^6	6/10	0/10	1(3/10) 3(5/10) 4(6/10)	NA*
10^5	3/10	0/10	3(4/10) 4(6/10)	NA*
10^4	0/10	0/10	NA*	NA*
10^3	0/10	0/10	NA*	NA*
control	0/10		NA*	
LD value(CFU/ml)	6 x10^5	>10^9		

*NA, not applicable: no death due to *A. hydrophila* infection during the experiment.

2009). To explore the role of quorum sensing system in regulating the extracellular virulent factors secretion and biofilm formation, we constructed an *ahyI* mutant strain of a fish-clinical isolate YJ-1, named YJ-1ΔAhyI. Inactivation of the *ahyI* gene of *A. hydrophila* did not result in noticeable changes in growth patterns compared with those of the wild-type strain. This finding indicates that *ahyi* has no significant effect on basic cellular metabolic processes required for growth of *A. hydrophila in vitro*. This is in contrast to some other bacteria in which *luxS* had an effect on growth, Lyon et al. (2001) reported that disruptions of *Streptococcus pyogenes, s luxS* shown a media-dependent growth defect, and the effect of quorum sensing on *Vibrio harveyi* growth rate can be either positive or negative (Nackerdien et al., 2008).

Decreased virulence has been seen in ΔluxS mutants of several pathogenic bacteria (Winzer and Williams, 2001). A *Vibrio cholerae luxO* mutant is severely defective in colonization of the small intestine in an infant mouse model, inactivation of the *rhlA* gene in *P. aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes (Van et al., 2009), and quorum sensing is necessary for the virulence of *P. aeruginosa* during urinary tract infection (Kumar et al., 2009), and *Vibrio alginolyticus* luxO-luxRval regulatory system control the expression of alkaline serine protease (Rui et al., 2009). In contrast, the *S. epidermidis luxS* mutant shows increased virulence in a model of catheter-associated infection. Most likely, the increased virulence may be partly attributed to the increased synthesis of PIA and more-intense biofilm formation. In this study, virulence factors were detected by patching bacteria on LB agar plates supplemented with different substrates, and the results shown that the virulence factors were decreased in the *ahyI* mutant strain, and on the PAGE, many proteins bands were lost or decreased, this phenomena suggest that QS control the extracellular proteins production, these band not only include the virulence factors, this results is consistent with the report on the protease.

The biofilm formation has been documented as survival strategy of pathogens, regulation of biofilm formation by quorum sensing systems has been shown in a number of bacteria. *In vitro* biofilm formation in *A. hydrophila* has been demonstrated using crystal violet staining assays as well as SEM. We could detect the difference between the parent strain and the QS mutant. Our data showed that the *ahyI* mutant strain was unable to develop a complete biofilm. This effect on biofilm formation by *luxS in vitro* was also observed in *Streptococcal* (Cvitkovitch et al., 2003), *Streptococcus* (Kong et al., 2006) and *V. cholerae* (Waters et al., 2008), while the luxS mutant of *Edwardsiella tarda* (Xiao et al., 2009) and *Streptococcus mutans* (Huang et al., 2009) were considerably increased biofilm formation. Thus, the quorum sensing signaling molecules have contrasting effects on biofilm formation in different strains.

In conclusion, we show quorum sensing system in *A. hydrophila* is functional for the secretion of extracellular virulence factors, the formation of mature biofilm and its pathogenicity, and these findings indicate that disrupt quorum sensing systems of pathogenic bacteria is a promising alternative for antibiotics in fighting bacterial infections.

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REFERENCES

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

Cahill MM (1990). Virulence factors in motile *Aeromonas* species: a review. J. Appl. Bacteriol., 69: 1-16.

Chu WH (2001). Studies on the pathology and control of the bacterial hemorrhage disease in a hybrid crussian carp. Reservoir Fisheries, 21: 40.

Croxatto A, Chalker VJ, Lauritz J, Jass J, Hardman A, Williams P, Camara M, Milton DL (2002). VanT, a homologue of *Vibrio harveyi* LuxR, regulates serine, metalloprotease, pigment, and biofilm production in *Vibrio anguillarum*. J. Bacteriol., 184: 1617–1629.

Cvitkovitch DG, Li YH, Ellen RP (2003). Quorum sensing and biofilm formation in *Streptococcal* infections. J. Clin. Invest., 112: 1626-1632.

Daskalov H (2006). The importance of *Aeromonas hydrophila* in food safety. Food Control, 17: 474-483.

Dennis JJ, Zylstra GJ (1998). Plasposons: Modular self-cloning minitransposon derivatives for rapid genetic analysis of Gram-negative bacterial genomes. Appl. Environ. Microbiol., 64: 2710-2715.

Fuqua WC, Winans SC, Greenberg EP (1994). Quorum sensing in bacteria-the LuxR-LuxI family of cell density-responsive transcriptional regulators. J. Bacteriol., 176: 269–275.

Hänninen ML, Oivanen P, Hirvelä-Koski V (1997). *Aeromonas* species in fish, fish-eggs, shrimp and freshwater. Int. J. Food Microbiol., 34: 17-26.

Henke JM, Bassler BL (2004). Quorum sensing regulates type III secretion in *Vibrio harveyi* and *Vibrio parahaemolyticus*. J. Bacteriol., 186: 3794–3805.

