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Identification of N-Acyl Homoserine Lactone-Degrading Bacteria Isolated from Rainbow Trout (*Oncorhynchus mykiss*)

Abbreviated running headline: "Isolation of AHL Degrading Bacteria"

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ABSTRACT

Aims:

A variety of pathogens use quorum sensing (QS) to control the expression of their virulence factors. QS interference has hence been proposed as a promising anti-virulence strategy. The specific aim of this study was to isolate bacteria from trout tissue able to degrade N-Acyl Homoserine Lactones (AHL), a QS molecule family.

Methods and Results:

In total 132 isolates were screened for AHL degradation using *Chromobacterium violaceum* CV026 as a biosensor. Twenty four quorum quenching (QQ) isolates were identified biochemically and characterized using 16S rDNA sequencing. They belong to *Bacillus*, *Enterobacter*, *Citrobacter*, *Acinetobacter*, *Agrobacterium*, *Pseudomonas* and *Stentrophomonas* genera. Four *Bacillus* spp. showed the highest and fastest QQ activity. AHL degradation proved to be enzymatic in most isolates (except for *Stentrophomonas* spp. and *Pseudomonas* sp) as QQ activity could be destroyed by heat and/or proteinase K treatments. All QQ activity proved to be cell-bound except for *Pseudomonas* sp., where it could be detected in the supernatant. The results of *aiiA* gene homology analysis revealed the presence of *aiiA* gene encoding AHL lactonase in all examined isolates except *P. syringae* and *E. cloacae*. The HXHXDH motif conserved in all AHL lactonases and considered to be essential for AHL degradation was detected in all AiiAs after sequence alignment.

Conclusions:

Some known and novel QQ bacteria were isolated from trouts and characterized in terms of enzymatic or non-enzymatic AHL degradation activity and their extracellular or intracellular location. In addition, an *aiiA* gene and its HXHXDH motif were detected in most isolates.

Significance and Impact of Study:

We could isolate and identify some novel QQ bacteria including *E. hormachea*, *A. radioresistens,* and *C. gillenii*. The *aiiA* gene was detected for the first time in these strains as well as in *S. maltophilia.* Our QQ isolates could be used for biocontrol of bacterial infections in aquaculture.

Keywords:

Aquaculture; Biocontrol; Degradation; Identification; Quorum sensing

INTRODUCTION

Bacteria are able to communicate and sense population density via a diverse set of signals. The process is generally called quorum sensing (QS) (Swift *et al*. 1993; Fuqua *et al*. 2001; Reading and Sperandio 2006). Through the QS system, they produce, release and sense small diffusible molecules (Kaplan and Greenberg 1985). These signal molecules have distinct structural features and are applied by different microbial classes. A wide range of Gram-negative bacteria produce N-Acyl Homoserine Lactone (AHL) as signal molecules in cell to cell communication. A generic AHL molecule contains an acyl chain with variable numbers of carbon and a consistent homoserine lactone ring (Czajkowski and Jafra 2009). It is assumed that at a critical threshold density, a cascade of biological events will be initiated inside the bacterial cells, resulting in regulated expression of specific genes such as virulence factors (Fuqua *et al*. 1994; Eberl 1999). Indeed, some physiological behaviors such as biofilm formation, swarming motility, bioluminescence, and secretion of virulence factors, but not growth, are regulated by QS mechanism (Miller and Bassler 2001; Chen *et al*. 2013).

Since antibiotic resistance has become a major concern in human and animal health care, quorum sensing interference (QSi) has been suggested to be an alternative strategy, with quorum quenching (QQ) being at the basis of an anti-virulence therapy (Chen *et al*. 2013). In contrast to antibiotic therapy, in QSi, pathogenic bacteria are not killed nor is cell growth inhibited; rather virulence factor production can be suppressed. QSi can be accomplished through three mechanisms: 1) interference in

synthase of signal molecules, 2) signal molecule degradation and 3) blockage of receptors of signal molecules (Geske *et al.* 2008; Galloway *et al.* 2011).

