Probiotics and Antimicrobial Proteins (2020) 12:160–171
https://doi.org/10.1007/s12602-018-9495-7

The Quorum Quenching Bacterium *Bacillus licheniformis* T-1 Protects Zebrafish against *Aeromonas hydrophila* Infection

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Published online: 4 February 2019

Abstract

Quorum sensing, bacterial cell-to-cell communication via small signaling molecules regulates virulence in many bacterial pathogens, and is a promising target for antivirulence therapy, which may inhibit virulence rather than cell growth and division. Herein, *Bacillus* strains capable of degrading QS molecules from freshwater environments were screened as potential aquaculture probiotics. A total of 34 *Bacillus* strains were isolated. Strain T-1 was selected with "H" streaking and double layer agar plate methods using *Chromabacterium violaceum* ATCC12472 as reporter, and eventually identified as *Bacillus licheniformis* based on biochemical and molecular identification. Quorum quenching by T-1 was confirmed using *C. violaceum* CV026. T-1 was non-hemolytic in vitro. In biocontrol experiments, T-1 reduced the pathogenicity of *Aeromonas hydrophila* cb15 in zebrafish co-injected intraperitoneally with both strains, achieving a relative percentage survival of 70%. Determination and analysis of the T-1 draft genome using the Illumina Hiseq 2500 platform identified the quorum quenching gene *ytnP*, encoding an acyl-homoserine lactone metallo-β-lactamase, as a potential QS quencher in T-1. In conclusion, *B. licheniformis* T-1 could be a safe and effective quorum quenching bacterium for protecting hosts against pathogenic bacterial infections in aquaculture.

Keywords Quorum quenching · *Bacillus licheniformis* · *Aeromonas hydrophila* · Zebrafish · *ytnP*

Introduction

Antibiotics are crucial for preventing, controlling, and treating diseases in aquatic animals, and many act by disrupting processes such as bacterial cell wall synthesis, DNA proliferation, and protein synthesis [1, 2]. However, excessive and inappropriate use of antibiotics has accelerated the evolution of bacteria that are resistant to antibiotics. As a consequence, many antibiotic treatments are no longer effective for treating humans and aquatic animals, which has implications for fish health [3]. Thus, developing novel strategies for controlling bacterial diseases without fear of further stimulating the evolution of antibiotic-resistant strains is important for human and veterinary medicine.

Aeromonas hydrophila, a Gram-negative bacterial pathogen found mainly in aquatic environments, causes a wide variety of symptoms in fish including tissue swelling, necrosis, ulceration, and hemorrhagic septicemia, and is strongly resistant to multiple antibiotics, resulting in significant economic losses to freshwater aquaculture [4]. The pathogenicity of *A. hydrophila* depends on the production of potential virulence factors, such as exoproteases and exotoxin [5]. Production of exoproteases is under the control of quorum sensing (QS). Evidence suggests that *A. hydrophila* harbors the AhyI/AhyR QS system, utilizes *N*-acyl-homoserine lactone (AHL)-dependent QS to regulate the expression of virulent genes [6, 7], and mediates microbial infection and colonization in the host, suggesting AHLs could be potential targets for controlling virulence. Therefore, inhibiting QS signals such as AHLs, a process referred to as quorum quenching (QQ), could decrease bacterial virulence, making it a promising strategy for fighting against pathogenic bacteria in aquaculture.

Quorum quenching mechanisms have been identified in many prokaryotic and eukaryotic organisms [8], where they regulate the antimicrobial activities of hosts by interfering with bacterial quorum sensing. The first quorum quenching gene, *aiiA*, was

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identified in *Bacillus subtilis* [9], and other quorum quenching genes have since been discovered, such as *ahlD* (*Arthrobacteria* sp.), *aiiM* (*Microbacterium testaceum*), *attM* (*Agrobacterium fabrum*), *aidC* (*Chryseobacterium* sp.), and *ytnP* (*B. subtilis*) [10–13]. Previous research indicates that QS inhibitors including bacteria or AHL-lactonase can reduce pathogenicity and protect aquatic animals against infection by Gram-negative pathogenic bacteria [14, 15]. *Bacillus* QSI-1 was isolated from the intestine of *Carassius auratus gibelio*, and fish fed a diet supplemented this organism displayed good survival [15]. Expression of the *Bacillus cereus aiiA* gene in *Pichia pastoris* can decrease mortality, and delay mortality in fish by quenching the QS signals of *A. hydrophila* [13]. Exactly how bacteria or AHL-lactonase disrupt QS is being investigated, and could provide new opportunities for therapeutic applications. *Bacillus licheniformis* produces a wide range of extracellular enzymes, and is reportedly a probiotic bacterium for animals, but little is known about the potential applications of *B. licheniformis* related to quorum sensing for the prevention and treatment of *A. hydrophila.*

Bacillus spp. are a natural resource bank for screening new quorum quenching bacteria, and are commonly regarded as safe strains for use in aquaculture as agents for improving water quality and preventing/controlling diseases [16]. However, recent studies indicate that *Bacillus* strains might contain toxin-producing genes [17]. Consequently, these results have given rise to concern about the safety of *Bacillus* products. A more rigorous selection process is thus required for *Bacillus* probiotic candidates.

The purpose of the present study was to identify safe and effective *Bacillus* spp. that degrade AHL molecules from freshwater culture ponds, and explore their potential mechanisms from the perspective of quorum sensing.

Materials and Methods

Bacterial Strains and Culture Conditions

The quorum quenching strain T-1 was isolated from a freshwater culture pond sediment, and *Escherichia coli* and *Bacillus subtilis* were obtained from the microbiology lab of Shanghai Ocean University. Strains were cultivated in Luria-Bertani (LB) broth consisting of 1% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.4 ± 0.2), and incubated for 24 h at 30 °C. The target pathogen *A. hydrophila* cb15 was isolated from necrosis of *Carassius auratus gibelio* in Shanghai and cultivated in LB broth 150 rpm in a shaker overnight at 28 °C for 24 h. *Chromabacterium violaceum* ATCC12472 was used as a biosensor to detect potential QS inhibitors of C4-AHL and C6-AHL that are synthesized via the autoinducer synthase CviI, which binds to the receptor CviR to form complexes, resulting in the production of violacein [18]. Additionally, *C. violaceum* CV026 was used as an AHL biosensor to detect exogenous AHLs (C6-HSL). Both *C. violaceum* strains were cultured in LB broth at 26 °C for 24 h. Where necessary, growth media were supplemented with kanamycin (50 μg ml^{-1}) [19].

