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Isolation and characterization of AHL-degrading bacteria from fish and pond sediment*

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Abstract Quorum sensing (QS) disruption is considered as a potential alternative strategy to combat bacterial diseases in aquaculture. In this study, we isolated and identified bacteria degrading QS molecules from pond sediment and fish intestine. A total of 132 strains were obtained in the enrichment culture, of which two strains were identified as *Enterobacter* sp. f003 and *Staphylococcus* sp. sw120, being isolated from the fish intestine and pond sediment, respectively. We found that strains f003 and sw120 could degrade acyl-homoserine lactones (AHLs) and cause no hemolysis of sheep red blood cells. The AHL lactonase (*aiiA*) homologous gene in the two strains was detected in PCR amplification and the high-degrading activity to N-hexanoyl-L-homoserine lactone (C6-HSL) and AHLs secreted from pathogenic *Aeromonas hydrophila* was assessed. Meanwhile, the artificial infection of cyprinid *Carassius auratus gibelio* with intraperitoneal injection showed that the two strains were avirulent. Therefore, the obtained indigenous bacteria are candidate probiotics against pathogenic *A. hydrophila* in aquaculture.

Keyword: quorum sensing (QS); *Aeromonas hydrophila*; probiotic; *Enterobacter*; *Staphylococcus*

1 INTRODUCTION

Aquaculture has grown dramatically over the past 30 years to around 51.42 million tons in 2016 accounting for 74.5% of fish production in China. However, the disease is one of the important constraints to the sustainable growth of modern fish farming. Infections caused by opportunistic bacteria, such as *Aeromonas hydrophila*, *Flavobacterium columnare*, and *Vibrio parahaemolyticus* leads to a severe loss in the fish and shrimp farming. Antibiotic treatment is the conventional measure to prevent and treat fish diseases. However, the increasing concerns about drug resistance of pathogens, food safety, and environment pollution have driven researchers to explore new disease control strategy.

Quorum sensing (QS) is well known in many bacteria, which is a type of bacterial cell-cell communication depending on small signal molecules (also called autoinducers), and can regulate bacterial cell population responding collectively to the ambient environment (Smith et al., 2003; Waters and Bassler, 2005; Norizan et al., 2013; Takayama and Kato, 2016). QS was firstly discovered in the marine bacterium *Vibrio fischeri* (Norizan et al., 2013), and subsequently reported from Gram-positive and Gram-negative bacteria involving different signal molecules. At present, four QS systems were identified in bacteria, i.e. LuxI/LuxR system using acyl-homoserine lactones (AHLs) as signal molecules in most gram-negative bacteria; luxS system with autoinducer 2 (AI-2), two-component system, consisting of a membrane bounding histidine kinase and a response regulator, which use modified oligopeptides as signals, and CqsA/S system involving cholerae autoinducer 1 as signals (Defoirdt et al., 2004; Boyen et al., 2009; Ghani et al., 2014; Zhao et al., 2015). The AHLs are most common and well-

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known QS signals, and pronouncedly enhance the production of virulence factors in pathogenic bacteria, including protease, lipase, aerolysin, and hemolysin (Lynch et al., 2002; Chu et al., 2011; Norizan et al., 2013). AHLs possess highly conserved molecular structure, and most have similar homoserine-lactone moiety with different acyl side chains (Chong et al., 2012; Lau et al., 2013).