Many bacteria are able to produce different enzymes to degrade AHL molecules. These enzymes are divided into four main groups: AHL acylase, AHL lactonase, oxidoreductase and paraoxonase (Chen *et al*. 2013). AHL acylase can irreversibly hydrolyze the amide bonds between the homoserine lactone ring and the acyl chain (Leadbetter *et al*. 2000; Lin *et al.* 2003). AHL lactonase enzymes cleave the homoserine lactone ring deactivating the QS molecules (Dong *et al.* 2000; Dong *et al.* 2001). The hydrolysis of lactone ring also occurs at a pH above 7 and can be reversed at acidic pH (Yates *et al.* 2002). In a few numbers of bacterial species, oxidoreductase enzymes oxidize or reduce the acyl side chain without degrading the AHL structure (Chowdhary *et al.* 2007; Bijtenhoorn *et al*. 2011). The last QQ enzyme class, paraoxonase (PONs), exists in mammalian sera and possesses a function similar to AHL lactonase (Yang *et al.* 2005).

In previous years, many bacteria with AHL degrading ability, isolated from a wide range of aquatic animals and environments, have been identified (Tinh *et al*. 2007; Chu *et al*. 2010; Christiaen *et al*. 2011; Noorashikin *et al*. 2016). In the present study, QQ bacteria from rainbow trout were isolated and characterized with the aim to use them in the biocontrol of bacterial infections in rainbow trout aquaculture.

MATERIALS AND METHODS

Bacterial strains, media and culture conditions

Chromobacterium violaceum strain CV026, a mini-Tn5 mutant derived from the *C. violaceum* strain ATCC31532 (McClean *et al.* 1997; Chu *et al*. 2010) was used to detect QS signal molecules. This reporter is able to synthesize the purple pigment violacein in the presence of exogenous acyl homoserine lactone (AHL) molecules with acyl side chain of four to eight carbons. P3/pME6863 and P3/pME6000 strains were used as positive and negative controls in AHL degradation assays, respectively (Reimmann *et al*. 2002; Maurhofer *et al*. 1998). P3/pME6863 is a recombinant *Pseudomonas fluorescence* carrying the *aiiA* gene from *Bacillus* sp. A24 encoding a lactonase enzyme

(Molina *et al.* 2003). *Yersinia ruckeri* strain CCUG14190, a pathogenic bacterium in aquaculture was provided by the Laboratory of Aquaculture and Artemia Reference Center (ARC), Belgium. Standard HHL, N-hexanoyl L-homoserine lactone [C6-HSL], used in this study, was purchased from Sigma (Sigma-Aldrich). Tryptone Soy (TS) agar or broth was used as a medium to grow all strains at 28 °C. The CV026 strain was grown in TS medium supplemented with kanamycin (20 mg l^{-1}) to maintain the plasmid harboring the gene responsible for violacein production. 3-[N-morpholino] propane sulfonic acid (MOPS) was added to all media used in AHL assays to buffer the medium at pH 6.8 preventing chemical degradation (Yates *et al.* 2002).

Sample collection and preparation

A total of 35 healthy rainbow trouts (*Oncorhynchus mykiss*) (76 ± 10 g) were collected from five aquaculture farms in Fars province, Iran. Upon transfer to the Aquatic Animals Health and Diseases Department, Shiraz University, all fish were euthanized using a high dose of tricaine methanesulfonate (200 mg L^{-1}) (MS222; Sigma Aldrich). Mucosal samples taken by swabbing gills and skin were directly inoculated onto tryptone soy agar (TSA) plates. Subsequently, the ventral surface of the fish were disinfected with alcohol (70%) and then their intestines were taken out aseptically and rinsed with physiological saline (0.9% NaCl solution, pH 7.4). One gram of the intestine was homogenized with sterilized 0.9% NaCl solution (10:1, v⁄w). Upon serial dilution up to 10^{-6}, 0.1 ml of the dilutions 10^{-4} - 10^{-6} were spread onto TSA plates in triplicates. The plates were incubated for 48 h at 28 °C. Subsequently, colonies with different morphologies were separated, sub-cultured on TSA plates, grown in TSB, supplemented with sterile glycerol (30%, v/v) and stored at -80 °C.

Screening for QQ isolates

In order to verify for a QQ ability in the isolated strains, *Y. ruckeri* as a natural AHL producer and CV026 strain were separately cultured on TSA plates and streaked as two parallel lines on TSA plate. Each isolate was then placed as a short streak between CV026 and *Y. ruckeri*, maintaining a 6-7 mm distance between them. The plates were checked for QS inhibition after 48 h incubation at 28 °C. P3/pME6863 and P3/pME6000 strains were used as positive and negative controls, respectively. The

isolates capable to inhibit purple pigment production were selected and stored in 30% sterile glycerol (v/v) and kept at -80 °C for further examinations.