Isolation of *Bacillus* spp. Candidates for Inhibiting Quorum Sensing

The isolation method was employed as described previously [12]. In brief, sediment from freshwater culture ponds was diluted to 10^{-5} with normal saline and incubated at 80 °C for 10 min, then 0.1 ml samples were plated in duplicate on LB agar and cultured at 28 °C for 24–48 h. Hemolytic testing of isolates was carried out on nutrient agar supplemented with Columbia CNA agar and 5% sheep blood. Isolates were inoculated onto hemolytic plates and incubated at 28 or 37 °C for 24 h. Plates were observed for hemolytic reaction, and strains not producing a change around colonies were considered to be non-hemolytic, while strains displaying a clean hemolysis zone around colonies were considered hemolytic [20]. Moreover, in order to test the safety of *B. licheniformis* T-1 on animals, and the impact on the aquatic environment, *C. auratus gibelio*, zebrafish, and mice were selected for safety evaluation. *Chlorella vulgaris*, *Daphnia magna*, and *Brachionus calyciflorus* were used as test organisms for ecological environment evaluation. Animal safety tests and ecology environment evaluation of *B. licheniformis* T-1 were conducted according to the National Standard (GB) Acute Toxicity methods [21].

Two methods of isolating *Bacillus* spp. for inhibiting QS were performed as described previously [22, 23]. Firstly, *Bacillus* strains were spread onto the center of an agar plate using a sterilized cotton swab, and *C. violaceum* ATCC12472 was spread in parallel along both sides where two strains formed an "H" shape, and plates were incubated overnight (24 h) at 26 °C. Next, 5 ml of molten soft LB agar was inoculated with 0.05 ml of *C. violaceum* ATCC12472 grown overnight in LB broth. The agar-culture solution was immediately poured onto LB agar and *Bacillus* spp. was spread onto the center of the plate. Plates were incubated overnight at 26 °C and examined for violacein pigment production. QS inhibition was detected by a colorless, opaque, but viable halo around discs (indicating loss of pigmentation).

To verify the AHL-degrading activity of T-1, *C. violaceum* CV026 was used as an AHL biosensor [24]. Agar plates were prepared by mixing overnight cultures of *C. violaceum* CV026 in 20 ml of LB agar which was then poured into petri dishes containing C6-HSL (200 μg ml^{-1}). Four sterile Oxford cups were placed on LB agar plates, and 0.2 ml enrofloxacin (0.64 μg ml^{-1}) was added to one cup as a negative control, while another received 0.2 ml sterile water as a blank control. The remaining two cups received 0.2 ml of strain T-1 as the experimental groups. Plates were cultivated for 24 h at 26 °C. The color of colonies around the Oxford cup zone was observed, and differences between the four cup zones were compared. All experiments were repeated twice.

To verify the potential bactericidal ability of T-1 against *A. hydrophila*, agar plates were prepared by mixing overnight cultures of *A. hydrophila* cb15 in 20 ml of LB agar, which was then poured into petri dishes. Five sterile Oxford cups were used, one receiving 0.2 ml sterile water (negative control), one receiving 0.2 ml enrofloxacin (0.64 μg ml^{-1}) as a positive control, and the other three receiving 0.2 ml T-1 culture following centrifugation at 5000 rpm for 5 min and diluted at the concentration of 10^0, 10^{-1}, and 10^{-2}. Plates were cultivated for 24 h at 30 °C. Transparent circles around the Oxford cup zone were observed, and differences between the five Oxford cup zones were compared. All experiments were repeated twice.

Identification of T-1

Molecular identification of T-1 was performed by extracting genomic DNA and amplifying the 16S rDNA gene by PCR using universal primers 27 F (5'-AGAGTTTGATCATG GCTCAG-3′) and 1492 R (5'-GGTTACCTTGTTAC GACTT-3′). Sequences were analyzed by Invitrogen Co. Ltd., and homology was compared using BLAST searches against the GenBank database. A phylogenetic tree was constructed with the Mega 5.05 software [25].

Physical and chemical identification was based on the Biolog method described previously [26]. *Bacillus* sp. was incubated on LB agar for 12 h, a single colony was transferred to GN/GP IFB inoculum with a sterile cotton swab, and the turbidity of the culture was adjusted to 92–96%. The sample was transferred into a GENIII Microplate 96-well plate with a pipette (0.1 ml per well) and incubated at 33 °C in a constant temperature incubator. Bacteria were identified by the Biolog method following culturing for 6 or 20 h.

The morphology of T-1 was observed using a scanning electron microscope. T-1 was cultured in nutrient broth overnight, the culture was centrifuged at 4480 g for 2 min, the supernatant was discarded, and the cell pellet was washed three times with distilled water. Deposited *Bacillus* cells were fixed with 2.5% glutaraldehyde for 2–4 h; washed three times with distilled water; dehydrated for 15 min with 60, 70, 80, 95, and 100% ethanol in series; and the last two steps were repeated. A 0.05-ml sample of bacteria was placed on a coverslip and dried overnight. The sample was attached to the sample stage using silver conductive glue, coated with an ion sputter device, and observed and imaged with a scanning electron microscope [27].

Determination of the Protective Effects of T-1 on Zebrafish

Determination of the LD_{50} against *A. hydrophila* cb15 in Zebrafish

For 50% lethal dose (LD_{50}) determination, *A. hydrophila* cb15 cells were cultured in LB broth at 28 °C for 24 h, and seven groups of 10 fish per group were challenged with 0.02 ml *A. hydrophila* cb15 at cell densities of 2.9×10^9, 2.9×10^8, 2.9×10^7, 2.9×10^6, 2.9×10^5, or 2.9×10^4 CFU ml^{-1}. Controls were injected with normal saline. Fish were monitored for mortality for 96 h. During this period, activity and behavior were recorded at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h. LD_{50} values were calculated by linear interpolation [14].