Quorum quenching (QQ), namely the enzymatic interruption of QS, refers to the process in which autoinducer-based quorum sensing be interfered and disrupted (Huang et al., 2016), and has recommended as a promising non-antibiotics strategy for bacterial diseases therapy (Tang and Zhang, 2014). Quorum quenching (QQ) includes three main methods (Huang et al., 2016), (1) inhibition of AHL synthesis, (2) degradation of AHLs, (3) blockage of AHL-LuxR interaction. Many microorganisms were demonstrated that can produce AHL degrading enzymes which were classified into three major types, AHL lactonase (lactone hydrolysis), AHL acylase (amid hydrolysis), and AHL oxidase/reductase (oxidoreduction) (Torres et al., 2016). The AHL-degrading bacteria have been widely found in plants, sewage, fish and bivalve hatchery (Chan et al., 2009; Chu et al., 2011; Torres et al., 2016). *Aeromonas hydrophila* is an important pathogenic bacterium in freshwater culture (Austin and Austin, 2012), possesses the typical AHL/LuxR/LuxI quorum sensing system, and produces two types of AHLs namely N-3-butanoyl-DL-homoserine lactone (C4-HSL) and N-3-hexanoyl-DL-homoserine lactone (C6-HSL), of which C4-HSL was the predominant type (Khajanchi et al., 2009). Thus, quorum quenching may provide an alternative efficient strategy to control *A. hydrophila* infection. The main purpose of this work was to screen and identify potential indigenous bacteria from the fish intestine and pond sediment, which can degrade AHLs secreted by the pathogenic *A. hydrophila*.

2 MATERIAL AND METHOD

2.1 Bacterial strains and culture media

Aeromonas hydrophila NJ-35, donated by Prof. Yong-Jie Liu (Nanjing Agricultural University, China) and originally isolated from diseased *Carassius auratus* (Wang et al., 2015), was used as test pathogenic strain. *Chromobacterium violaceum* CV026 from our laboratory was used as reporter strain to detect AHL for its inducible by short chain AHL compounds with N-acyl side chains ranging from C4 to C8, with different sensitivity (McClean et al., 1997). Unless stated otherwise, all bacteria were routinely cultured at 28°C in Luria-Bertani (LB) medium.

To screen AHL-degrading bacteria, KG medium containing C6-HSL (Sigma-Aldrich, St. Louis, Missouri, USA) was used following the previous report of Chan et al. (2009). In brief, 1-L KG medium contains 1.0 g NaCl, 0.5 g KCl, 0.4 g $MgCl_2$, 0.1 g $CaCl_2$, 0.15 g Na_2SO_4, 5.0 g KH_2PO_4, 1.0 g 2-N-morphlino-ethanesulfonic acid (Sigma, USA), 1 mg $FeCl_3$, 0.1 g $MnCl_2$, and 0.46 g $ZnCl_2$. Before use, fresh prepared C6-HSL was added in KG medium as final concentration 500 μg/mL.

2.2 Sample collection and isolation of AHL-degrading bacteria

The soil from carp pond sediment was collected and suspended in KG medium (10 g soil diluted in 40 mL KG) with vigorous vortex for 10 min. The suspension was firstly centrifuged at 2 000 r/min for 1 min; and then the supernatant was collected and centrifuged (12 000 r/min, 1 min) again. The pellet was resuspended in 2 mL KG medium, and the resuspension (100 μL) was inoculated into 3-mL KG medium containing C6-HSL (500 μg/mL) and incubated for 48 h at 28°C with shaking (220 r/min). The same 10% (V/V) transfer was conducted three times to KG medium containing C6-HSL. After the final cultivation, the bacteria were isolated as single colonies and purified on the LB agar.

Twenty crucian carp (*C. auratus gibelio*), 53±5 g, were collected from a fish farm in Wuxi, China, and anaesthetized with tricaine methanesulfonate (100 mg/L, MS222, Sigma). Fish were dissected aseptically, and the intestinal mucosa was collected and homogenized with KG medium. The bacterial isolation and enrichment were conducted as the above-mentioned method.

2.3 Screening AHL-degrading strains with *A. hydrophila*

To confirm the obtained strains can degrade the AHL produced by pathogenic *A. hydrophila* NJ-35, the degrading activity was detected in the well-diffusion assay. LB agars were first spread with 100 μL CV026 culture, punched for 4–5 wells (2 mm in diameter), and then added 10 μL sterilized PBS (negative control), 10-μL C6-HSL (0.1 μg/μL,

positive control), 10 μL *A*. *hydrophila* NJ-35 culture, the mixture of isolated strains (5 μL) and *A*. *hydrophila* NJ-35 (5 μL) in the wells, respectively. Plates were incubated at 28°C for 48 h to observe the zone of purple pigment produced by CV026.