AHL degradation assays

A dose-response curve between the hexanoyl L-homoserine lactone [C6-HSL] and the violacein diameter

One mg of C6-HSL was dissolved in 1 ml of dimethyl sulfoxide (DMSO) to prepare a stock solution of C6-HSL. CV026 was grown in TSB containing 20 mg l^{-1} kanamycin until 0.1 OD at 600 nm. CV026 was then spread on a buffered (pH 6.5) TSA plate. Ten µl C6-HSL solution was dropped into a well in the plate agar. Plates were put in an incubator at 28°C for 24 h. This assay was performed with five different concentrations of C6-HSL including 10, 5, 2.5, 1, and 0.5 mg l^{-1}. The diameter of purple-pigmented halos and the C6-HSL concentration displayed a linear regression line. A standard curve was obtained with the following equation: diameter of purple-pigmented halo = 0.386 [C6-HSL] + 1.0734 and regression coefficient $R^2 = 0.953$ (**Figures 1b, 1c**).

Kinetic of AHL degradation by agar well diffusion assay

Each QQ isolate was grown in a 10 ml buffered TSB (pH 6.5) supplemented with 5 mg l^{-1} C6-HSL. P3/pME6863 and P3/pME6000 strains were cultured under the same conditions as positive and negative controls, respectively. The mixture solutions were incubated at 28 °C with constant agitation (120 × *g*). One ml of each culture medium was taken and filtered (0.22-µm) at 12 h and 24 h. Ten µl of the filtrate was spotted into a well in a TSA plate spread with 100 µl bacterial suspension of CV026. After 24 h incubation, the diameter of purple halos was measured and converted into the residual concentration of C6-HSL in the filtrates.

Quorum quenching characterization

The positive isolates for AHL degradation were examined for enzyme- or non-enzyme-bound AHL degradation by heat and proteinase K treatments. One hundred µl of a proteinase K solution (500 µg ml^{-1}) (Sigma Aldrich) was supplemented to 850 µl of TSB overnight isolate cultures, followed by incubation at 28 °C for 24 h. 228 µl (1 mM) of Pefabloc SC plus (AEBSF) [4-(2-Aminoethyl)-benzenesulfonylfluoride hydrochloride] solution (1 mg ml^{-1}) (Sigma Aldrich e) was added into the reaction mixture to terminate proteinase K activity for 1 hour at room temperature. Alternatively, the

isolates were subjected to autoclaving at 121 °C, 20 min. The proteinase K and heat treated samples were mixed with C6-HSL and QQ activity was evaluated by the diffusion assay as described above.

Determination of extracellular/intracellular AHL degradation in QQ isolates

AHL degrading isolates cultured in TSB were centrifuged at 6000 × *g* for 15 min and cell-free supernatants, as an extracellular fraction, were obtained by filter sterilization (0.2 µm). C6-HSL was added to reach 5 mg l^{-1} and incubated at 28 °C for 24 h. Ten µl of mixture reactions was spotted into a well in a TSA plate seeded with 100 µl overnight CV026 culture. The plates were incubated for 24 h to allow the purple pigmented halo to develop.

Identification of QQ isolates

Biochemical characterization

Morphological properties such as shape, size, and pigmentation of QQ colonies were investigated on TSA plates after purification. Furthermore, Gram staining was done to verify Gram reaction, size, shape and type arrangement of cells, microscopically. AHL degrading bacteria were biochemically characterized using the following tests: oxidase and catalase activity, motility test, citrate utilization test, gas production from glucose, indole production, nitrate reduction, gelatin hydrolysis, oxidative fermentation, production of hydrogen sulfide, urease and hemolytic activity, aesculin hydrolysis, voges–proskaüer test (acetoin production), l-arabinose fermentation, glucose and mannitol fermentation.

PCR assay and 16S rDNA sequencing

Total DNA was extracted from AHL degrading bacteria by the boiling method. Amplification of partial 16S rDNA gene of bacteria was performed using FUP-5'- ACGGCTACCTTGTTACGACTT-3' and RUP-5'-AGAGTTTGATCCTGGCTCAG-3' as a universal set of forward and reverse primers, thermocycler apparatus (MJ mini, BioRad, USA). The PCR reaction was set up with an initial denaturation at 95 °C for 5 min, 37 cycles at 94 °C for 1 min, annealing at 59 °C for 1 min, extension at 72 °C for 1 min and an eventual extension at 72 °C for 5 min. PCR products were analyzed by 1.2 % agarose gel containing Red Safe (Intron Biotechnology, Korea) staining through electrophoresis. All PCR products were purified and sequenced bi-directly using a capillary DNA analyzer (ABI3730; Applied Biosystems, Foster City, CA, USA). Continuous sequences were assembled with the BioEdit

software version 7.0.5 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Results of DNA sequencing were compared to the GenBank database using Basic Local Alignment Search Tool Nucleotide (BLAST-N) server at NCBI (National Center for Biotechnology Information databases) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to find the closest sequences and establish a tentative identification (acknowledging that 16s rDNA information might not always be sufficient to allow unambiguous identification at the species level). Sequences of the identified isolates were submitted to NCBI with an accession code.