Huang Z, Meric G, Liu Z, Ma R, Tang Z, Lejeune P (2009). luxS-based quorum-sensing signaling affects Biofilm formation in *Streptococcus mutans*. J. Mol. Microbiol. Biotechnol., 17: 12-19.

Keller L, Surette MG (2006). Communication in bacteria: an ecological and evolutionary perspective. Nat. Rev. Microbiol., 4: 249–258.

Khajanchi BK, Sha J, Kozlova EV, Erova TE, Suarez G, Sierra JC, Popov VL, Horneman AJ, Chopra AK (2009). N-Acylhomoserine 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. Microbiol. 155: 3518-3531.

Kim SY, Lee SE, Kim YR, Kim CH, Ryu PY, Choy HE, Chung SS, Rhee JH (2003). Regulation of *Vibrio vulnificus* virulence by the LuxS quorum-sensing system. Mol. Microbiol., 48: 1647–1664.

Kjelleberg S, Molin S (2002). Is there a role for quorum sensing signals in bacterial biofilms? Curr. Opin. Microbiol., 5: 254–258.

Kong KF, Cuong V, Michael O (2006). *Staphylococcus* quorum sensing in biofilm formation and infection. Int. J. Med. Microbiol., 296: 133-139.

Kumar R, Chhibber S, Harjai K (2009). Quorum sensing is necessary for the virulence of *Pseudomonas aeruginosa* during urinary tract infection. Kidney Int., 76: 286-292.

Lilley BN, Bassler BL (2000). Regulation of quorum sensing in *Vibrio harveyi* by LuxO and sigma-54. Mol. Microbiol., 36: 940–954.

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

Lyon WR, Madden JC, Levin JC, Stein JL, Caparon MG (2001). Mutation of luxS affects growth and virulence factor expression in *Streptococcus pyogenes*. Mol. Microbiol., 42: 145–157.

Manefield M, Harris L, Rice SA, de Nys R, Kjelleberg S (2000). Inhibition of luminescence and virulence in the black tiger prawn (Penaeus monodon) pathogen *Vibrio harveyi* by intercellular signal antagonists. Appl .Environ. Microbiol., 66: 2079–2084.

March JC, Bentley WE (2004). Quorum sensing and bacterial cross-talk in biotechnology. Cur. Opin. Biotechnol., 15: 495–502.

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. Microbiol., 143: 3703–3711.

Nackerdien ZE, Keynan A, Bassler BL, Lederberg J, Thaler DS (2008). Quorum Sensing Influences *Vibrio harveyi* Growth Rates in a Manner Not Fully Accounted For by the Marker Effect of Bioluminescence. PLoS ONE 3(2): e1671. doi:10.1371/journal.pone.0001671.

O'Toole GA, Kolter R (1998). Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol., 30: 295-304.

Pemberton JM, Kidd SP, Schmidt R (1997). Secreted enzymes of Aeromonas. FEMS Microbiol Lett. 152: 1-10.

Penfold RJ, Pemberton JM (1992). An improved suicide vector for construction of chromosomal insertion mutations in bacteria. Gene., 118: 145–146.

Reed LJ, Muench H (1938). A simple method of estimating fifty percent endpoints. Am. J. Hyg., 27: 493–497.

Rubires X, Saigí F, Pigué N, Climent N, Merino S, Albertí S, Tomás JM, Regué M (1997). A gene (*wbbL*) from *Serratia marcescens* N28b (O4) complements the *rfb-50* mutation of *Escherichia coli* K-12 derivates. J. Bacteriol., 179: 7581-7586.

Rui HP, Liu Q, Wang QY, Ma Y, Liu H, Shi CB, Zhang YX (2009). Role of alkaline serine protease, Asp, in *Vibrio alginolyticus* virulence and regulation of its expression by LuxO-LuxR regulatory system. J. Microbiol. Biotechnol., 19: 431-438.

Sambrook J, Fritsch E, Maniatis T (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed, Cold Spring Harbor Laboratory, Press Cold Spring Harbor, New York, U.S.A.

Simon R, Priefer U, Pühler A (1983). Vector plasmids for *in vivo* and *in vitro* manipulations of Gram-negative bacteria. In *Molecular Genetics of the Bacteria–Plant Interaction*, pp. 98–106. Edited by A. Pühler. Heidelberg: Springer.

Swift S, Karlyshev AV, Fish L, Durant EL, Winson MK, Chhabra SR, Williams P, Macintyre S, Stewart GS (1997). Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate N-acylhomoserine lactone signal molecules. J. Bacteriol., 179: 5271-5281.

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

Van GM, Christensen LD, Alhede M, Phipps R, Jensen PØ, Christophersen L, Pamp SJ, Moser C, Mikkelsen PJ, Koh AY, Tolker-Nielsen T, Pier GB, Høiby N, Givskov M, Bjarnsholt T (2009). Inactivation of the rhlA gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes. APMIS., 117: 537-546.

Waters CM, Lu W, Rabinowitz JD, Bassler BL (2008). Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of vpsT. J. Bacteriol., 190: 2527-2536.

Winzer K, Williams P (2001). Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria. Int. J. Med. Microbiol., 291: 131-143.

Xiao J, Wang Q, Liu Q, Xu L, Wang X, Wu H, Zhang Y (2009). Characterization of *Edwardsiella tarda* rpoS: effect on serum resistance, chondroitinase activity, biofilm formation, and autoinducer synthetases expression. Appl. Microbiol. Biotechnol., 83: 151-60.