2.4 Hemolysis of the obtained strains

To determine the hemolytic activity, the obtained strains were grown in LB broth overnight at 28°C with shaking (220 r/min). The 20-μL culture was spotted on the sheep blood agar plate (Nanjing SenBeiJia Biological Technology Co., Ltd.), and incubated overnight at 28°C to measure the hemolytic zone.

2.5 Strain identification

The morphological and biochemical characteristics of obtained isolates were tested with traditional methods (Supl. Table S1), and use the bacterial micro biochemical reaction tube (Hangzhou Binhe Microorganism Reagent Co., Ltd.). The data was compared with the Bergey's Manual of Determinative Bacteriology (Holt, 1994).

Genomic DNA was extracted with an Ezup column bacterial genome extraction kit (Sangon Biotech, Shanghai, China). 16S ribosomal DNA (rDNA) sequences were amplified with primers 27F and 1525R (Weisburg et al., 1991; Dewhirst et al., 1999). The PCR reaction was conducted with initial denaturation at 94°C for 5 min, 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min 30 s, and a final extension at 72°C for 5 min. Amplified products were purified with electrophoresis and then sequenced directly with primers 27F and 1525R using ABI 3100 Genetic Analyzed automated DNA sequencer (Applied Biosystems). Homologous sequences were determined by performing BLAST search at NCBI. Sequences alignment was conducted by using MEGA7.0 software with the default setting, and phylogenetic analyses were conducted with maximum likelihood (ML) method.

2.6 PCR amplification of *aiiA* homologue gene

The *aiiA* (AHL lactonase) homologue gene of the obtained isolates were amplified with primers designed by Huma et al. (2011), and the PCR program consisted of initial denaturation at 94°C for 10 min, 35 cycles of 94°C (30 s), 55°C (30 s) and 72°C (1 min), and final extension at 72°C for 5 min. The PCR products were purified and sequenced.

The sequence open reading frame (ORF) was determined by using the tBlastn and ORF analyses in NCBI. Predicted amino acid sequences aligned with DNAMAN 7.0 software. The *aiiA* sequences of *Bacillus* sp. A24 (AF397400.1), *Bacillus* sp. 240B1 (AF196486.1) and *Bacillus thuringiensis* (EF379241.1.) were used as reference sequences.

2.7 AHL-degrading activity

AHL degrading assays were conducted as previously report (Chan et al., 2009). *E*. *coli* DH5α was used as a control group. The cells of obtained isolates were collected with centrifugation, resuspended in PBS (OD_{600}=1). C6-HSL was dissolved in the bacterial cell suspension solution (0.1 μg/μL). The mixtures were incubated for 0, 3, and 6 h at 28°C with gently shaking in a hybridization oven. Heat-denatured suspension (10 μL) was inoculated on LB agar, which has been spread with CV026. Degradation of C6-HSL in the mixture was assessed by the loss of purple pigmentation of CV026.

2.8 Lethal doses (LD_{50}) of the isolate

One hundred twenty healthy *C*. *auratus gibelio*, weight 50±3 g, were divided into 6 groups and maintained at 25°C in a water recirculating system. The obtained strains were harvested and then serially diluted with PBS. Fish were injected intraperitoneally with the suspensions at the doses ranging (10^5, 10^7, 10^9, 10^{11}, and 10^{13} cfu/mL). The infected fish were observed for 7 days, and the death was recorded daily. The LD_{50} was calculated following the method of Karber (1931). All animal experiments in the present study were approved by Nanjing Agricultural University in accordance with the recommendations under the Guide for the Care and Use of Laboratory Animals of China.