PCR amplification and homology analysis of *aiiA* gene

The presence of the AHL-lactonase gene (*aiiA*) in AHL degrading bacteria was evaluated by amplification of the *aiiA* gene as previously described by Dong *et al.* (2002) and Nusrat *et al.* (2011) with some modifications. The *aiiA* gene was amplified using forward and reverse primers, *aiiA* F-5'-ATGACAGTAAAGAAGCTTTA-3' and *aiiA* R1-5'-TATATATTCAGGGAACACTT-3', respectively. The PCR conditions were as follows: initial denaturation (at 95 °C for 6 min), 40 cycles for denaturation (at 94 °C for 1 min), annealing (at 58 °C for 45 sec), extension (at 72 °C for 50 sec) and a final extension (at 72 °C for 15 min). The amplified samples were resolved by 1.2% agarose gel electrophoresis. Appropriate PCR products for several samples (n=10) were directly sequenced as described above. Sequencing data were processed with the BioEdit software and then the BLAST program was used for comparison with the information in GenBank. Phylogenetic analyses were conducted using the obtained AHL lactonases sequences together with known AHL lactonases on NCBI using maximum likelihood (ML) method by MEGA 6. To assess the robustness of the branches, bootstrap test with 1,000 pseudoreplicates was performed, following the rule of branch consistency (Tamura *et al.* 2013). At the end, the nucleotide sequences of the *aiiA* genes were registered in the GenBank database. In addition, genetic similarity and multiple amino acids alignment were performed using the CLC Main Workbench 5 software for different AHL lactonase.

Statistical analysis

In the present study, SPSS 16.0 software was applied for statistical analysis. Independent samples t-test and one way ANOVA were chosen for the comparison of test samples and control groups with a *p*-value of 0.05.

RESULTS

Isolation and screening of bacteria for QQ activity

In total 132 isolates were obtained from skin, gill, and intestine of rainbow trout. They were screened for AHL degradation (QQ) activity. QQ activity was verified by a plate agar assay using the CV026 strain as a biosensor for AHLs and *Y. ruckeri* as a natural AHL producer. The negative control, P3/pME6000, was completely purple (**Figure 1aA**). The presence of a non-purple zone in the CV026 strip adjacent to the QQ isolate was used as an initial criterium for QQ ability **(Figure 1aB)**. In total twenty four QQ isolates were selected for further examination.

Determination of the AHL degradation kinetics

Kinetic of AHL degradation by each QQ isolate was investigated by adding a C6-HSL solution to the isolate cultures. Remaining AHL concentration were verified in samples taken after 12 h and 24 h incubation. All selected isolates degraded C6-HSL in comparison with negative control and almost all QQ bacteria were able to lower the C6-HSL concentration below 1 mg l^{-1} after 24 h incubation. Four QQ isolates 7q, 18q, 26q (*Bacillus thuringiensis*) and 24q (*B. cereus*) along with the positive control completely degraded AHL during 24 h incubation. A comparison of QQ activity in different genera revealed that *Bacillus* strains degraded C6-HSL faster ($p < 0.05$) than non-*Bacillus* strains during the first 24 h such as for strains belonging to *Stenotrophomonas*, *Enterobacter*, *Citrobacter* and *Pseudomonas* genera. No chemical degradation was observed in the negative control (P3/pME6000 treatment, buffered at 6.5). The results are shown in **Figure 2a**.

Determination of mechanism of action in QQ isolates

To verify whether AHL degradation in the isolates was enzymatic, heat and proteinase K treatments were applied. Both treatments significantly reduced AHL degradation activity in most QQ isolates compared with the untreated controls ($p < 0.05$; **Figure 2b**). However, isolates 2q, 32q, 43q belonging to *Stenotrophomonas maltophilia*, and isolate 31q, *Pseudomonas syringae,* still displayed some AHL degradation activity after heat treatment, indicating that they might produce heat stabile enzymes. Besides, *S. maltophilia* strains retained their QQ ability although they were partly sensitive to proteinase K.