Assaying the Protective Effects of T-1 on Zebrafish

The protective effects of T-1 on zebrafish were determined as described previously [25]. Healthy, energic zebrafish were divided into five groups (10 zebrafish per group). Group I was the blank control, and experimental groups II, III, and IV were separately co-injected intraperitoneally with 0.02 ml of *A. hydrophila* cb15 at a cell density of 7.2×10^7 CFU ml^{-1}, or T-1 at a cell density of 2.6×10^8, 2.6×10^7, or 2.6×10^6 CFU ml^{-1}. Group V was injected intraperitoneally with normal saline. Fish mortality was recorded at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h after injection.

Protective Effects of T-1 on Zebrafish at Different Timepoints after *A. hydrophila* Infection

A total of 60 zebrafish were divided into six groups, and groups I, II, III, IV, and V were injected intraperitoneally with 0.02 ml of *A. hydrophila* cb15 at a cell density of 7.2×10^7 CFU ml^{-1}. Fish in groups I, II, III, and IV were then injected intraperitoneally with 0.02 ml of T-1 at a cell density of 2.6×10^8 CFU ml^{-1} at 0, 4, 8, or 16 h. Fish in group VI were injected with normal saline. Fish mortality was recorded at 1, 2, 4, 6, 8, 12, 24, 48, 72, and 96 h after injection.

Sequencing and Assembly of the T-1 Genome

Sequencing and assembly of the T-1 genome was completed by Shanghai Meiji Biological Company. Sequencing was performed using an Illumina Miseq 2500 sequencing platform to construct an Illumina PE library (400 bp library), and quality control was carried out using bioinformatics analysis following genome scans.

Sequence Analysis of the Quorum Quenching-Related T-1 Gene *ytnP*

Genomic DNA was extracted using a Bacterial Genome Extraction Kit (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. The *ytnP* gene was amplified with forward and reverse primers F (5'-ATGAAGCTGATTCA GGTTGCATTT-3′) and R (5′- CTAGCGGCTTTCTCTTTTTA CTGA-3′). Amplification involved denaturation for 5 min at 98 °C, followed by 34 cycles at 98 °C for 30 s, 55 °C for 30 s,

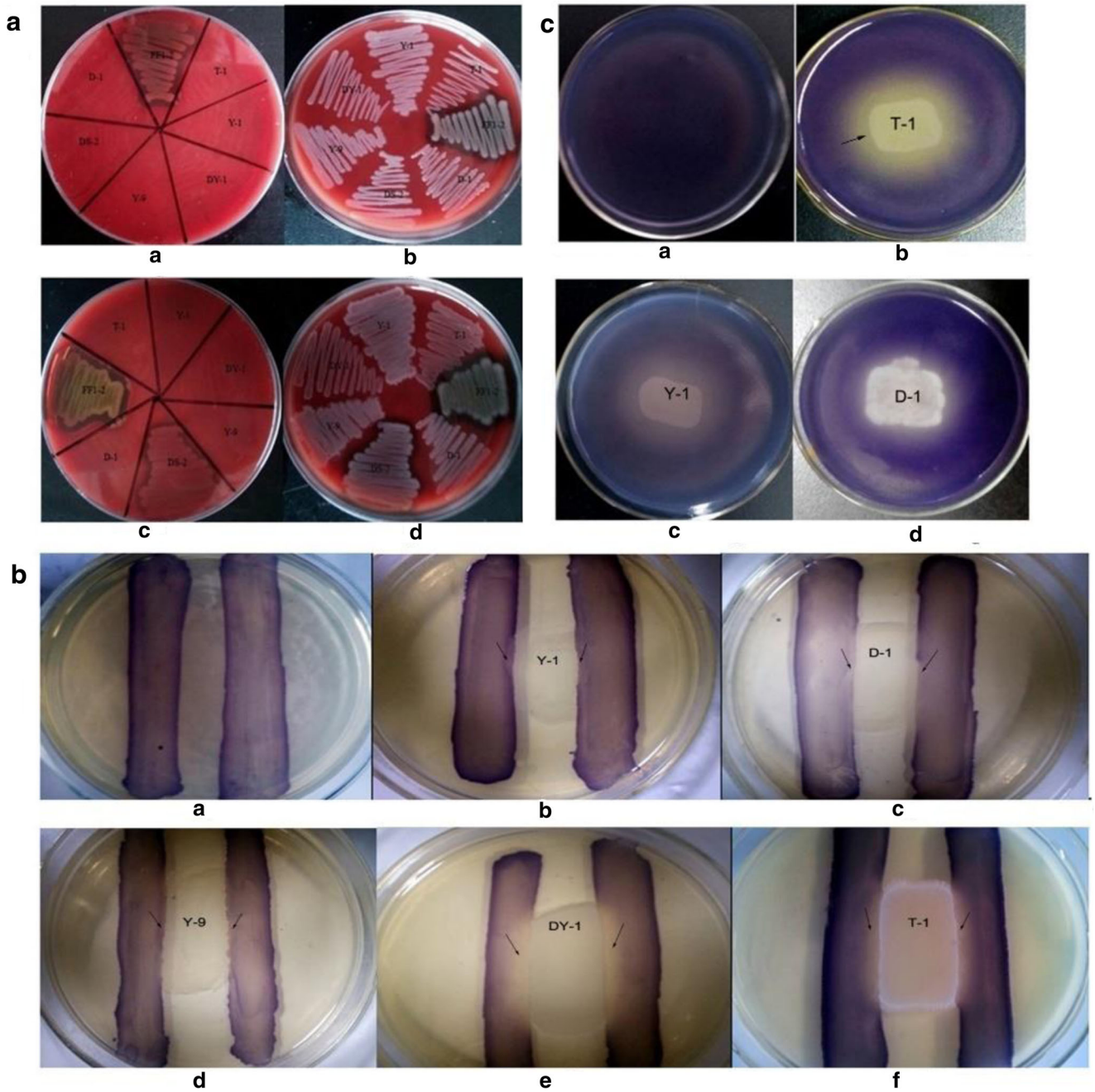

Fig. 1 Quorum quenching of *Bacillus* sp. strain T-1. **a** Hemolytic analysis of different *Bacillus* strains: a, b: hemolytic analysis at 28 °C; c, d: hemolytic analysis at 37 °C. **b** QQ effects of T-1 by method of 'H' streaking: a: negative control; b: Y-1; c: D-1; d: Y-9; e: DY-1; f: T-1. **c** QQ effects of T-1 by method of double layer agar. a: blank control, b: T-1; c: Y-1; d: D-1. The arrows showed the ability of the isolated quorum quenching strains interfered with violacein of *Chromabacterium violaceum* ATCC12472

and 72 °C for 1 min, and a final extension at 72 °C for 5 min. PCR products were purified using a TIANgel Midi Purification Kit (Tiangen Biotech) and cloned into the PMD19-T vector by overnight ligation at 16 °C. Ligated plasmids were transformed into competent *E. coli* cells using the heat-shock method, and 20 mg ml^{-1} 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal) and 50 mg ml^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) were used to select positive clones. After PCR amplification, 1% agarose gel electrophoresis was carried out and products confirmed by comparison with DL2000 DNA markers. A positive clone was selected and sequenced by Sangon Biotech Co. Ltd. (Shanghai, China). The *ytnP* sequence was compared with closely related sequences using BLASTn and the NCBI website (http://blast.ncbi.nlm.nih.gov/). Neighbor-joining and bootstrap analysis with 1000 replicates were performed to construct a phylogenetic tree using MEGA 5.05 [25].