3 RESULT

3.1 Isolation and identification of AHL-degrading bacteria

After the selective and enrich culture with KG medium containing C6-HSL, 132 isolates were obtained from fish gut and sediment. AHLs secreted by *A*. *hydrophila* NJ-35 can cause purple pigmentation production on the CV026 lawn. Therefore, the disappearance of purple pigmentation production was observed the quorum quenching activities of the isolates on the CV026 lawn. Seven of these isolates

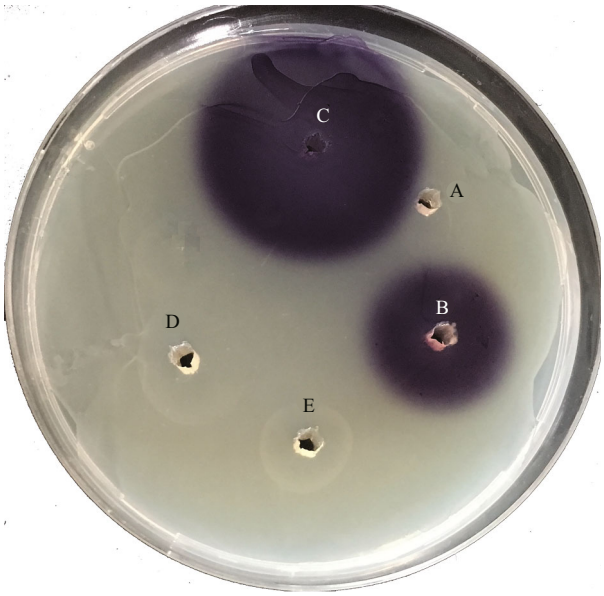

Fig.1 Degradation of AHL (acylated homoserine lactone) secreted by *Aeromonas hydrophila* strains and *Chromobacterium violaceum* CV026 used as a biosensor

A. sterilized PBS (negative control); B. *A. hydrophila* NJ-35; C. C6-HSL (positive control); D. *A. hydrophila* NJ-35 and the obtained strain sw120; E. *A. hydrophila* NJ-35 and the strain f003.

were detected can degrade AHLs secreted by *A. hydrophila* NJ-35 (Fig.1); however, five of the seven isolates caused the sheep blood hemolysis (Supl. Fig.S1). Regarding the safety of fish culture, only the isolates f003 and sw120 were used for further analyses.

3.2 Identification of the obtained isolates

The 16S rRNA of the obtained stains f003 and sw120 were sequenced and deposited in the GenBank (Accession Nos. KY810611 and KY810613). BLAST analyses showed that the sw120 was highly similar to *Staphylococcus saprophyticus* subsp. *saprophyticus* (NR074999.2, 98.6%), and the f003 was highly similar to *Enterobacter tabaci* (NR146667.2, 98.9%). Phylogenetic analyses indicated that the isolates f003 and sw120 were firmly clustered in the branches of *Staphylococcus* and *Enterobacter*, respectively (Figs.2 and 3). Based on the 16S rRNA sequences and biochemical characters (Supl. Table S1), the obtained isolates were identified as *Staphylococcus* sp. strain sw120 and *Enterobacter* sp. strain f003, respectively.