Determination of extracellular/intracellular QQ activity

The supernatants of isolates were used to evaluate whether AHL degradation activity was intracellularly or extracellularly localized. None of the supernatants of QQ isolates showed AHL degradation activity (**Figure 2c**), indicating intracellular QQ activity except for isolate 31q identified as *Pseudomonas syrignae.*

Identification of QQ isolates

Biochemical characterization

The isolates were subjected to some biochemical tests and the results are tabulated in **Table S1**.

16S rDNA gene sequencing

The 16S rDNA gene in QQ positive strains was amplified and sequenced. BLAST analysis of 16S rDNA sequences demonstrated AHL degrading isolates most closely belonged to *Bacillus* (13 isolates), *Stentrophomonas* (3 isolates), *Enterobacter* (4 isolates), *Acinetobacter*, *Citrobacter*, *Agrobacterium* and *Pseudomonas* (each one 1 isolate) genera**.** AHL degradation activity *in E. hormachea* (similarity 100%), *A. radioresistens* (similarity 98%) and *C. gillenii* (similarity 100%) has not been described before. Identified 16S rDNA sequences were submitted to the GenBank database with an accession number **(Table 1).**

Analysis of the *aiiA* homologous genes

The observations in the agar well diffusion assay and inactivation of degradation caused by heat and proteinase K treatments suggest the presence of QQ genes in the isolates. QQ bacteria were investigated for *aiiA* homologous genes coding for AHL lactonase enzyme by PCR amplification using specific primers for *aiiA* gene. An amplicon of 750 bp was obtained in *B. thuringiensis*, *B. cereus*, *Bacillus* sp., *S. maltophilia*, *E. hormachea*, *A. radioresistens*, *Ag. tumefaciens* and *C. gillenii* as can be expected for an AHL lactonase gene **(Figure 3)**. Using the same primer set, an *aiiA* homologous gene seems to be absent in *P. syringae* and *E. cloacae* (data not shown).

DNA sequencing and alignment of lactonase gene

Molecular analysis of *aiiA* sequences in the QQ isolates demonstrated that at least two different AHL lactonase genotypes (genotypes A and B) are present among the isolates. Comparisons of the *aiiA* sequences with other known AHL lactonase sequences available in the GenBank database (*aiiA*, *attM*,

ahlD and *ahlK*) revealed high similarity (up to 99%). Although the sequences of the genotypes A and B are not completely similar to the previously described sequences in the GenBank, both of them phylogenetically belong to the known *aiiA* type lactonases major group (**Figure 4**). The degree of similarity among the isolated *aiiA* genes and the accession numbers are listed in **Table 2**.

In accordance with the phylogenetic results, the comparison of deduced amino acids sequence showed a high degree of similarity (up to 99%) to the protein data available in the GenBank database for AiiA group. Also, multiple alignments of AHL-lactonase amino acids sequence indicated the presence of the motif "HXHXDH" and zinc-binding sites in both *aiiA* genotypes. The conserved Histidine (H) at the positions 104, 106 and 169 in metal site 1 and Aspirate acid (D) 108, His 109 and His 235 in metal site 2 were present. Moreover, Tyrosine (Y) residue was found at position 194 in all sequences (**Figure 5**).

DISCUSSION

Various bacteria utilize QS systems to estimate their cell density and regulate genes (e.g. virulence factors) in response to that. There are also many bacteria which are able to degrade signal molecules, such as acyl homoserine lactones (AHL). In this study, AHL degrading bacteria were isolated from various trout tissues (gills, skin, and intestines). Many of the isolated species have already been described for having AHL degrading capacity, while for some species this capacity is novel (Carlier *et al.* 2003; Shepherd and Lindow 2009; Ma *et al.* 2013; Chu *et al.* 2014; Noorashikin *et al.* 2015; Lopez *et al.* 2017).