Table. 1 Colony morphology and origin of 34 strains of *Bacillus* spp.

Strains	Colony morphology	Origin
B-1	Light yellow and flat	River water out of school
B-2	White and flat	River water out of school
Bc	White and flat	Preservation in the laboratory
Bs	Light yellow and plicated	Preservation in the laboratory
D-1	White with irregular margin	Shrimp pond from Fenxian district
DY-1	Light yellow and flat	The bottom mud from Dishui Lake
DS-1	White and flat	The water from Dishui Lake
DS-2	White with a heave in the middle	The water from Dishui Lake
FF1-2	White and flat	Preservation in the laboratory
H-1	White and flat	River water from Lingang
H-2	Light yellow and plicated	River water from Lingang
J-1	Light yellow and semitransparent	The bottom mud from Xialian River
J-2	Light yellow with a heave in the middle	The bottom mud from Xialian River
J-3	White and plicated	The bottom mud from Xialian River
J-5	Light yellow with irregular margin	The bottom mud from Xialian River
T-1	Light yellow with irregular margin	The bottom mud from culture pond in Binhai
T-2	White and flat	The bottom mud from culture pond in Binhai
T-4	Light yellow and semitransparent	The bottom mud from culture pond in Binhai
T-5	Light yellow with a heave in the middle	The bottom mud from culture pond in Binhai
W-1	Light yellow with a heave in the middle	Sewage water out of school
W-2	Light yellow and plicated	Sewage water out of school
W-3	White with irregular margin	Sewage water out of school
X-1	White and flat	The bottom mud of the river
X-2	Light yellow and semitransparent	The bottom mud of the river
X-3	Light yellow with a heave in the middle	The bottom mud of the river
X93	White and flat	Intestinal tract of *Larimichthys crocea*
X3914	White and flat	Intestinal tract of *L. crocea*
X77	Light yellow and flat	Intestinal tract of *L. crocea*
Y-1	White with irregular margin	Intestinal tract of *Carassius auratus*
Y-2	Light yellow and semitransparent	Intestinal tract of *C. auratus*
Y-3	Light yellow with a heave in the middle	Intestinal tract of *C. auratus*
Y-8	White and plicated	Intestinal tract of *C. auratus*
Y-9	Light yellow with irregular margin	Intestinal tract of *C. auratus*
FF1-2	White and flat	Strain preserved in lab

Statistical Analysis

Data were subjected to one-way analysis of variance (ANOVA) to confirm significant differences among experimental groups at $p < 0.05$.

Results

Isolation of Candidate *Bacillus* spp. Strains for Inhibiting Quorum Sensing

C. violaceum synthesizes the violet pigment as a result of QS. Loss of purple pigmentation in *C. violaceum* ATCC12472 in the vicinity of *Bacillus* spp. was indicative of QS inhibition (Fig. 1). Where *Bacillus* inhibited the growth of *C. violaceum*, clear inner zones of inhibition were observed. The outer colorless zone of inhibition was opaque and not transparent, indicating that the halos around discs were caused by inhibition of QS, rather than inhibition of cell growth (Fig. 1). A total of 34 *Bacillus* spp. strains were isolated from river water, sediment, fish intestinal tract, or strains preserved in the lab (Table 1). The results of hemolysis experiments indicated that *Bacillus* strains T-1, D-1, Y-1, DY-1, and Y-9 lacked hemolytic properties, while *Bacillus* strains FF1-2 and DS-2 possessed hemolytic activity (Fig. 1a). T-1 displayed the strongest ability to inhibit quorum sensing among isolates identified by the "H" streaking method (Fig. 1b), and the results of double layer

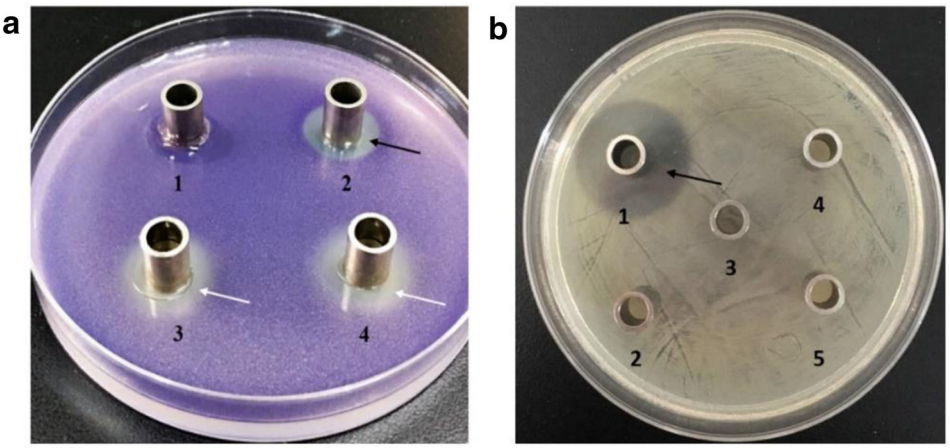

Fig. 2 **a** AHL-degradation assay on Luia-Bertani agar. (1. blank control: sterile water; 2. negative control: enrofloxacin; 3 and 4. experimental groups: strain T-1. The blank arrow showed that there was no bacteria in the area of *C. violaceum* CV026 around Oxford Cup and it inhibits the growth of bacteria. The white arrow around 3 and 4 showed the bacteria appeared and the area near T-1 of *C. violaceum* CV026 has no purple pigment indicates the ability to degrade AHL molecules). **b** Antibacterial effect of T-1 on *Aeromonas hydrophila* (1. positive control: enrofloxacin; 2. blank control: sterile water; 3. 10^{0} of T-1; 4. 10^{-1} of T-1; 5. 10^{-2} of T-1. The blank arrow showed that there was no bacteria in the area of enrofloxacin around Oxford Cup and it inhibits the growth of bacteria, and the *A. hydrophila* cells grew well in the presence of T-1 of 3, 4, and 5)

agar experiments further confirmed the inhibitory effects of T-1 (Fig. 1c).