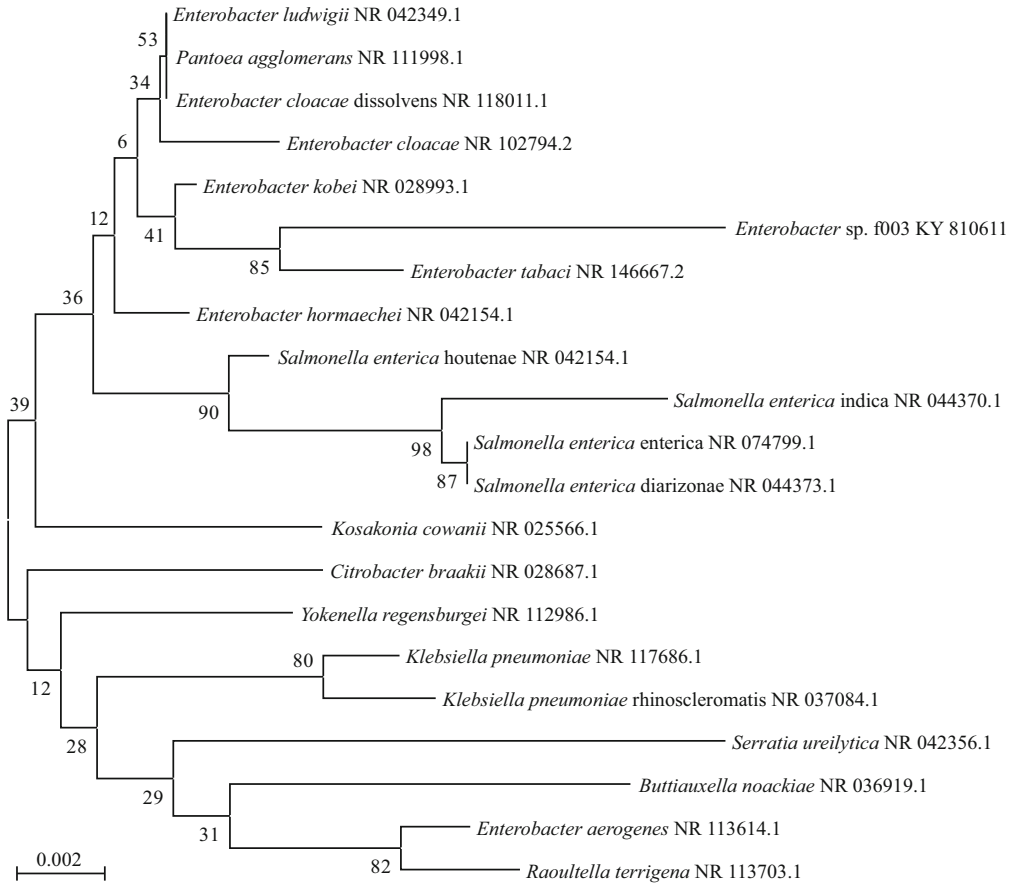

Fig.2 Phylogenetic relationship of the strain f003 with 16S rDNA gene

Bootstrap values showing at branch nodes.

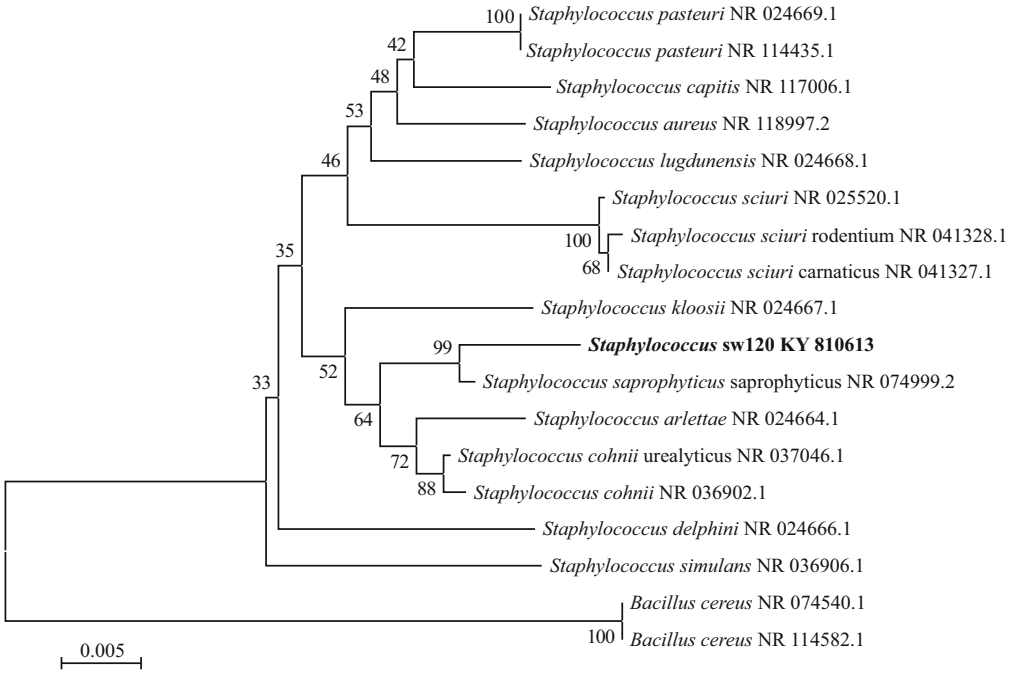

Fig.3 Phylogenetic relationship of the strain sw120 with 16S rDNA gene

Bootstrap values showing at branch nodes.