There are few publications reporting AHL degradation by *Stenotrophomonas maltophilia* (here the putative identification of strains 2q, 32q and 43q). Singh *et al.* (2013) reported for the first time the QQ capacity in *S. maltophilia* isolated from the roots of a grass species. We identified three different putative species of *Enterobacter* with QQ activity including *E. hormachea*, *E. cloacae,* and *Enterobacter* sp. Although the QQ ability in *E. hormachea* is reported here for the first time, some research previously reported AHL degradation in other species of *Enterobacter* (Rajesh and Rai 2014a; Noorashikin *et al.* 2015; Gopu *et al.* 2016). AHL degradation activity has already been observed in *Acinetobacter* spp. (Kang *et al.* 2004; Kim *et al.* 2014; Lopez *et al*. 2017) and our current

finding revealed that *A. radioresistens* shows AHL degradation capacity. However, we could not find any publication referring QQ activity in this species. There is no published evidence for AHL degradation by *Citrobacter,* while the *C. gillenii* strain isolated here, could degrade AHL. Finally, the capacity of *Bacillus* species to degrade AHL has been described by Dong *et al*., (2002), Momb *et al*., (2008), Chu *et al*., (2010), Defoirdt *et al*., (2010), an observation that was also made in this study. AHL degrading *Bacillus* species might have the technological advantage of having the capacity to form spores, which make them more suitable for inclusion in for instance aquaculture feed. The *Bacillus* strains isolated here tended to degrade AHL faster than some non-*Bacillus* strains, which make them additionally attractive in QQ applications.

Except for *P. syringae* (as in other reports on the *Pseudomonas* genus; Christiaen *et al*. 2011; Cheong *et al*. 2013), no AHL degrading capacity was found in the supernatants. Additionally, heat treatment resulted in the loss of QQ ability in most isolates (*S. maltophilia* and *P. syringae* displayed less sensitivity to heat treatment than other isolates; Singh *et al*. (2013) and Christiaen *et al.* (2011) observed the same phenomenon in *S. maltophilia* and *Pseudomonas* strains). Taken together this illustrates that in most isolates AHL degradation is done by cell-bound enzymes as it is the case for AHL lactonases (Czajkowski and Jafra 2009; Christiaen *et al*. 2011; Rajesh and Rai 2014b). Heat-resistant compound(s) have also been described by Mandrich *et al*. (2010) in thermophilic archaea. The extracellular QQ activity of *P. syringae* might have some technological advantages in a QQ strategy. For instance, fast degrading *Bacillus* strains (producing cell bound lactonases) could be combined with *P. syringae* strains producing extracellular AHL degrading enzymes.

Additionally, treatment with proteinase K led to the loss of AHL degrading ability in the QQ isolates, suggesting degradation of AHLs by lactonase or acylase enzymes. The limited effect of proteinase K on *S. maltophilia* AHL degrading activity might be caused by different phenomena. E.g. proteinase K might be degraded or proteinase K might not enter the cells, or the AHL degrading enzyme is resistant to proteinase K activity. Further investigations are required to understand the exact mechanisms of QQ in this strain.

In the lactonase family, AiiA lactonase enzyme is the first well-studied group (Dong *et al.* 2001; Riaz *et al*. 2008). In fact, AiiA lactonase homologs have been characterized in numerous bacteria. In the present study, the PCR products of *aiiA* gene, responsible of encoding AHL lactonase, for ten bacterial species showed high genetic similarity (up to 99%) with other *aiiA* homologous gene existing in NCBI database. Conversely, PCR products from *P. syringae* and *E. cloacae* were free of *aiiA* gene. Previously, it has been indicated *Pseudomonas* spp often utilizes AHL acylase to degrade signal molecules (Huang *et al.* 2003; Huang *et al.* 2006; Shepherd and Lindow 2009). Based on these observations, it could be suggested that acylase enzyme is probably the main enzyme to degrade AHLs in *P. syringae* pathovar *syringae.* PCR amplification failed to reveal *aiiA* gene in *E. cloacae* in our research. To our knowledge, there is no report for the existence of lactonase or even acylase enzyme in this species and it seems further studies are required to unravel the AHL degradation mechanism in *E. cloacae.* Nevertheless, it has been demonstrated for some other species of *Enterobacter*, such as *E. ludwigii*, *E. aerogenes,* and *E. asburiae* PT39 that they are able to interfere with QS using AiiA homologs lactonase (Rajesh and Rai 2014a; Rajesh and Rai 2014b; Gopu *et al.* 2016). Interestingly, we could also indicate for the first time that *E. hormaechei* encodes *aiiA* homologous gene. The current study is also the first note of QQ ability by an *aiiA* homologous gene in *S. maltophilia*. Previous studies have demonstrated that the QQ activity in *Ag. tumefaciens* is lactonase based as encoded by *hqiA*, *attM* and *aiiB* genes (Carlier *et al.* 2003; Carlier *et al.* 2004; Torres *et al*. 2017). Here the existence of an *aiiA* gene homologue was demonstrated in *Ag. tumefaciens*. Furthermore, *C. gillenii* also displayed the presence of *aiiA* gene which is the first report for this strain.