To verify the AHL-degrading activity of T-1, *C. violaceum* CV026 was used as an indicator strain. After incubation with T-1, the purple pigment around the Oxford cup zone on the plate disappeared (Fig. 2), while the area around the Oxford cup zone on the sterile water negative control plate remained purple. Additionally, there were no bacteria in the area around the Oxford cup zone on the plate treated with enrofloxacin. These results demonstrate that C6-HSL was degraded by T-1, and it did not affect the growth of *C. violaceum* CV026, which indicates that T-1 has the ability to degrade AHLs. Moreover, the results of animal safety and ecological environment evaluation experiments indicate that *B. licheniformis* T-1 inhibits quorum sensing and is nontoxic to *C. auratus gibelio*, zebrafish, and mice, and has no adverse effects on plankton in a water ecological environment. Furthermore, the results in Fig. 2b showed that there were no bacteria in the area around the Oxford cup zone on the plate treated with enrofloxacin, but *A. hydrophila* cb15 cells grew well in the area around the Oxford cup treated with T-1, comparable with the negative control plate.

Table. 2 Identification results of T-1 in Biolog system (20 h)

Group	Identification results	PROB	SIM	DIS	Type
1	*Bacillus licheniformis*	0.610	0.610	5.639	GP-RodSB
2	*Bacillus sonorensis*	0.150	0.150	6.015	GP-RodSB
3	*Bacillus subtilis*	0.087	0.087	6.342	GP-RodSB
4	*Bacillus atrophaeus*	0.075	0.075	6.434	GP-RodSB

Bacterium was incubated at 33 °C for 4–6 h, similarity (SIM) ≥ 0.75; for 16–24 h, similarity (SIM) ≥ 0.50, and it will be identified as species

PROB probability, *SIM* similarity, *DIS* distance

Identification of T-1

Analysis of 16S rDNA Sequences

The 16S rDNA sequences of T-1 were compared with homologous sequences in GenBank. The phylogenetic tree based on 16S rDNA sequences of T-1 revealed *B. licheniformis* 55P3-1 as the closest homolog (accession number JN366777.1). Thus, T-1 was preliminarily identified as *B. licheniformis* (GeneBank accession number KP117098).

Analysis of T-1 Using the Biolog Microbial System

According to the Biolog system, if a bacterial strain incubated at 33 °C for 4–6 h has a similarity (SIM) ≥ 0.75, and a SIM ≥ 0.50 when incubated for 16–24 h, the species can be considered identified (SIM indicates the reliability, and a larger value represents greater reliability). T-1 was incubated at 33 °C for 20 h, and the SIM value was 0.610, confirming T-1 as *B. licheniformis* (Table 2).

Analysis of T-1 by Scanning Electron Microscopy

Scanning electron microscopy (SEM) analysis of T-1 in solid and liquid mediums revealed short rods with dimensions of 3 ± 0.21 μm × 10 ± 0.34 μm, arranged in a linear manner, and displaying morphological characteristics typical of *Bacillus* spp. (Fig. 3).