Fig.4 AiiA amino acid sequence alignment of the obtained strains sw120 and f003

3.3 *aiiA* homologue gene sequence analysis

The isolated strains *Staphylococcus* sw120 and *Enterobacter* f003 were all found carrying the *aiiA* homologue gene. Sequence alignment showed their predicted amino acid sequences presented high homology with that of *Bacillus* sp. A24, *Bacillus* sp. 240B1, and *Bacillus thuringiensis* (sequence similarity 93.9% and 92.3%) (Fig.4).

3.4 AHL-degrading activity

To detect the AHL degrading activity, the bacterial cells of the obtained strains *Staphylococcus* sw120 and *Enterobacter* f003 were first incubated with C6-HSL (0.1 mg/mL) for 0, 3, and 6 h, and then inoculated on the LB plate with CV026. PBS and *E. coli* were used as blank and negative control groups. *Staphylococcus* sw120 (Supl. Fig.S2), and *Enterobacter* f003 (Fig.5) all showed a high activity and can degrade nearly all C6-HSL in 6 h, and no apparent AHL degradation was observed in the negative control *E. coli* DH5α. The supernatant of f003 had no AHL-inactivating activity, and the diameter of the purple pigmentation had no significant difference between the f003 and *E. coli* DH5a well.

3.5 Lethal doses (LD50) of the isolates

The virulence of the obtained strains f003 and sw120 were tested in crucian carp (Table 1). The LD50 of f003 was 7.9×10^{13} CFU to *C. auratus gibelio* and the sw120 was 1.6×10^{13} CFU. In addition, there is no death observed when the bacterial cells

Table 1 Death of *C. auratus gibelio* injected with the strains sw120 and f003 in 7 days

Group	Injection concentration (cfu/mL)	Number of fish in the experiment	Number of deaths							Cumulative mortality (%)
			1 d	2 d	3 d	4 d	5 d	6 d	7 d	
f003	1×10^{5}	20	0	0	0	0	0	0	0	0%
	1×10^{7}	20	0	0	0	0	0	0	0	0%
	1×10^{9}	20	0	0	0	0	0	0	0	0%
	1×10^{11}	20	0	0	0	0	0	0	0	0%
	1×10^{13}	20	0	1	0	0	0	0	0	5%
sw120	1×10^{5}	20	0	0	0	0	0	0	0	0%
	1×10^{7}	20	0	0	0	0	0	0	0	0%
	1×10^{9}	20	0	0	0	0	0	0	0	0%
	1×10^{11}	20	0	0	0	0	0	0	0	0%
	1×10^{13}	20	0	4	3	1	0	0	0	40%

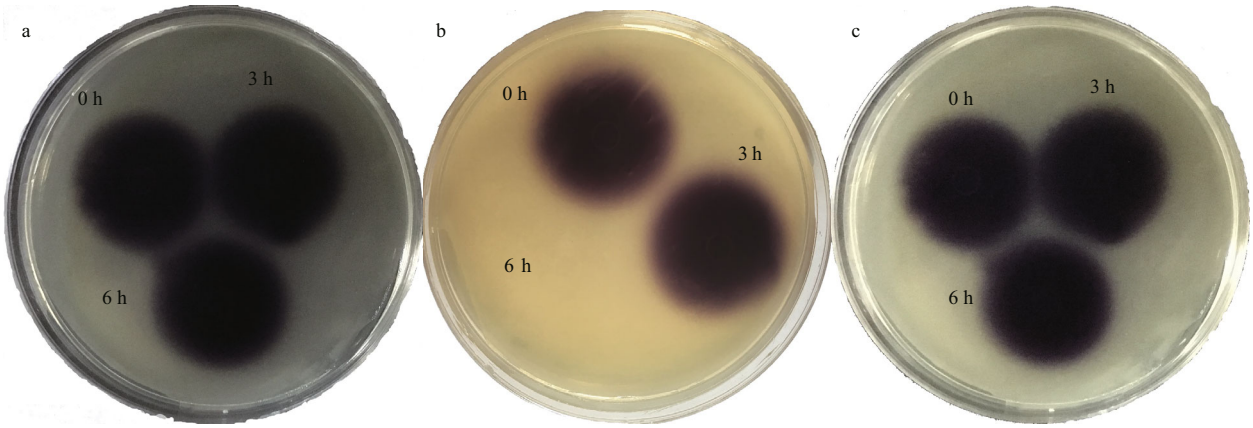

Fig.5 C6-HSL degrading activity of the obtained strain f003

a. PBS (blank control); b. f003; c. *E. coli* DH5α (negative control).