The AHL lactonases are divided into three main types including the Metallo-lactamase superfamily, the α/β-hydrolase-fold family (Mei *et al*. 2010; Wang *et al*. 2010) and the phosphotriesterase (PTE) family (Uroz *et al.* 2008). It seems that zinc-binding motifs are conserved in almost all of the Metallo-lactamase superfamily enzymes (Momb *et al.* 2008; Liao *et al.* 2009). In our study, the motif "HXHXDH" was observed in both types of identified genotypes, confirming AHL lactonase activity, as the conserved motif "HXHXDH" is essential for zinc-binding and AHL degradation (Thomas *et al.* 2005). Besides, we observed Tyrosine residue (Y) at the position 194 in the sequences. Elias *et al.*

(2008) reported the Tyrosine residue is conserved in all lactonase enzymes and has a key role in the positioning of lactone ring of the substrate.

In conclusion, 24 known and novel AHL degrading strains were isolated from rainbow trout. These bacteria were able to deactivate natural and synthetic AHL *in vitro* tests and most of them showed cell-bound QQ activity. Heat and proteinase K treatments inhibited QQ capacity in nearly all isolates, indicating enzymatic activity. Various studies have demonstrated that QQ bacteria can inhibit QS *in vivo* experiments and improve the resistance of hosts against pathogens (Tinh *et al*. 2007; Chu *et al*. 2014). Hence, the current isolates could be considered and evaluated for the treatment and prevention of diseases in aquaculture.

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ETHICAL APPROVAL

All animal experiments were approved by the State Committee on Animal Ethics, Shiraz University, Shiraz, Iran (IACUC no: 4687/63).

CONFLICT OF INTEREST

All authors declare that they have no conflict of interest.

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Table 1 Identified AHL degrading isolates following BLAST-N search in GenBank based on 16S rDNA sequences

Isolate code	Source	Location	Closest species	Similarity (%)	Accession no.
1q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter hormaechei*	100%	MF687147
2q	Gill of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	99%	MF687148
5q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter hormaechei* subsp. *hormaechei*	100%	MF687149
7q	Gill of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687150
8q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus sp.*	100%	MF687151
10q	Intestine of healthy rainbow trout	Fars, Iran	*Citrobacter gillenii*	100%	MF687152
11q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687153
12q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687154
17q	Skin of healthy rainbow trout	Fars, Iran	*Enterobacter cloacae*	99%	MF687155
18q	Skin of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	99%	MF687156
20q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687157
22q	Gill of healthy rainbow tout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687158
23q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687159
24q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	99%	MF687160
26q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis*	100%	MF687161
27q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	100%	MF687162
29q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus cereus*	100%	MF687163
30q	Intestine of healthy rainbow trout	Fars, Iran	*Enterobacter sp.*	99%	MF687164
31q	Gill of healthy rainbow trout	Fars, Iran	*Pseudomonas syringae pv. tomato*	100%	MF687165
32q	Intestine of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	99%	MF687166
40q	Gill of healthy rainbow trout	Fars, Iran	*Acinetobacter radioresistens*	98%	MF687167
41q	Gill of healthy rainbow trout	Fars, Iran	*Agrobacterium tumefaciens*	99%	MF687168
42q	Intestine of healthy rainbow trout	Fars, Iran	*Bacillus thuringiensis* strain	100%	MF687169
43q	Skin of healthy rainbow trout	Fars, Iran	*Stenotrophomonas maltophilia*	100%	MF687170

Table 2 Sequences homology of *aiiA* gene from QQ isolates using BLAST-N analysis

Isolate Code	Closest Species Based on 16S rDNA	References Strains (Database GenBank)	Similarity (%)	Accession no
2q	*Stenotrophomonas maltophilia*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687171
5q	*Enterobacter hormaechei*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687172
7q	*Bacillus thuringiensis*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687173
8q	*Bacillus sp*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687174
10q	*Citrobacter gillenii*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687175
12q	*Bacillus cereus*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687176
18q	*Bacillus thuringiensis*	*Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (Genotype B)	98%	MF687177
24q	*Bacillus cereus*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687178
40q	*Acinetobacter radioresistens*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687179
41q	*Agrobacterium tumefaciens*	*Bacillus mycoides* strain ATCC 6462 plasmid pBMX_1 (Genotype A)	99%	MF687180