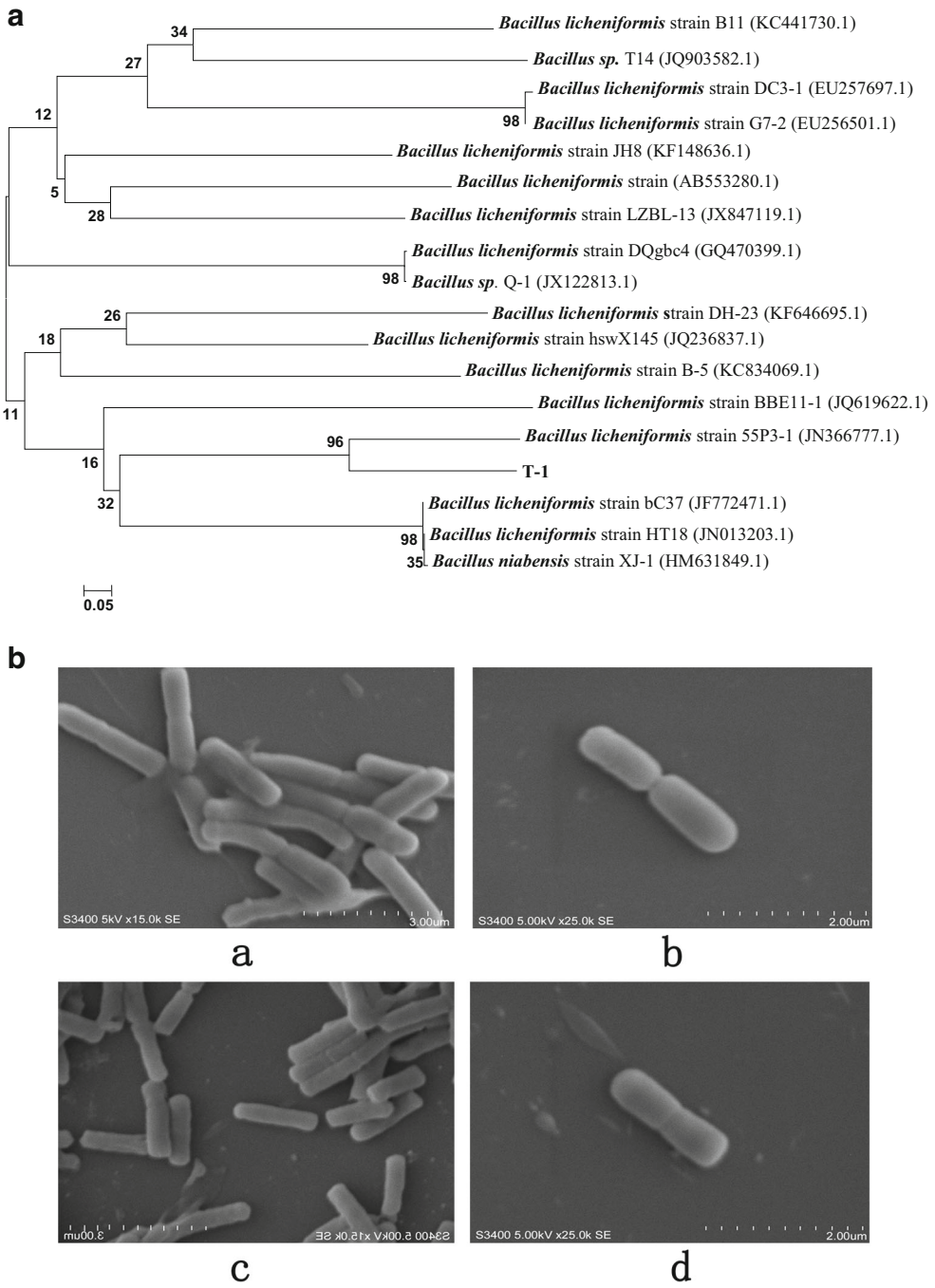

Fig. 3 **a** Phylogenic relationships based on 16S rDNA sequences of T-1.The branching pattern was generated by the Neighbor-joining method. Bootstrap values of 1000 or more are indicated at the nodes. Sequence accession numbers are listed in parentheses. **b** SEM of *Bacillus* sp. strain T-1. a, b on solid medium (20 h) (a × 15,000; b × 25,000); c, d on liquid medium (20 h) (c × 15,000; d × 25,000)

The LD_{50} of *A. hydrophila* cb15 in Zebrafish

The LD_{50} of *A. hydrophila* cb15 in zebrafish was 1.45×10^7 CFU ml^{-1}.

Protective Effects of T-1 on Zebrafish Challenged with *A. hydrophila*

Experimental analysis of protection ability showed that no fish died after being injected with normal saline, and the percentage survival of the group injected with 7.2×10^7 CFU ml^{-1} *A. hydrophila* cb15 was below 20%. The results also showed that the protective effects of T-1 on zebrafish challenged with *A. hydrophila* cb15 were dependent on the dose of T-1 (Fig. 4a); survival of zebrafish reached 70% when co-injected with T-1 at a dose of 2.6×10^8 CFU ml^{-1} at 96 h after injection, which was significantly higher than control and other groups ($p < 0.05$). T-1 significantly attenuated *A. hydrophila* cb15 infection. Besides, at the same injected T-1 dose, the protective effects were correlated with injection time. Survival of zebrafish was beyond 60%

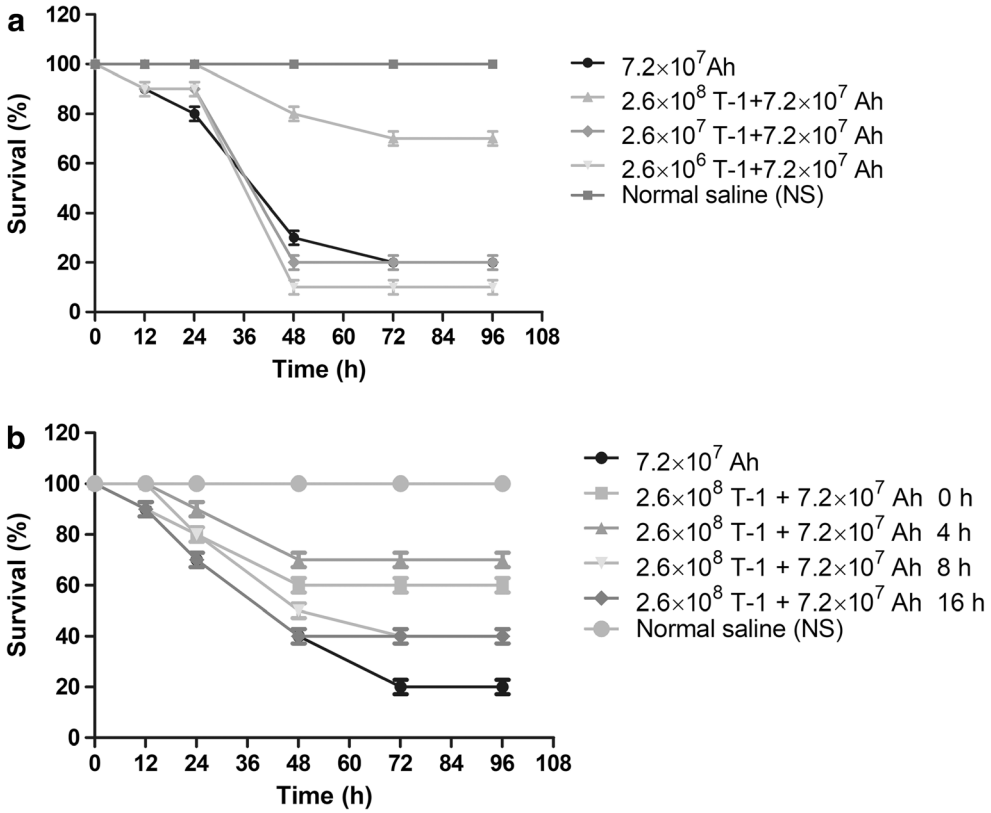

Fig.4 **a** Protective effects of T-1 on zebrafish with *A. hydrophila* cb15. Abdominal injections of *A. hydrophila* cb15 result in significant levels of mortality in zebrafish reared in a closed aquaculture system. However, co-injection with T-1 significantly increases the survival over a period of 96 h. Shown are the cumulative mortalities for replicate groups of zebrafish injected with different concentrations of T-1. **b** Protective effects of T-1 on zebrafish at different timepoints after *A. hydrophila* infection. Survival of zebrafish was beyond 60% when injected T-1 at the 0 and 4 h and survival of zebrafish was less than 40% when injected T-1 at the 8 and 16 h

following injection with T-1 at 0 and 4 h after *A. hydrophila* cb15 infection, and 4 h was the optimal injection time. Compared with survival of zebrafish in the positive control (only *A. hydrophila* cb15) and other experimental groups (8 and 16 h), the protective effects were better after injection at 4 h after infection (Fig. 4b).