concentration was not above 10^{11} cfu/mL, while the death was found at an extremely high concentration 10^{13} cfu/mL. The results suggest that the obtained f003 and sw120 were low virulent to crucian carp *C. auratus gibelio* and no death occurred below 10^{11} cfu/mL.

4 DISSCUSSION

To interfere with bacterial quorum sensing by degrading AHL molecules is an important survival competition strategy between different strains, and widely distributed in the natural ecosystem (Chu et al., 2011; Pande et al., 2015). Because of the potential to control bacterial infection, AHL-degrading bacteria have been isolated from the various environment, and mostly belong *Bacillus* (Dong et al., 2000, 2001; Cao et al., 2012). In this study, we isolated and identified two novel and indigenous AHL-degrading bacterial strains, *Enterobacter* sp. f003 from the fish intestine, and *Staphylococcus* sp. sw120 from the pond sediment. To our knowledge, this is the first report that *Staphylococcus* strain could degrade AHL molecules.

Quorum sensing signals can be inactivated or degraded through enzymatic degradation or metabolization of bacteria. Two major classes of AHL-inactivating enzymes have been described: lactonase, which is capable of degrading a wide range of AHLs, cleaving the lactone ring into acylated homoserine, and varying in acyl chain length and substitution; acylases, which exhibits substrate specificity and can cleave AHLs into homoserine lactone and fatty acid (Huang et al., 2006). In this work, 132 strains were isolated from KG medium containing C6-HSL; however, only 7 strains could degrade the AHLs produced by *A. hydrophila*. Those isolates showed different AHL-degrading property and specificity. In the seven strains, five were detected caused the sheep blood hemolysis and not used for further analyses. In the other two strains *Enterobacter*

sp. f003 and *Staphylococcus* sp. sw120, AHL lactonase gene (*aiiA*) was found by PCR amplification. However, whether another AHL-degrading mechanism involving the AHLs produced by *A. hydrophila* was not clear.

Aeromonas hydrophila is a typically opportunistic bacterial pathogen in freshwater environments and causes severe hemorrhagic septicemia disease in cultured and wild fishes, such as carp, catfish, perch, and tilapia (Hossain et al., 2014). *A. hydrophila* uses a conventional acylated homoserine lactone (AHL) mediated quorum-sensing system in cell-to-cell communication (Bruhn et al., 2005; Garde et al., 2010). C4-HSL and C6-HSL are the predominant quorum-sensing signals and regulate different virulence factors of *A. hydrophila*, including flagella, a serine protease, biofilm, and hemolysins (Swift et al., 1997; De Kievit and Iglewski, 2000; Khajanchi et al., 2009). The obtained strains *Enterobacter* sp. f003 and *Staphylococcus* sp. sw120 could effectively degrade the C6-AHL in the KG medium and AHLs produced by *A. hydrophila*. Therefore, use of the obtained strains in aquaculture would interfere with the virulence of pathogenic bacteria and increase the fish survival with infection. The potential use for aquaculture needs to be carefully assessed in further research.

5 CONCLUSION

In this study, two indigenous bacterial strains *Enterobacter* sp. f003 and *Staphylococcus* sp. sw120, isolated from the cultured fish intestine and pond sediment, were obtained and were considered as potential probiotics used in fish culture.

6 DATA AVAILABILITY STATEMENT

The datasets supporting the conclusions of this article are available in the GenBank international nucleotide sequence repository under accession numbers KY810611 and KY810613.

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Electronic supplementary material

Supplementary material (Supplementary Table S1 and Supplementary Figs.S1–S2) is available in the online version of this article at https://doi.org/10.1007/s00343-019-8137-6.