Figure Legends

Figure 1. a) (**A**) Quorum quenching (QQ) activity assay on TSA plate by *Pseudomonas* sp. P3/pME6000 an AHL-degrading negative strain; (**B**) QQ activity by one of the QQ isolates in the present study. *Yersinia ruckeri* and CV026 were used as a natural AHL producer and AHL biosensor strains, respectively. The part adjacent to QQ isolate (middle streak) of CV026 shows no pigment, indicating AHL degradation. P3/pME6000, as a negative control, was not able to inhibit QS; **b)** Production of the pigment by *Chromobacterium violaceum* CV026 in the presence of different concentrations of *N*-hexanoyl homoserine lactone (C6-HSL); **c)** Standard curve resulted from the relationship between concentrations of C6-HSL and diameter of zones induced by *C. violaceum* CV026. The error bars indicate the standard deviation of 3 replicates.

Figure 2. a) Kinetic of the degradation of *N*-hexanoyl homoserine lactone (C6-HSL) by the QQ isolates in reaction mixtures containing 5 mg l^{-1} of C6-HSL at 12 h (white bars) and 24 h (grey bars) incubation (averages ± S.D.); **b)** AHL degradation in heat (white bars), proteinase K (grey bars) and untreated (black bars) cell cultures (averages ± S.D.). All isolates lost degradation ability after both treatments. Exceptions include *Stenotrophomonas maltophilia* strains (exposure to heat and proteinase K) and *Pseudomonas syringae* (exposure to heat); **c)** Residual C6-HSL level after treatment with the extracellular matrix of QQ isolates (average ± S.D.). Supernatant of isolate 31q, *Pseudomonas syringae,* contained extracellular C6-HSL degradation activity. All other isolates did not display extracellular C6-HSL degradation activity. *Pseudomonas* sp. P3/pME6000 and P3/pME6863 were applied as negative and positive controls, respectively. The error bars indicate the standard deviation of 3 replicates.

Figure 3. PCR amplification of *aiiA* gene coding lactonase enzyme. Bands with 750 bp on electrophoresis gel agar showing the existence of *aiiA* gene in QQ isolates. The first and second lanes indicate 100kb DNA ladder (M) and negative control (NC), respectively. The numbers in the top holes show the AHL degrading strains: 2q: *S. maltophilia*; 5q: *E. hormaechei*; 7q: *B. thuringiensis*; 8q: *Bacillus* sp.; 10q: *C. gillenii*; 12q: *B. cereus*; 18q: *B. thuringiensis*; 24q: *B. cereus*; 40q: *A. radioresistens* and 41q: *Ag. tumefaciens*

Figure 4. Phylogenetic tree resulting from likelihood presenting the relationship among *aiiA* genotype A and *aiiA* genotype B enzymes and known AHL degrading enzymes: *aiiA* 240 (*Bacillus* sp., accession number: AF196486), *aiiA* 8010 (*B. thuringiensis*, accession number: AY943832); *aiiA* B65 (*B. weihenstephanensis*, accession number: KC823046); *aidC* (*Chrysemobacterium* sp., accession number: AP014624); *aidC* (*Chrysemobacterium* sp., accession number: AB73663); *ahlD* (*Arthrobacter* sp., accession number: AF525800); *ahlk* (*Klebsiella pneumoniea*, accession number: AY222324); *attM* (*Agrobacterium tumefaceins*, accession number: U59485)

Figure 5. Alignment of amino acid sequences of AHL lactonases of A and B genotypes with amino acid sequences of known sequences: AHL lactonase of AiiA 240 (*Bacillus* sp., accession number: AF196486), AiiA 8010 (*B. thuringiensis*, accession number: AY943832); AiiA B65 (*B. weihenstephanensis*, accession number: KC823046); AidC (*Chrysemobacterium* sp., accession number: AP014624); AidC (*Chrysemobacterium* sp., accession number: AB73663); AhlD (*Arthrobacter* sp., accession number: AF525800); Ahlk (*Klebsiella pneumoniea*, accession number: AY222324); AttM (*Agrobacterium tumefaceins*, accession number: U59485). The motif of HXHXDH and Tyrosine (194) were put into boxes; Conserved sequences were marked by gray shadow; Metal

ligands for the dinuclear zinc form proposed by Thomas *et al.* (2005) were represented with an asterisk

Supporting Information Legends:

Table S1 Biochemical characteristics of quorum quenching strains