Prediction and Analysis of the T-1 Quorum Quenching Gene

Sequencing of the T-1 genome was performed using an Illumina Miseq 2500 sequencing platform, an Illumina PE library (400 bp library) was constructed, and quality control was carried out using bioinformatics analysis following genome scanning.

Four AHL lactonase metallo-β-lactamase genes (*ytnP, yycJ*, *yobT*, and *yrkH*), were predicted from the T-1 draft genome framework. A BLAST search using the AHL-lactonase metallo-β-lactamase gene revealed 99% sequence identity between the *ytnP* gene of T-1 and its homolog in *B. subtilis* subsp. BSP1 (Fig. 5a), but lower homology with *yycJ*, *yobT*, and other metallo-β-lactamases. As shown in Fig. 5b, proteins encoded by *ytnP* and *yobT* contain the conserved HXHXDH motif that is essential for AHL lactone enzyme activity. However, proteins encoded by *yycJ* and *yrkH* lack key histidine residues, which likely render them unable to perform this function.

PCR Amplification of the *ytnP* Gene from T-1

An autoinducer inactivation gene (*ytnP*) has been identified previously in Gram-positive *Bacillus* sp. [12]. T-1, D-1, *B. subtilis*, and *E. coli* were tested for the presence of a *ytnP* homolog by PCR, and three of the four bacteria were positive for amplification of *ytnP* (the expected amplicon was ~ 900 bp), while *E. coli* was negative. A positive clone from T-1 was selected, subjected to PCR amplification, and sequenced by Sangon Biotech Co. Ltd. The results revealed 100% sequence identity, confirming the presence of *ytnP* in T-1.

Discussion

In the present study, we isolated the novel *B. licheniformis* strain T-1 from a freshwater environment that can effectively degrade AHLs (Figs. 1 and 2). AHL-producing and AHL-degrading bacteria co-exist in ecosystems, and have developed different strategies to gain a competitive advantage [28, 29]. Recently, the ability to degrade AHLs has been identified in several *Bacillus* genera, including *B. subtilis*, *B. cereus*, and *Bacillus thuringiensis* [30–32]. *B. licheniformis* is a Gram-positive, oxidase-positive, and catalase-positive endospore bacterium belonging to the genus *Bacillus* [33, 34]. It produces a wide range of extracellular enzymes and has been reported as a probiotic bacterium for animals [35, 36].

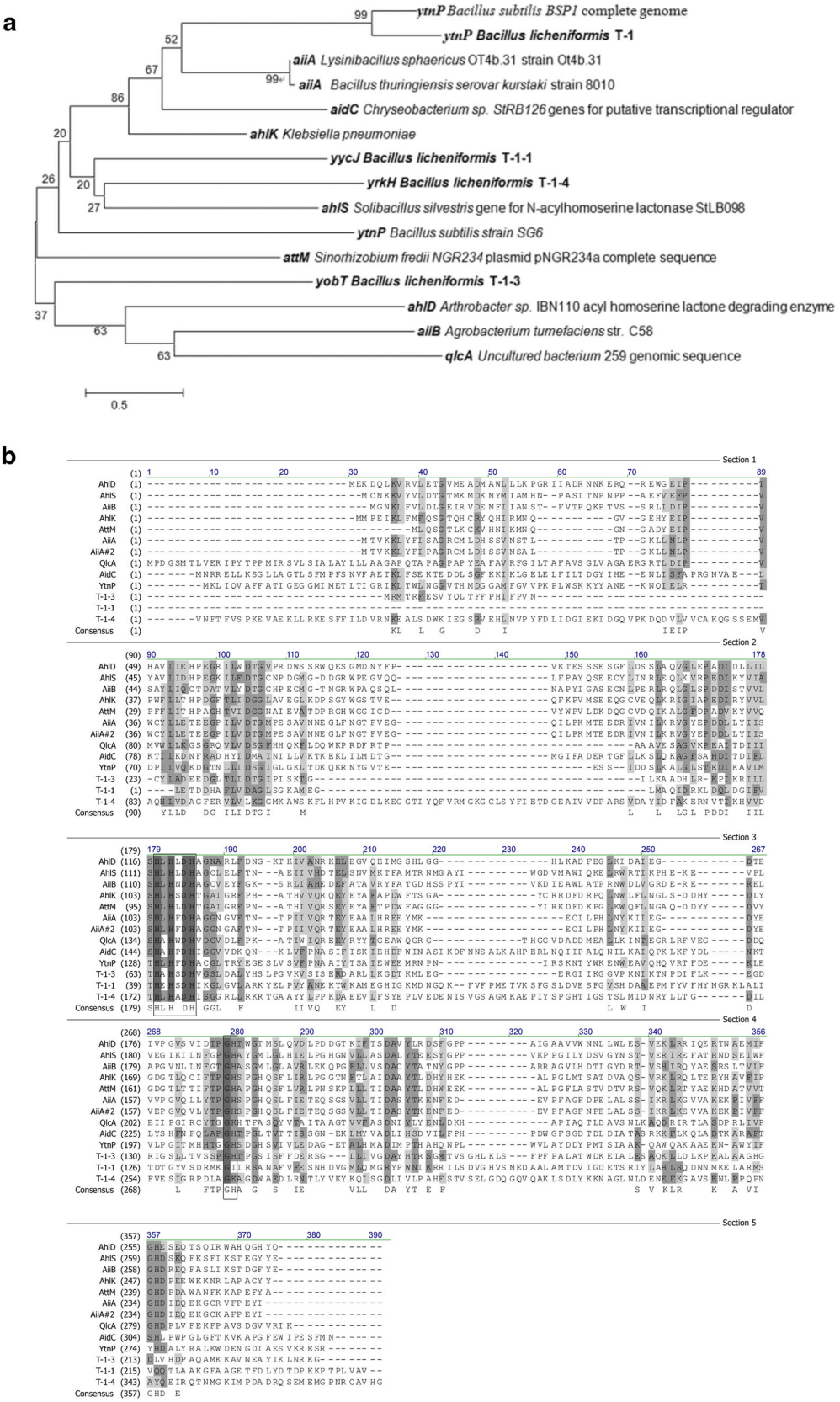

Fig. 5 Evolutionary tree with acyl-homoserine lactone (AHL) metallo-β-lactamase gene and protein sequence analysis of AHL-lactonase metallo-β-lactamase, *yycJ* (T-1-1), *yrkH* (T-1-4), and *yobT* (T-1-3) (Fig. 5a). The similar and identical residues are shaded in gray and black, respectively. The conserved motif HXHXDH is boxed (Fig. 5b).

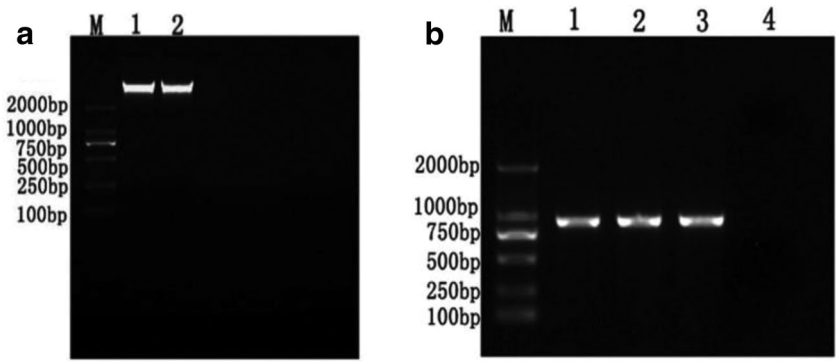

Fig.6 **a** Extraction of total DNA. Lane M, 2000 bp DNA Ladder (Invitrogen); lane1 and lane 2, genome DNA of T-1. **b** PCR detection of the *ytnP* gene. Lane M, 2000 bp DNA ladder (Invitrogen); lane 1, T-1; lane 2, D-1; lane 3, *Bacillus subtilis*; lane 4, *E. coli*, negative control

Although research is in progress, there are few reports on the quorum quenching of *B. licheniformis*. In addition, recent studies indicate that *Bacillus* might contain toxin-producing genes [17], which could impact their food, environmental, and clinical applications [16, 37]. Herein, we screened *B. licheniformis* T-1, which can be considered non-hemolytic (Fig. 1) and is nontoxic to *C. auratus gibelio*, zebrafish, and mice, and has no adverse effects on plankton in a water ecological environment [21]. Thus, T-1 could have potential as a safe probiotic for use in aquaculture.

B. licheniformis is used as a dietary supplement to significantly increase the growth rate of animals. Administration of *B. licheniformis* can inhibit *Vibrio* spp. by competitive exclusion, and improve the intestinal microflora and immune ability of *Litopenaeus vannamei* [38]. Similar improvement in immune parameters and reduction in gut pathogenic bacteria have been reported for *Penaeus japonicus* when *B. licheniformis* was fed in combination with *B. subtilis* and isomaltooligosaccharide [39]. Additionally, *B. licheniformis* KADR5 has been isolated from the gut of healthy *Labeo rohita* fish injected with *A. hydrophila* (in the case of subcellular components) and orally infected (in case of live cells, at a cell density of 10^8 CFU g^{-1} of feed) [40]. In the present study, *B. licheniformis* T-1 significantly decreased the mortality of zebrafish infected with *A. hydrophila* cb15, and increased survival to 70%. Compared with the results of AHL-lactonase AiiA expressed in *E. coli* or *P. pastoris*, supplementation of $AiiA_{AI96}$ into fish feed by oral administration significantly attenuated *A. hydrophila* infection in zebrafish, with survival < 60% [14, 41]. In addition, survival of zebrafish was > 60% at 0 and 4 h after *A. hydrophila* injection, but lower at 8 and 16 h (Fig. 4). This suggests that T-1 might have much stronger prophylactic and therapeutic effects during the early phase of *A. hydrophila* infection. Thus, these results indicate that T-1 is safe and effective as a potential probiotic and biocontrol agent for disease prevention and control in aquaculture.

Quorum quenching is considered a promising strategy for controlling bacterial diseases as an alternative to antibiotics to reduce the risk of drug resistance [42, 43]. There are several kinds of hydrolytic enzymes in *Bacillus* spp. that degrade AHLs and block pathogenicity. However, in the case of *B. licheniformis*, there are few studies on the quorum quenching mechanism or quorum quenching genes. One *B. licheniformis* strain isolated from seawater that can degrade AHLs possesses the *aiiA* gene [44]. In the present study, the T-1 draft genome was determined and analyzed using the Illumina Hiseq 2500 platform (unpublished). We annotated a typical AHL lactonase gene, *ytnP*, encoding an AHL lactonase metallo-β-lactamase, and three other potential genes (*yobT*, *yrkH*, and *yycJ*; Fig. 5). Gene *ytnP* was subsequently successfully amplified, but attempts to amplify *aiiA* were unsuccessful (Fig. 6), and *aiiA* was not found in the T-1 genome. Analysis of protein sequences corresponding to *ytnP* and comparison with homologs *aiiA* (*B. thuringiensis*), *ahlD* (*Arthrobacteria* sp.), *aiiM* (*Microbacterium testaceum*), *attM* (*Agrobacterium fabrum*), and *aidC* (*Chryseobacterium* sp.) [10–13] revealed the presence of a typical zinc finger motif, including the HXHXDH consensus sequence containing key histidine residues, in the protein encoded by *ytnP*, confirming the presence of the necessary lactonase architecture. Meanwhile, Schneider and colleagues reported that addition of 20 nM purified Ytnp, encoded by the quorum quenching gene *ytnP*, to cultures of *Pseudomonas aeruginosa* inhibited biofilm formation, which indicates that Ytnp may block the signaling pathways controlled by quorum sensing in *P. aeruginosa* [12]. Thus, *ytnP* might be an important gene for quorum quenching in T-1. Besides, we speculated that *B. licheniformis* strains isolated from different environments might possess different quorum quenching systems. T-1 was isolated from freshwater, whereas the strain in the previous study was from seawater [44]. The environments of bacteria in freshwater and seawater are clearly different, and this could affect microbial growth and genetic variation, but this hypothesis needs further investigation.

In conclusion, the present study revealed that *B. licheniformis* T-1 is a safe quorum quenching bacterium and a potential probiotic. Survival of zebrafish challenged with *A. hydrophila* reached 70% when intraperitoneally co-injected with T-1, demonstrating its potential as a biocontrol agent for disease prevention and control in aquaculture. The *ytnP* gene might be important in quenching QS, but further studies on the quorum quenching mechanism of T-1 are needed.

Acknowledgments This work was supported by the Innovation Fund of Nanjing Government Jiangsu Province, China.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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